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Voltage-dependent modulation of rat sensory neurone calcium channel currents by G protein activation: effect of a dihydropyridine antagonist

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The ability of a depolarizing prepulse to increase the rate of activation of I_{Ba} has been examined in cultured sensory neurones of the rat. Both in control neurones and in the presence internally of the guanine nucleotide analogue, guanosine 5'-O-3-thiotriphosphate (GTPγS) which markedly slows the rate of activation of I_{Ba} and reduces its amplitude, a depolarizing prepulse increased the rate of activation of I_{Ba}, but did not increase its amplitude measured at the end of the 100 ms voltage step. The calcium channel antagonist (−)-202-791, which we have previously shown to increase the amplitude of I_{Ba} in the presence of GTPγS, did not occlude the response to a depolarizing prepulse, suggesting that the mechanism of action of (−)-202-791 is not to disrupt the interaction of the channels with activated G proteins.

Introduction It has been observed in several cell types that the effect of internal guanine nucleotide analogues on calcium currents is to slow the rate of activation and reduce the current amplitude (Scott & Dolphin, 1986; Lewis *et al.*, 1986). We have previously shown that calcium channel antagonist ligands, including the dihydropyridine (−)-202-791 (2,6-dimethyl - 3 - carbomethoxy - 5 - nitro - 4 - (2 - trifluoromethyl - phenyl)-1,4-dihydropyridine) increase rather than decrease calcium channel currents (I_{Ba}) in the presence of internal guanine nucleotide analogues (Scott & Dolphin, 1987; Dolphin & Scott, 1989). Several explanations for this phenomenon are possible. Activated G protein may interact with the calcium channel ligand binding site such that either agonist binding or its efficacy is increased. It is also possible that the dihydropyridines reverse the effect of activated G proteins on the calcium channel, thus increasing current amplitude.

It has recently been suggested by Grassi & Lux (1989) that the kinetic effect of the neurotransmitter γ-aminobutyric acid (GABA) and the non-hydrolysable GTP analogue guanosine 5'-O-3-thiotriphosphate (GTPγS) in slowing calcium current activation in chick dorsal root ganglion neurones (DRGs) is due to gradual recovery of the calcium channels from the voltage-dependent block conferred by their interaction with activated G proteins.

In the present study we have confirmed the voltage-dependence of the kinetic effect of GTPγS on I_{Ba} in rat DRGs. We have further shown that the amplitude inhibition of I_{Ba} is not restored by a depolarizing prepulse, and that the agonist response to (−)-202-791 does not prevent a prepulse from reversing the kinetic effect of GTPγS.

Methods Neonatal rat DRGs were dissociated and maintained in culture as previously described, (Dolphin *et al.*, 1986), except that they were plated on polyornithine-laminin coated coverslips and maintained in a serum-free defined medium (Bottenstein *et al.*, 1980; Winter, 1987). Calcium channel currents were recorded from cells between 1 day and 2 weeks in culture.

The whole-cell recording technique (Hamill *et al.*, 1981) was used to examine Ca^{2+} channel currents (I_{Ba}). Low resistance pipettes (2–4 MΩ) were used, and cells were voltage-clamped with an Axoclamp-2 switching amplifier, operated at a sampling rate of 25–30 kHz. The recording medium contained (mM): NaCl 130, KCl 3, $MgCl_2$ 0.6, $NaHCO_3$ 1.0, HEPES 10, glucose 4, $BaCl_2$ 2.5, tetra-ethylammonium bromide (TEA) 25, and tetrodotoxin 0.0025. It was adjusted to pH 7.4 with NaOH and to 320 mosM with sucrose. Patch electrodes were filled with a solution containing (mM): Cs acetate 140, $CaCl_2$ 0.1, EGTA 10, $MgCl_2$ 2, ATP-Na 2 and HEPES 10. The pH was adjusted to 7.2 with Tris and the osmolarity to 310 mosM with sucrose. In some experiments GTPγS (100 or 500 μM was included in the patch pipette). Initial current-voltage relationships were generated, and only cells showing adequate space clamp were used.

The maximum I_{Ba} was recorded by a 100 ms test depolarization from V_H −80 mV to −10 or 0 mV. Leak subtraction was carried out using hyperpolarizing steps to −130 mV performed immediately beforehand. When a prepulse was applied the prepulse current was not leak subtracted. Analysis was carried out by use of programmes supplied by Dr J. Dempster, Strathclyde University.

Results The maximum I_{Ba} examined over the first minute in a cell recorded with a pipette containing GTPγS, generally showed a transient component, before the effect of the GTP analogue was manifest (Figure 1a, initial current). GTPγS then slowed the activation of I_{Ba}, and reduced its amplitude over 2–5 min (Figure 1a, GTPγS). As shown by Grassi & Lux (1989), a prepulse (pp) of 40 ms to +30 mV, terminating 5 ms before activation of the maximum inward current, reversed the GTPγS-induced slowing of activation of I_{Ba} (Figure 1a, +pp; $n = 7$). However, in all cells in which the initial current was recorded, the prepulse did not restore the amplitude of the current to its initial level before the response to GTPγS had taken effect. The initial current was 2.5 ± 0.2 nA (mean ± s.e.mean; $n = 6$); in the presence of GTPγS it was reduced to 1.9 ± 0.2 nA and following the prepulse the amplitude remained at 1.9 ± 0.2 nA. In all cases I_{Ba} was measured at the end of the 100 ms step. Larger depolarizing prepulses to up to +100 mV did not increase the current amplitude at the end of the 100 ms step, although the initial peak amplitude was increased.

It is unlikely that the inability of the prepulse to reverse the inhibition by GTPγS of the amplitude of I_{Ba} is due to current rundown, because none is observed in these cells over the first 5–10 min of recording (Dolphin *et al.*, 1986). In a proportion of control neurones, in the absence of GTPγS, an identical prepulse also enhanced the initial rate of activation of the control I_{Ba} (5/8 control neurones; e.g. Figure 1b). Again the amplitude of I_{Ba} at the end of the 100 ms voltage step was not increased. In no case did the prepulse reduce the amplitude of the maximum I_{Ba}.

Since it is possible that the agonist response to (−)-202-791 observed in the presence of GTPγS is due to a reversal of the

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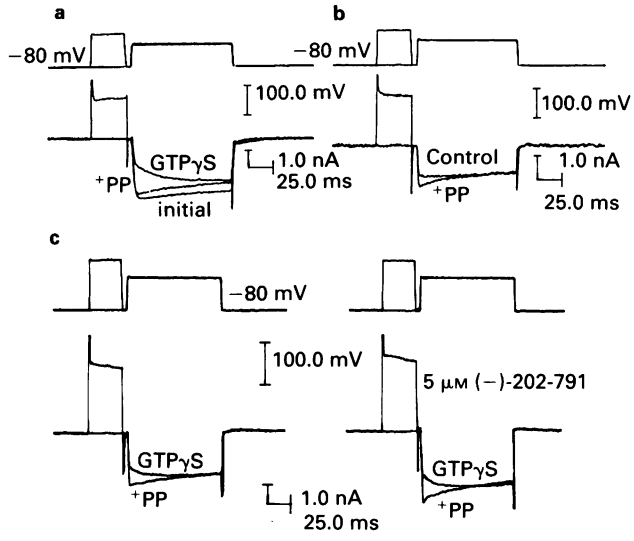


Figure 1 The effect of depolarizing prepulses on guanosine 5'-O-3-thiotriphosphate (GTPγS)-induced inhibition of I_{Ba}. The maximum I_{Ba} was evoked by a 100 ms step from V_H −80 mV, to V_C 0 mV. Leak subtraction was performed using steps to −130 mV immediately beforehand. The effect was examined of a 40 ms prepulse to +30 mV, terminating 5 or 10 ms before activation of I_{Ba}. Currents are superimposed, and the prepulse current is not leak subtracted. (a) An initial maximum I_{Ba} was recorded immediately after establishing whole cell clamp using a pipette containing 100 μM GTPγS. After 5 min the effect of GTPγS is maximal and a prepulse (+pp) restores the rate of activation of I_{Ba} to that of the initial current, although the amplitude remains reduced. (b) A control I_{Ba} was recorded in the absence of GTPγS in the patch pipette. A prepulse to +40 mV increased the initial transient component of I_{Ba} (+pp), but did not affect the amplitude at the end of the 100 ms step. (c) I_{Ba} was recorded in the presence of 500 μM GTPγS with or without a prepulse (+pp) (left panel). (−)-202-791 (5 μM) was then applied by low pressure ejection from a blunt micropipette placed 100 μm from the cell. An increase in I_{Ba} was observed in the currents recorded both with and without a prepulse (right panel).

effect of activated G protein on I_{Ba}, we examined the ability of the prepulse protocol to reverse the kinetic effect of GTPγS before and during application of 5 μM (−)-202-791. In the cell shown in Figure 1c, the prepulse increased the control peak I_{Ba} in the presence of GTPγS (Figure 1c, left panel). Application of (−)-202-791 to this cell then produced an agonist response, 30 s after the start of application, enhancing the amplitude of the current by 25%. However, the kinetic effect of GTPγS remained present and the activation of I_{Ba} was still increased to the same extent by a depolarizing prepulse (Figure 1c, right panel). Further application of (−)-202-791 for 2 min did not abolish this effect of GTPγS, although I_{Ba} was further enhanced. This experiment was repeated in 5 other cells with identical results. The amplitude of I_{Ba} in the presence of GTPγS was 2.0 ± 0.6 nA (n = 6), and the increase in amplitude of I_{Ba} due to (−)-202-791, both in the absence and in the presence of a prepulse, was 0.8 ± 0.1 nA.

Discussion In these experiments we have confirmed the finding of Grassi & Lux (1989) that the slowed activation of the calcium channel current by GTPγS is reversed by a depolarizing prepulse. However, the amplitude of the current is not restored, when measured at the end of the 100 ms depolarizing voltage step. The inability of the prepulse to restore the current amplitude is not because of prepulse-induced inactivation, or current run-down, since neither phenomenon was observed in these experiments. The voltage-dependence of the effect of agonists on neuronal calcium currents was previously suggested by Bean (1989) who showed that noradrenaline no longer inhibited outward calcium channel currents activated by large depolarizing steps in frog DRGs. However we have observed that both the $GABA_B$ agonist baclofen and GTPγS reduce the amplitude of the outward calcium channel currents in rat DRGs, although the rate of activation of the outward current in the presence of these agents is much faster than the rate of activation of the inward I_{Ba} (Dolphin & Scott, 1990). Taken together with the present result, this points to two effects of G protein activation by agonists or GTPγS, a kinetic effect on activation of the calcium current, and an additional effect on the current amplitude. The finding that a prepulse also increased the rate of current activation in a proportion of control cells suggests that the current may be under partial tonic inhibition by GTP-activated G proteins.

The finding that (−)-202-791 remained an agonist in the presence of GTPγS when I_{Ba} was recorded with a depolarizing prepulse, and that (−)-202-791 did not reverse the kinetic effect of GTPγS, indicates that the mechanism of action of (−)-202-791 is unlikely to involve displacement of GTPγS binding or prevention of interaction of activated G protein with the calcium channel.

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5-Hydroxytryptamine and arachidonic acid metabolites modulate extensive platelet activation induced by collagen in cats *in vivo*

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1 The pathways contributing to the platelet adhesion/aggregation reaction elicited by collagen microfibrils, administered to cats *in vivo*, were analysed.

2 The intra-aortic infusion of collagen (100 $\mu g\,kg^{-1}$ in 1 min) caused an extensive activation of platelets, as evidenced by the time-dependent drop of free platelet numbers in whole blood, and the increases of 5-hydroxyindoles (5-HI), 5-hydroxytryptamine (5-HT) and thromboxane B_2 (TXB_2) levels in plasma, prepared from effluent venous blood sampled from the inferior caval vein.

3 5-HT_2 receptor blockade with ketanserin (0.63 $mg\,kg^{-1}$ i.v., 10 min) and cyclo-oxygenase inhibition with aspirin (10 $mg\,kg^{-1}$ i.v., 10 min) slightly attenuated the peak reduction of free platelets in whole blood in response to collagen without affecting changes in plasma 5-HI. Aspirin, but not ketanserin, reduced the collagen-induced changes in plasma TXB_2, prostaglandin E_2 (PGE_2) and 6K-$PGF_{1\alpha}$.

4 Dual TXA_2 synthetase inhibition/TXA_2-prostaglandin endoperoxide receptor antagonism with ridogrel (5 $mg\,kg^{-1}$ i.v., 10 min) halved the drop in free platelets, reduced the release of platelet 5-HI, inhibited the increase in plasma TXB_2 and elevated that of 6K-$PGF_{1\alpha}$ and PGE_2 in response to collagen.

5 Combined treatment with ketanserin and aspirin reduced the collagen-induced drop of free platelets and the release of platelet 5-HI to a similar extent as ridogrel alone; plasma prostanoids were affected as with aspirin alone.

6 Combined administration of ketanserin and ridogrel virtually eliminated the collagen-induced platelet adhesion/aggregation response and release of 5-HI; prostanoids were affected as with ridogrel alone.

7 The results indicate that the interplay between 5-HT and arachidonic acid metabolites is causally involved in the platelet reaction to activation induced by collagen in cats *in vivo*.

Introduction

Subsequent to an initial activation on a vessel wall lesion, blood platelets form a mural thrombus and release several mediators. These include adenosine 5'-diphosphate (ADP), 5-hydroxytryptamine (5-HT) and trace amounts of catecholamines, stored in their granules, as well as the prostaglandin endoperoxides PGG_2 and PGH_2 and thromboxane A_2 (TXA_2), derived from arachidonic acid (Holmsen, 1977; Vermylen *et al.*, 1983). Together with plasma coagulation products such as thrombin, pressor hormones such as catecholamines, adenine nucleotides released from sheared red blood cells, and phospholipid derivatives produced by white blood cells, the platelet-derived products contribute to the further recruitment of more cells which build up a thrombus, eventually occluding the damaged artery (De Clerck *et al.*, 1984b; Schmitz *et al.*, 1985; Ashton *et al.*, 1986).

Among the various mediators, 5-HT as well as prostaglandin endoperoxides and TXA_2 have been identified as platelet-derived products, contributing significantly to this secondary recruitment phase. Indeed, these autocoids are released during platelet aggregation and thrombus formation in damaged coronary arteries, both in experimental animals (Schmitz *et al.*, 1985; Hook *et al.*, 1985; Ashton *et al.*, 1986) and in man (FitzGerald *et al.*, 1986; Van den Berg *et al.*, 1989). Moreover, specific blockade of platelet 5-HT_2 receptors (Bush *et al.*, 1984; Golino *et al.*, 1988b), TXA_2/PGH_2 receptors (Golino *et al.*, 1988b), inhibition of platelet cyclo-oxygenase (Folts *et al.*, 1976) or TXA_2 synthetase activity (Aiken *et al.*, 1981) substantially reduces arterial thrombus formation in experimental animals, in particular when discrete endothelial cell injury is the precipitating cause (De Clerck & de Chaffoy de Courcelles, 1989).

By contrast, arterial thrombus formation over deep intimal lesions (Hook *et al.*, 1985) or subsequent to recombinant tissue plasminogen activator (rt-PA) fibrinolysis (Golino *et al.*, 1988a) often are not altered by such single pharmacological interventions; this may be due to simultaneous mobilization of various pathways, acting in concert to produce extensive platelet activation through the amplification phenomenon (De Clerck & de Chaffoy de Courcelles, 1989).

We here present evidence that 5-HT_2 receptor blockade with ketanserin (De Clerck *et al.*, 1982; 1984a,b), added to cyclo-oxygenase inhibition with aspirin and, in particular, to dual TXA_2 synthetase inhibition/TXA_2-PGH_2 receptor antagonism with ridogrel (De Clerck *et al.*, 1989a,b), provides enhanced inhibition of extensive platelet activation induced by collagen in cats *in vivo*.

Methods

Experimental procedures

Experiments were performed in cats of either sex, weighing 3 to 5 kg. Anaesthesia was induced and sustained with ketamine hydrochloride (15 $mg\,kg^{-1}$ and 7.5 $mg\,kg^{-1}$ increments i.m. as required). After tracheotomy, respiration was supported by pressure-monitored ventilation (air 35% : oxygen 65%) before the administration of succinylcholine (0.1 $mg\,kg^{-1}$, i.v.) (De Clerck *et al.*, 1986a).

Under radioscopic guidance, a catheter was positioned via the left common carotid artery in the lower aorta with its tip protruding distally from the renal arteries, just above the trifurcation.

In a first set of experiments, animals ($n = 6$) were allowed to stabilize for at least 30 min, then treated with saline (1 $ml\,kg^{-1}$ of NaCl 0.15 M via a catheter in a jugular vein), and 10 min

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thereafter infused with a suspension of collagen microfibrils ($100\,\mu g\,kg^{-1}$ in 1 min in 2 ml via the catheter in the aorta). Blood was sampled via a catheter, inserted through the saphenous vein of the right hindleg into the inferior vena cava and collected into appropriate anticoagulants, before the injection of saline, 9 to 10 min thereafter and at various time intervals after the start of the infusion of collagen. Blood samples were analysed for numbers of free platelets in whole blood, plasma levels of 5-hydroxyindoles (5-HI), including 5-HT, and of TXB_2 and 6K-$PGF_{1\alpha}$.

In a second set of experiments, animals were treated i.v. with saline ($1\,ml\,kg^{-1}$ with a catheter in a jugular vein, $n = 13$), ketanserin $0.63\,mg\,kg^{-1}$ ($n = 13$), aspirin $10\,mg\,kg^{-1}$ ($n = 13$), ridogrel $5\,mg\,kg^{-1}$ ($n = 13$), ketanserin plus aspirin ($n = 13$) or ketanserin plus ridogrel ($n = 13$). Ten minutes later, a suspension of collagen microfibrils ($100\,\mu g\,kg^{-1}$ in 1 min) was infused intra-aortically. Blood was sampled from the catheter in the caval vein before the intravenous administration of the compounds, and again just before the administration of collagen; thereafter, it was appropriately processed for the counting of free platelets in whole blood, the measurements of plasma 5-HI and the determination of plasma prostanoid levels. Blood samples taken 1.5 and 3 min after the start of the collagen infusion were analysed for free platelet number and plasma 5-HI levels (1.5 min samples) and for plasma prostanoid levels (3 min samples) respectively.

For the determination of the number of free platelets in whole blood, 0.5 ml of blood was drawn into a syringe containing 2 ml of buffered formol-citrate, fixed for 30 min at 37°C, centrifuged (10 min at $180\,g$) to a red blood cell-poor supernatant and platelet numbers ($\times 10^3$ cells μl^{-1}) counted under a microscope (De Clerck et al., 1983). Platelet numbers after challenge with collagen are expressed as a percentage of those in blood sampled after the administration of solvent or compounds, just before the administration of collagen (pre-challenge = 100%) in the same animal.

In order to estimate the extent of pharmacological effects on the platelet adhesion/aggregation reaction induced by collagen, the mean percentage reduction of free platelets was calculated from these values (V) for each treatment group [100 − V = % aggregation (A)]. The latter value in solvent-treated animals (AS) was taken to reflect the intensity of the reaction in control conditions. The values obtained in the treatment groups (AT) were expressed as a percentage of that in solvent conditions (AT × 100/AS = AT1) and used to calculate the mean percentage inhibition produced by the compound relative to solvent (100 − AT1 = % inhibition of platelet adhesion/aggregation).

For the determination of 5-HI levels in plasma, 1 ml of blood was collected in a syringe containing 0.125 ml of anticoagulant mixture A and 0.125 ml of anticoagulant mixture B, and centrifuged (10 min at $350\,g$) to platelet-free plasma; the resultant supernatant (0.25 ml) was diluted with EDTA (1×10^{-2} M in NaCl 0.15 M; 0.25 ml) and, after conjugate formation with *o*-phthaldialdehyde, used for the fluorimetric determination of 5-hydroxyindoles ($ng\,ml^{-1}$) in plasma (De Clerck et al., 1983). Plasma 5-HI levels after challenge with collagen are expressed as a percentage of those in samples taken after drug treatment, just before challenge in the same animals (pre-challenge value = 100%). In order to validate that the fluorimetrically assessed changes in plasma 5-HI levels are representative of those in 5-HT levels in the present experimental conditions, plasma samples taken from a separate group of animals ($n = 5$) before, 1.5 min and again 5 min after the administration of collagen were processed additionally for the specific determination of 5-HT, by high performance liquid chromatography (h.p.l.c.) analysis. For the latter purpose, 0.5 ml of platelet-free plasma was mixed with 50 μl of 4 M $HClO_4$ containing hydroxy-indolecarboxylic acid 4×10^{-6} M. After centrifugation (20 min × 10,000 g), 40 μl samples of acidified supernatants were injected directly to the h.p.l.c. apparatus (Varian HPLC model 5060), equipped with a microcomputer-controlled reciprocating single piston pump system; electrochemical detection (BAS model 4B, Bioanalytical Systems) using a glassy carbon electrode was performed as described by Leysen et al. (1988).

For determination of plasma prostanoid levels, 1.65 ml of blood was drawn into a syringe containing EDTA 77 mM (150 μl) and suprofen 1×10^{-4} M (200 μl), centrifuged (5 min at $350\,g$) to platelet-free plasma, and stored at −25°C; plasma levels (pg $100\,\mu l^{-1}$) of TXB_2, 6K-$PGF_{1\alpha}$, PGE_2 were determined on unextracted samples by radioimmunoassay, using specific antisera and tritiated traces (De Clerck et al., 1989b). Plasma prostanoid levels after challenge with collagen are expressed as a percentage of those in samples taken after medication, just before the challenge in the same animals (pre-challenge value = 100%).

Data analysis

Results are expressed as mean ± s.e.mean of the stated number of experiments. Data obtained before and after the administration of compounds or collagen in the same animals were analysed for differences by the Wilcoxon Matched-Pairs Signed-Rank test. Data after collagen administration relative to their pre-challenge values (% of pre-challenge value) in the various treatment groups were compared by the Mann-Whitney U-test for intergroup comparisons. P values equal to or smaller than 0.05 were considered to reflect a statistically significant difference. The relationship between the values of 5-HI, assessed by fluorimetry and those of 5-HT assessed by h.p.l.c. on the same samples was evaluated by calculation of a product-moment correlation coefficient according to Pearson (Siegel, 1956).

Chemicals

Ketanserin for platelet 5-HT_2 receptor blockade (De Clerck et al., 1984a,b), and ridogrel (R 68 070, or (E)-5-[[[(3-pyridinyl)[3-(trifluoromethyl)phenyl]-methylen]amino]oxy] pentanoic acid, Janssen Research Foundation, Belgium) for dual TXA_2 synthetase inhibition/TXA_2-PGH_2 receptor antagonism (De Clerck et al., 1989a,b) were dissolved and diluted in NaCl 0.15 M. Aspirin as lysine acetylsalicylate was obtained commercially as an injectable formulation (Aspégic, Synthélabo, Brussels). Collagen microfibrils (Hormon Chemie, München; suspension in acid medium at $1\,mg\,ml^{-1}$) were dispersed in appropriate volumes of NaCl 0.15 M at 24°C.

Anticoagulant mixture A used for the collection of blood to be analysed for plasma 5-HT/5-HI levels consisted of acid-citrate-dextrose, enriched with PGE_1 1×10^{-5} M (Sigma), adenosine 1×10^{-3} M (Merck), EDTA 1×10^{-3} M (Titriplex III, Merck), imipramine 2×10^{-5} M (De Clerck et al., 1982). Acid-citrate-dextrose solution consisted of trisodium citrate $2H_2O$ 0.085 M, citric acid 0.065 M and dextrose 2% in water. Anticoagulant mixture B consisted of EDTA 0.4 M in NaCl 0.15 M, enriched with sodium metabisulphite 5.3×10^{-5} M as an additional anti-oxidant (Molyneux & Clarke, 1985).

The buffered formol-citrate solution used for the fixation of venous blood prior to the counting of free platelets consisted of 0.47% trisodium citrate $2H_2O$, dissolved in phosphate-buffered saline (1 part of phosphate buffer 0.15 M and 9 parts of NaCl 0.15 M), supplemented with formol 0.1% (De Clerck et al., 1983). For the collection of blood samples to be analysed for plasma prostanoid levels, suprofen 1×10^{-4} M (200 μl), as a cyclo-oxygenase inhibitor (De Clerck et al., 1984a; 1989b) and EDTA 77 mM (150 μl) were used for each 1.65 ml of blood to be anticoagulated (De Clerck et al., 1983). All biochemical reagents were of analytical grade.

Results

Collagen-induced platelet activation

Intra-aortic infusions of collagen microfibrils ($100\,\mu g\,kg^{-1}$ in 1 min) induced an extensive activation of circulating blood

platelets. This was demonstrated by the pronounced, time-dependent reduction of free platelets in whole blood [before challenge: $167.9 \pm 33.2 \times 10^3$ cells μl^{-1}; after collagen (1.5 min): $10 \pm 2.04 \times 10^3$ cells μl^{-1}; $n = 6$] and increase in 5-HI [before challenge: 133.3 ± 14 ng ml^{-1}; after collagen (1.5 min): 475 ± 37.4 ng ml^{-1}; $n = 6$], TXB_2 [before challenge: 62.2 ± 31.5 pg $100 \mu l^{-1}$; after collagen (2.5 min) 1787.3 ± 445.2 pg $100 \mu l^{-1}$; $n = 6$] and 6K-$PGF_{1\alpha}$ [before challenge: 14.7 ± 2 pg $100 \mu l^{-1}$; after collagen (5 min): 419.6 ± 86.4 pg $100 \mu l^{-1}$; $n = 6$] in plasma, prepared from effluent venous blood at various intervals after challenge (Figure 1). Specific measurement of 5-HT by h.p.l.c. in samples obtained before (51 ± 26.5 ng ml^{-1}), 1.5 min (195 ± 75.7 ng ml^{-1}) and 5 min (53.9 ± 25.8 ng ml^{-1}) after the injection of collagen (100 μg kg^{-1}, i.a.) in a separate group of animals ($n = 5$) confirmed the transient increase of the plasma monoamine levels after challenge, as suggested by the fluorimetrically detected changes in plasma 5-HI (before: 230.2 ± 60.4 ng ml^{-1}); 1.5 min after collagen: 409 ± 122.7 ng ml^{-1}; 5 min after collagen: 239 ± 60.8 ng ml^{-1}). Calculation of the product-moment correlation coefficient between the amounts of genuine 5-HT assessed by h.p.l.c. and those of all 5-HI assessed by fluorometry revealed an acceptable correlation (Pearson correlation coefficient = 0.945) between the results of the two methods. Although not indicative of absolute values of 5-HT specifically (Vatassery *et al.*, 1981; Curzon *et al.*, 1981), the fluorimetric assay of plasma 5-HI thus can be used effectively to monitor changes in collagen-induced release of platelet intragranular products *in vivo*.

Pharmacological analysis

Ketanserin (0.63 mg kg^{-1} i.v., 10 min before the challenge) slightly (11.3% inhibition, $P < 0.05$ versus solvent) attenuated the peak reduction of the free platelet number, measured 1.5 min after the start of the collagen infusion, relative to that in solvent-treated animals. Peak increases of plasma 5-HI levels, as quantified in control conditions, were not significantly reduced by such a pharmacological intervention. Collagen-induced changes of plasma TXB_2, 6K-$PGF_{1\alpha}$, and PGE_2 at 3 min after the onset of the challenge were also not modified by ketanserin (Figure 2; Table 1).

Aspirin (10 mg kg^{-1} i.v., 10 min) markedly reduced the collagen-induced changes in plasma TXB_2, 6K-$PGF_{1\alpha}$, PGE_2 and 5-HI, but only slightly (16.3% inhibition; $P < 0.05$ versus solvent) inhibited the reduction of free platelets in whole blood (Figure 2; Table 1).

Ridogrel (5 mg kg^{-1} i.v., 10 min) markedly reduced the collagen-induced drop in free platelet number in whole blood (47.1% inhibition; $P < 0.05$ versus solvent) as well as the increase of the plasma 5-HI levels. Challenge-induced increases of TXB_2 in venous plasma were virtually eliminated; simultaneously, plasma levels of 6K-$PGF_{1\alpha}$ and PGE_2 increased (Figure 2; Table 1).

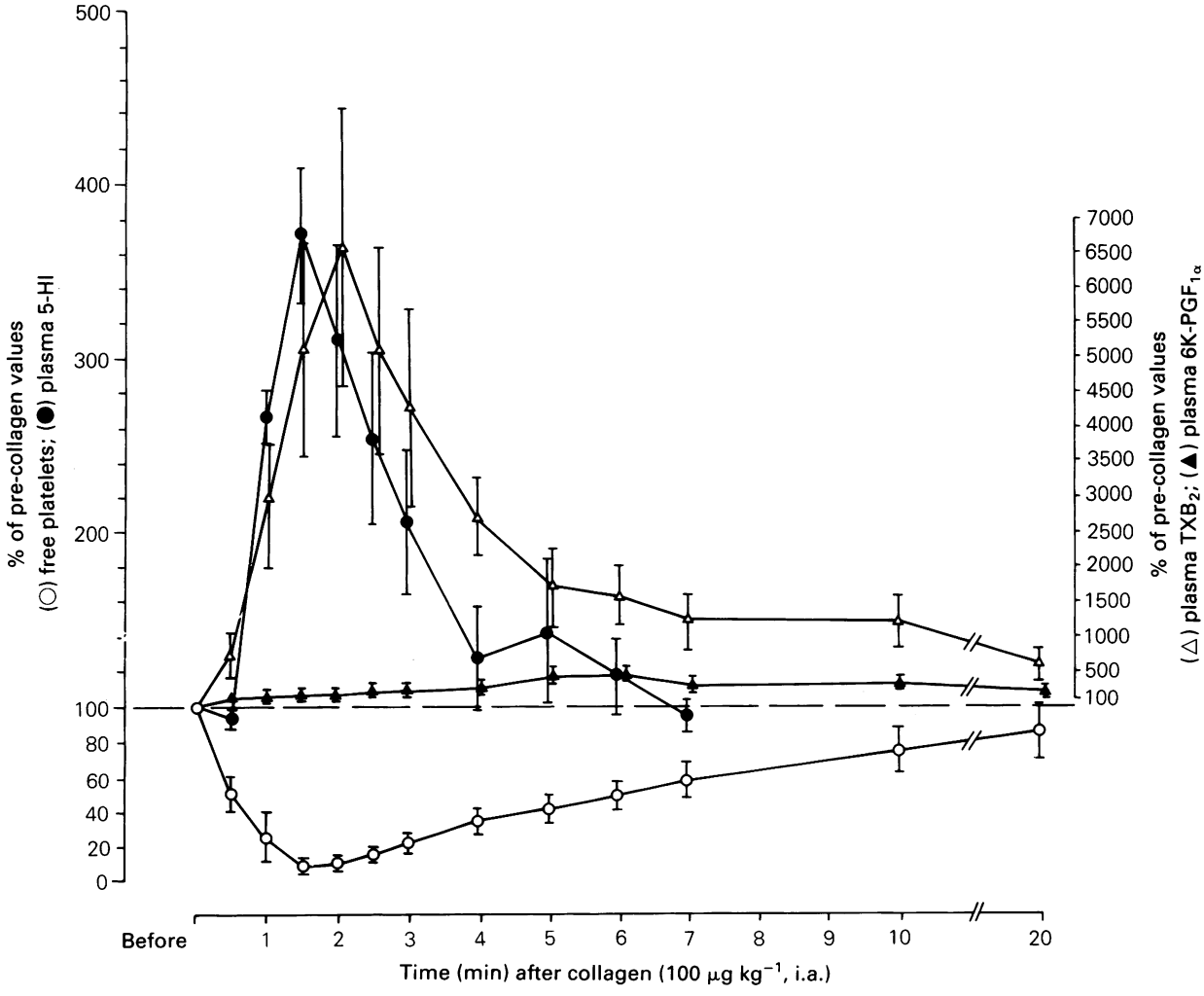


Figure 1 Platelet activation by collagen, administered into the aorta in cats. Blood sampled from the inferior caval vein at the indicated intervals after the onset of the infusion of collagen microfibrils (100 μg kg^{-1} in 1 min). Time-dependent reduction of free platelets in whole blood (○) and increase in plasma levels of 5-hydroxyindoles (5-HI, ●), thromboxane B_2 (TXB_2, △), 6K-$PGF_{1\alpha}$, (▲), indicative of extensive platelet activation by collagen. Mean values are shown with s.e.mean indicated by vertical bars, $n = 6$. $P < 0.05$ versus pre-challenge up to 6 min for free platelets, plasma TXB_2 and 6K-$PGF_{1\alpha}$, up to 3 min for plasma 5-HI.

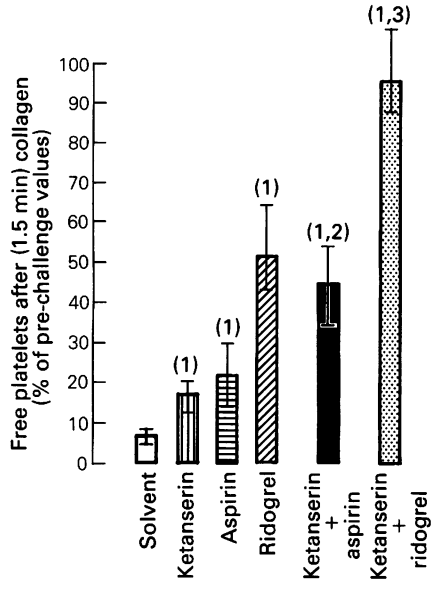


Figure 2 Pharmacological modification of the collagen-induced reduction of free platelets in whole blood in cats *in vivo*. Blood sampled from the inferior caval vein before and after (1.5 min) infusion of collagen microfibrils (100 $\mu g\,kg^{-1}$ in 1 min) into the lower aorta. Number of free platelets in whole blood after collagen expressed as a percentage of that before challenge (range 125 to 166.9 $\times 10^3$ cells μl^{-1}, $n = 78$) in animals treated (10 min i.v. before challenge) with solvent (open column; $n = 13$), ketanserin (vertically lined column; 0.63 $mg\,kg^{-1}$; $n = 13$), aspirin (horizontally lined column; 10 $mg\,kg^{-1}$; $n = 13$), ridogrel (hatched column; 5 $mg\,kg^{-1}$; $n = 13$), ketanserin plus aspirin (solid column; $n = 13$), ketanserin plus ridogrel (stippled column; $n = 13$). Results are mean with s.e.mean shown by vertical bars. (1) $P < 0.05$ versus solvent; (2) $P < 0.05$ versus ketanserin or aspirin alone; (3) $P < 0.05$ versus ketanserin or ridogrel alone.

Combined treatment with ketanserin and aspirin before challenge with collagen substantially reduced the drop of free platelets in whole blood (39.9% inhibition; $P < 0.05$ versus solvent); the inhibition of collagen-induced platelet adhesion/aggregation was significantly greater than that obtained with either ketanserin or aspirin singly but of a similar magnitude to that obtained with ridogrel alone. The effects of such a combined ketanserin/aspirin administration on changes of plasma prostanoid and 5-HI levels were comparable to those obtained with aspirin alone (Figure 2; Table 1).

Combined treatment with ketanserin and ridogrel before challenge almost completely eliminated the drop of free platelets in whole blood (94% inhibition; $P < 0.05$ versus solvent) and the increase of plasma 5-HI levels. Inhibition obtained with these combined agonists was significantly greater than that provided by the single drugs and by the combination of ketanserin with aspirin. Changes in plasma prostanoid levels after such a combined pharmacological manipulation were comparable to those obtained with ridogrel alone (Figure 2; Table 1).

Free platelet number in whole blood, plasma levels of prostanoids and 5-HI before and 10 min after the administration of the drugs, prior to challenge with collagen, were not significantly different.

Discussion

Collagen microfibrils, administered intra-aortically into cats, produce an extensive platelet activation. This is shown by the time-dependent reduction of free platelets in whole blood and the concomitant increases of 5-HI, 5-HT and TXB_2 levels in plasma, prepared from venous blood draining from the challenged vascular bed. These data are in agreement with earlier observations in other species, e.g. rats and guinea-pigs, reacting following intravenously administered collagen with transient thrombocytopenia and increases of plasma prostanoid levels (Vargaftig *et al.*, 1979; MacGuire & Wallis, 1983).

The elevation of plasma TXB_2 after the challenge is compatible with the concept that collagen activates platelet phospholipase A_2, liberating phospholipid-bound arachidonic acid for its subsequent enzymatic conversion into prostaglandin endoperoxides and TXA_2 (Vermylen *et al.*, 1983; De Clerck & de Chaffoy de Courcelles, 1989); the comparatively modest increase in plasma 6K-$PGF_{1\alpha}$ levels occurring simultaneously then may be due to the taking up by prostacyclin synthetase in monocytes and endothelial cells (Marcus *et al.*, 1980; De Freyn *et al.*, 1982) of prostaglandin endoperoxides, produced by activated platelets in excess of their capacity for endogenous TXA_2 synthesis.

The increase of plasma 5-HI levels observed are compatible with the capacity of collagen to induce release of intragranular platelet products, including 5-HT, ATP and ADP (Vermylen *et al.*, 1983; De Clerck & de Chaffoy de Courcelles, 1989). The extent of the peak reduction of free platelets and of the peak increases of both 5-HI and TXB_2 in plasma suggests that the collagen-induced changes are extensive ones, overwhelming the normal scavenging capacity of platelets, endothelial cells and other blood components for free 5-HT and the metabolic

Table 1 Changes in haematological values induced by collagen in cats *in vivo*

	Parameter[1]				
Treatment	*Free platelets*	*5-HI*	*TXB_2*	*6K-$PGF_{1\alpha}$*	*PGE_2*
Solvent ($n = 13$)	6.9 ± 2.2	202 ± 28.4	1996 ± 400	352 ± 63	101 ± 1
Ketanserin (0.63 $mg\,kg^{-1}$, i.v., $n = 13$)	17.4 ± 4.6[2]	147.6 ± 15.3	2246 ± 491	351 ± 78	160 ± 60
Aspirin (10 $mg\,kg^{-1}$, i.v., $n = 13$)	22.3 ± 8.47[2]	121.6 ± 14.5[2]	380 ± 21.3[2]	124 ± 9[2]	87 ± 9[2]
Ridogrel (5 $mg\,kg^{-1}$ i.v., $n = 13$)	50.7 ± 7.9[2]	127.8 ± 18.4[2]	125 ± 10[2]	2141 ± 434[2]	590 ± 134[2]
Ketanserin + aspirin ($n = 13$)	44 ± 10[2]	125 ± 10.5[2]	149 ± 18[2]	94 ± 10[2]	88 ± 8[2]
Ketanserin + ridogrel ($n = 13$)	94.4 ± 8.2[2]	92.8 ± 4.8[2]	172 ± 30[2]	1694 ± 409[2]	557 ± 151[2]

[1] Values of free platelets ($\times 10^3$ per μl whole blood at 1.5 min), plasma 5-HI ($ng\,ml^{-1}$ in 1.5 min), plasma TXB_2, 6K-$PGF_{1\alpha}$, PGE_2 (pg 100 μl^{-1} plasma at 3 min) after collagen (10 $\mu g\,kg^{-1}$, i.a.), expressed as a percentage of the pre-challenge ones. Mean ± s.e.mean. Plasma PGE_2 before challenge: 15.8 ± 0.2 to 27 ± 18.1 pg 100 μl^{-1}.

[2] $P < 0.05$ versus solvent.

degradation or circulatory wash-out of TXB_2 (De Clerck *et al.*, 1984a). This interpretation is reinforced by a comparison with earlier feline experiments (De Clerck *et al.*, 1986a) in which discrete platelet activation over a static thrombus in the aorta produced sufficient platelet 5-HT release to compromise tissue perfusion, but without measurable changes in the same haematological parameters as quantified in the present study.

The pronounced reduction of free platelets in whole blood occurring after the challenge with collagen in the present model could result from the combination of at least two mechanisms. First, adhesion of platelets to the collagen microfibrils via glycoprotein-dependent processes (Nurden, 1987) may initially reduce free platelet numbers and induce platelet arachidonic acid metabolism and extracellular release of intragranular products (MacIntyre, 1976; De Clerck & de Chaffoy de Courcelles, 1989). Secondly, platelet aggregation subsequent to a further activation by released endogenous mediators such as ADP, 5-HT, prostaglandin endoperoxides and TXA_2 may propagate the platelet response further into an almost complete reduction of free circulating platelets. Indeed, in cats, ADP (Tschopp, 1970) as well as 5-HT, acting on $5\text{-}HT_2$ receptors (De Clerck *et al.*, 1984b) and prostaglandin endoperoxides or TXA_2 (Meyers *et al.*, 1979) are potent inducers of the platelet aggregation/release reaction; *in vitro*, the latter two mediators contribute equally to the release-dependent platelet aggregation wave elicited by collagen in feline platelet-rich plasma (De Clerck *et al.*, 1984b).

The pharmacological analysis we applied with ketanserin, aspirin, ridogrel and the combinations ketanserin/aspirin or ketanserin/ridogrel points to differential contributions of 5-HT and of prostanoids to the platelet functional response to collagen in cats *in vivo*. Ketanserin is a potent $5\text{-}HT_2$ receptor blocker *in vitro*, so that it inhibits in low concentrations ($IC_{50} = 1.15 \times 10^{-8}$ M; >90% inhibition at 1×10^{-7} M) feline platelet aggregation in citrated platelet-rich plasma induced by 5-HT (5×10^{-6} M); moreover, it curtails the release reaction and subsequent cellular aggregation in response to collagen, without affecting the enzymatic conversion of arachidonic acid into eicosanoids (De Clerck *et al.*, 1984b). In the present experimental conditions, ketanserin produces a biologically modest reduction of the collagen-induced platelet adhesion/aggregation reaction *in vivo*. This observation contrasts to the strong inhibition by ketanserin of the platelet aggregation, induced by discrete endothelial cell injury and resulting in cyclic reductions of the blood flow in stenosed canine coronary arteries (Bush *et al.*, 1984; Golino *et al.*, 1988b); however, it matches its modest action against platelet aggregation, triggered by extensively damaged arteries and reinforced by rt-PA fibrinolysis (Golino *et al.*, 1988a). It suggests that 5-HT, alone, is not the major mediator responsible for the platelet adhesion/aggregation reaction in conditions of extensive activation.

Aspirin, a cyclo-oxygenase inhibitor reducing the formation of arachidonic acid-derived prostaglandin endoperoxides and TXA_2 (Smith & Willis, 1971; Pedersen & FitzGerald, 1985), can attenuate platelet aggregation and release of intragranular products, provided the reaction is elicited by comparatively low concentrations of collagen (Vargaftig *et al.*, 1981). In our experiments, aspirin profoundly attenuated the increases of plasma TXB_2, 5-HI, and also $6K\text{-}PGF_{1\alpha}$ in response to collagen, but only partly inhibited the thrombocytopenia. This suggests that mechanisms operating independently from prostaglandin endoperoxides and TXA_2 are also involved in the platelet adhesion/aggregation reaction to collagen.

Ridogrel combines specific TXA_2 synthetase inhibition with TXA_2-prostaglandin endoperoxide receptor antagonism (De Clerck *et al.*, 1989a,b). This dual action results in attenuation of the agonist action of prostaglandin endoperoxides and TXA_2 as well as in an increase of the amounts of the inhibitory PGD_2, PGE_2 and PGI_2 formed and yields an anti-platelet activity beyond the potential of aspirin, dazoxiben, sulotroban or ketanserin alone for cyclo-oxygenase inhibition, TXA_2 synthetase inhibition, TXA_2-prostaglandin endoperoxide receptor antagonism, or $5\text{-}HT_2$ receptor blockade respectively in experimental animals (De Clerck *et al.*, 1986b, 1989b; Golino *et al.*, 1988b) and in man (Hoet *et al.*, 1989). In cats challenged with collagen *in vivo*, ridogrel attenuates the increase of plasma TXB_2 and 5-HI, enhances that of PGE_2 and $6K\text{-}PGE_{1\alpha}$ and markedly reduces the platelet adhesion/aggregation response. In the latter aspect, its effect is significantly greater than that of ketanserin or aspirin, demonstrating the superior anti-platelet effect of TXA_2 synthetase inhibition added to TXA_2-prostaglandin endoperoxide receptor antagonism in comparison with $5\text{-}HT_2$ receptor blockade or with cyclo-oxygenase inhibition alone.

In contrast to single pharmacological interventions, combinations of ketanserin with aspirin or with ridogrel markedly reduces or virtually eliminates, respectively, the platelet adhesion/aggregation reaction in response to collagen. Our present data thus strengthen the concept (De Clerck *et al.*, 1986b; Golino *et al.*, 1988a) that the interplay between 5-HT and arachidonic acid metabolites, rather than the effects of these autocoids separately, determines the magnitude of the platelet functional response to extensive stimulation. This may have implications for future anti-platelet therapy in patients.

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The interaction of *para*fluorohexahydrosiladiphenidol at muscarinic receptors *in vitro*

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1 The antagonistic actions of *para*fluorohexahydrosiladiphenidol (pFHHSiD) at muscarinic receptors has been studied in cardiac muscle, smooth muscle and cell culture preparations. In this paper, the classification scheme of Doods *et al.* (1987) is employed. This scheme is based upon differential affinities of muscarinic antagonists. pFHHSiD exhibited high pA_2 values at M_3 receptors mediating contractions of guinea-pig ileum and oesophageal muscularis mucosae (7.8 and 8.2 respectively) whereas low values were determined at M_2 receptors mediating negative inotropic responses in guinea-pig atria (6.0). Intermediate pA_2 values were determined at M_1 receptors mediating contractions of the canine femoral and saphenous veins.

2 The pA_2 values of pFHHSiD at receptors mediating endothelial-dependent relaxation of rat aortic rings, rabbit jugular vein and canine femoral artery (7.6–7.9) were similar to those determined on the ileum. However, the pA_2 values of pFHHSiD at receptors mediating contractions of the guinea-pig trachea (7.1), which has been previously shown to possess M_3 receptors, were different from those determined in the ileum.

3 The similarity in pA_2 values of pFHHSiD between the M_3 receptors in guinea-pig ileum and the receptors mediating endothelial-dependent relaxations provide further evidence for the role of M_3 receptors in this vascular response. Taken together, pA_2 values for pFHHSiD range from 7.1 to 8.2, depending upon the M_3 preparation used. The selectivity of the compound therefore for the M_3 versus the M_2 muscarinic receptor ranged from 13 to 163 fold.

4 At muscarinic receptors mediating stimulation of phosphatidylinositol hydrolysis, pFHHSiD paradoxically displayed a high affinity for the M_1 receptor in the SH-SY5Y cell line ($pA_2 = 7.9$) as well as for the M_3 receptor in the human astrocytoma (1321 N1 cell line ($pA_2 = 7.6$). The value at the M_1 receptor in SH-SY5Y cells was greater than was observed at M_1 receptors mediating contractions of both the canine saphenous and femoral veins (7.1).

5 pFHHSiD, therefore, clearly delineated M_3 from M_2 muscarinic receptors, whilst the separation between M_1 and M_3 receptors was variable. The reason for the anomalous affinity estimates in some functional studies remains unclear. These data indicate that the pA_2 values for pFHHSiD appear to be tissue-dependent since the M_3 selectivity varies according to the preparations studied. As a result the utility of pFHHSiD in muscarinic receptor classification is limited.

Introduction

Genomic cloning studies (see Barnard, 1988 for review) have shown that heterogeneity of muscarinic receptors is due to the expression of a number of distinct, but related gene products (Peralta *et al.*, 1987; Bonner *et al.*, 1987). These have been denoted as m_1, m_2, m_3, m_4 and m_5 (Bonner *et al.*, 1987). Each gene product, on the basis of *in situ* mRNA hybridisation studies, exhibits a unique and specific distribution within the CNS (Buckley *et al.*, 1987) although equivalent studies in the periphery have yet to be reported. The nomenclature of these gene products differs between laboratories, i.e. the HM1, HM2, HM4 and HM3 human gene products identified by Peralta *et al.* (1987) correspond to the m_1, m_2, m_3 and m_4 genes identified by Bonner *et al.* (1987). In the present paper the nomenclature of Bonner *et al.* (1987) is used for purposes of clarity.

Muscarinic receptors have been divided on a pharmacological basis into three major subtypes, which have been denoted as M_1, M_2 and M_3 (Doods *et al.*, 1987; Hammer *et al.*, 1987) and the evidence for each subtype has been reviewed elsewhere (Eglen & Whiting, 1986; Mitchelson, 1988). The M_1 and M_2 subtypes most closely correspond to the m_1 and m_2 gene products (Peralta *et al.*, 1987), whilst the function of the m_3 and m_4 gene products remains unclear. Numa and his group, cited in a review by Barnard (1988), have suggested that the m_3 gene product most closely resembles the M_3 receptor. Buckley *et al.* (1989) have also shown that the radioligand binding profile of the m_3 gene product expressed in CHO-K1 cells was similar to M_3 receptors. Peralta *et al.* (1987) have shown that NG108-15 cells express m_4 receptor mRNA. In addition, Michel *et al.* (1989) have shown that the radioligand binding profile of the receptor present in NG108-15 and PC-12 cells was similar to that shown by Buckley *et al.* (1989) at m_4 receptors expressed in CHO-K1 cells.

M_1 receptors are most sensitive to the antagonists, pirenzepine and *o*-methoxy-silahexocyclium, whilst M_2 receptors are sensitive to methoctramine, AF-DX 116 and himbacine (Michel & Whiting, 1988; Melchiorre *et al.*, 1986; Eglen *et al.*, 1988; see Mitchelson, 1988 for review). Until recently, no antagonist has been available which exhibits a high affinity for the M_3 receptor only, and a low affinity for both M_1 and M_2 receptors. 4 Diphenyl acetoxy-N-methyl piperidine methiodide (4-DAMP; Barlow *et al.*, 1980), whilst differentiating M_3 receptors in smooth muscle from M_2 receptors in the atria, does not clearly distinguish M_1 receptors in the superior cervical ganglion from M_3 receptors in smooth muscle (Brown *et al.*, 1980). Similar conclusions may be drawn with regard to other putative M_3 antagonists such as silabenzhexol or hexahydrosiladiphenidol (see Mitchelson, 1988 for review). However, *para*fluorohexahydrosiladiphenidol (pFHHSiD) has recently been shown (Lambrecht *et al.*, 1988) to be a relatively selective M_3 receptor antagonist since it exhibits pA_2 values of 7.8, 6.7 and 6.1 at M_3, M_1 and M_2 receptors, respectively. The activity of pFHHSiD at expressed gene products has yet to be reported.

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We have undertaken a further analysis of this compound using functional techniques in a range of isolated tissues. In view of the relatively large M_3 vs M_2 receptor selectivity of the compound, in comparison to 4-DAMP, it has been used to characterize further a number of isolated tissues which have previously exhibited anomalous pA_2 values with respect to the current pharmacological classification (Doods *et al.*, 1987; Hammer *et al.*, 1987) i.e. the oesophageal muscularis mucosae, urinary bladder and the vascular endothelium.

A preliminary account of this work has been communicated to the British Pharmacological Society (Eglen *et al.*, 1989).

Methods

The muscarinic receptor subtypes present in the preparations used in this paper are summarised in Table 1.

Isolated tissue studies

Guinea-pigs (male, Dunkin-Hartley, 300–350 g) and rats (male, Sprague-Dawley, 200–250 g) were killed by CO_2 asphyxiation. Guinea-pig proximal ileum, oesophageal muscularis mucosae (OMM) and urinary bladder were isolated and placed in Tyrode solution (pH 7.4, 34°C). Guinea-pig left atria, trachea and rat aortic rings (2 mm) were isolated and placed in Krebs physiological salt solution (pH 7.4, 37°C). Rings (2 mm) of canine saphenous vein, femoral vein and artery, rabbit (male, New Zealand white, 2.5–3 kg) jugular vein were isolated from animals previously anaesthetized with sodium pentobarbitone (60 mg kg^{-1}, i.p.) and also placed in Krebs solution (pH 7.4, 37°C). The composition of the Tyrode and Krebs solution is given below.

The detailed methods for each preparation have been described previously i.e. ileum (Clague *et al.*, 1985), OMM (Kamikawa *et al.*, 1985), urinary bladder (Eglen & Whiting, 1987), left atria (Blinks, 1966) and trachea (Eglen & Whiting, 1988). Rings of femoral and saphenous veins, thoracic aorta or jugular vein were prepared according to the method of O'Rourke & VanHoutte (1987). The endothelium of the vascular rings was left intact, as judged by a relaxant response (to 1 μM acetylcholine) of a ring precontracted with 0.1 μM phenylephrine. Preliminary experiments showed that the canine femoral and saphenous veins exhibited only contractile responses to muscarinic agonists, irrespective of the presence or absence of an intact endothelium. The finding that removal of the endothelium in these tissues did not enhance the contracture to muscarinic agonists is in agreement with the report by Rubanyi & Vanhoutte (1988).

The smooth muscles and vascular rings were suspended under 1.0 g tension, whilst the left atria was suspended under 0.5 g tension. This latter tissue was electrically paced for the duration of the experiment, using parallel platinum electrodes (5 Hz, threshold voltage + 20%, 5 ms duration) after the method of Blinks (1966).

All preparations were allowed 60 min to equilibrate, during which time the bathing solution was replaced every 15 min. Concentration-response curves were then constructed, with carbachol as the agonist, in either a cumulative (OMM, urinary bladder, atria and vascular tissues; van Rossum 1963) or non-cumulative (ileum; 30 s exposure on a 5 min dose cycle; Clague *et al.*, 1985) manner. Endothelial-dependent relaxations of the femoral artery, jugular vein and aorta were determined in tissues precontracted with phenylephrine (0.1 μM) in all experiments except those using methoctramine. The α_1-adrenoceptor antagonist activity of this antagonist (Melchiorre *et al.*, 1986) precluded the use of phenylephrine. U46619 (0.1 μM) was used instead to raise the tension in the tissue (Dainty *et al.*, 1988).

All responses were determined as changes in isometric tension (mg), measured with either a Grass FT03 force transducer and displayed on a Grass Polygraph or a Hugo Sachs K30 transducer connected to a Graphtec-Watanabe linearecorder (WR3101).

After construction of the initial concentration-response curve the tissues were allowed 60 min to equilibrate at each antagonist concentration, during which time the bathing solution was replaced every 15 min. A second concentration-response curve was then constructed in the presence of the antagonist. Each tissue was exposed to only one concentration of antagonist.

The concentration-ratios calculated at each of 6 concentrations (0.1, 1, 3, 10, 30 and 100 μM) of pFHHSiD were determined using EC_{50} (concentration of agonist which elicited 50% of the maximal response) values in the absence and presence of the antagonist. The EC_{50} values were calculated by fitting the data to a non-linear iterative curve fitting procedure (Parker & Waud, 1971) using RS1 software (BBN Software Products Corp., Cambridge, MA, U.S.A.). The dissociation constants of the antagonists were estimated by determination of the pA_2 value, according to the method of Arunlakshana & Schild (1959) by determining the intercept of the resulting straight line with the abscissae and these parameters. In order to obtain an accurate estimation of the dissociation constant, the Schild slopes were determined by linear regression using least squares analysis. When the slopes were not significantly different from unity, they were constrained to unity (Kenakin, 1987).

Phosphatidylinositol hydrolysis studies

Human astrocytoma cells (1321 N1) were cultured and phosphatidylinositol hydrolysis studies were conducted as described previously (Kunysz *et al.*, 1989). Human SH-SY5Y cells were cultured at 37°C in a humidified atmosphere as a monolayer culture in a medium consisting of 45% Hams F12, 45% Dulbecco's modified Eagle's medium (DMEM) and 10% foetal calf serum. Cultures were passaged at a ratio of 1:4 and grown until confluent (usually 5–7 days). After 3 and 5 days of culture the media was replaced with fresh medium. Cells were harvested by incubation with 0.05% EDTA in phosphate buffered saline for 1 min at 37°C.

Phosphatidylinositol hydrolysis was studied in the SH-SY5Y cell line after the method of Kunysz *et al.* (1989) as follows: the cells were harvested as described above and incubated for 12 h, at a density of 5×10^6 cells ml^{-1}, in a medium consisting of Hams F10 and 0.1% BSA and 1 μCi ml^{-1} [^{3}H]-myo-inositol. After washing the cells twice with buffer to remove excess [^{3}H]-myo-inositol, the cells were resuspended in 0.75 ml of oxygenated modified Krebs-Bicarbonate buffer (see below for composition). The antagonist was added (100 μl) and, after a 45 min equilibration period, carbachol (50 μl) was added to the cells and allowed to equilibrate for 45 min at 37°C. The reaction was terminated by the addition of 3 ml of chloroform/methanol (1:2 vol:vol) followed by the addition of 1 ml of chloroform and 1 ml of water. The mixture was shaken for 20 min prior to determination of total inositol phosphate accumulation using a procedure based upon that of Minneman & Johnson (1984). Briefly, 2 ml of the aqueous layer were applied to Dowex columns (200–400 mesh in the formate form) and following two 10 ml washes with 5 mM myo-inositol, the inositol phosphates were eluted with 2 ml of 1 M ammonium formate in 0.1 N formic acid. Of this eluate 1.8 ml was transferred to a scintillation vial containing 10 ml Aquasol and counted by liquid scintillation spectrophotometry (Packard Tri Carb). The pA_2 values were calculated as described above for the isolated tissue preparations.

Statistics

All significant differences were assessed by the use of Student's t test, with a value of $P < 0.05$ being considered significant. All values are expressed as mean ± s.e.mean, $n = 6$–12.

Physiological salt solutions

The solutions used were as follows: Krebs solution (mM): NaCl 118.4, KCl 4.9, $MgSO_4 \cdot 7H_2O$, 1.2, KH_2PO_4 1.2, glucose 11.1, $NaHCO_3$ 25.0 and $CaCl_2 \cdot 6H_2O$ 2.5; Krebs-Bicarbonate buffer (mM): NaCl 118, $NaHCO_3$ 25, KCl 4.7, KH_2PO_4 1.2, $CaCl_2 \cdot 6H_2O$ 1.3, $MgSO_4$:$(H_2O)_7$ 1.1, glucose 11 and LiCl 10; Tyrode solution (mM): NaCl 136.9, KCl 2.7, $MgCl_2 \cdot 6H_2O$ 1.1, $NaH_2PO_4 \cdot 2H_2O$ 0.4, glucose 3.6, $NaHCO_3$ 11.9 and $CaCl_2 \cdot 6H_2O$ 1.8.

All physiological salt solutions were gassed with 5% CO_2/95% O_2, and maintained at pH 7.4.

Drugs used

[^{3}H]-myo-inositol (specific activity 15 Ci mmol^{-1}) was obtained from American Radiolabeled Chemicals Inc. *Para*-fluorohexahydrosiladiphenidol (pFHHSiD) and methoctramine were synthesized by Dr R. Clark, IOC, Syntex, Palo Alto, California, U.S.A. Pirenzepine was donated by Boots PLC. The remaining drugs were obtained from Sigma Chemical Co. Ltd.

Results

Isolated tissue studies

Carbachol, with the exception of the urinary bladder ($-\log EC_{50} = 6.1 \pm 0.08$), elicited concentration-dependent contractile responses in all smooth muscle preparations with $-\log EC_{50}$ values ranging between 6.8–7.0. These values were in good agreement with previous data from our laboratory (Eglen *et al.*, 1988). Similar $-\log EC_{50}$ values were determined at receptors mediating endothelial-derived relaxing factor (EDRF)-dependent relaxations of the vascular tissue (6.7–7.0).

The muscarinic receptors present in each tissue studied are summarised in Table 1. However, the canine femoral artery and vein have yet to be classified with regard to the muscarinic receptor subtype present. At receptors mediating contractions of the femoral vein, the pA_2 values (with Schild slopes shown in parentheses), for pirenzepine and methoctramine were 7.86 ± 0.16 (0.98 ± 0.11) and 6.07 ± 0.17 (1.17 ± 0.15), respectively. These values are consistent with responses being mediated through an M_1 receptor (Eglen & Whiting, 1986). The pA_2 values at receptors mediating relaxations of the femoral artery for pirenzepine and methoctramine were 6.26 ± 0.11 (1.07 ± 0.10), and 6.05 ± 0.09 (1.01 ± 0.07), respectively. The Schild slopes in these studies were not significantly different from unity. The concentrations of pirenzepine used were 0.03, 0.1, 1 and 3 μM whilst the concentrations of methoctramine used were 1, 3 and 10 μM. Concentrations of methoctramine above 10 μM were not used due to possible allosteric effects (Eglen *et al.*, 1988).

pFHHSiD (1 nM–10 μM) did not elicit responses itself in any of the preparations. Parallel dextral shifts, which were not accompanied by any diminution in the maxima of the concentration-response curves to carbachol were observed in all preparations studied, with the exception of the urinary bladder. In the latter tissue, the concentration-response curves were shifted dextrally in a non-parallel fashion and the maxima were also depressed by pFHHSiD in a concentration-dependent manner. The pA_2 values and slopes of the Schild regressions are shown in Table 2. The slopes were not significantly different from unity in the majority of tissues with the exception of those determined from experiments in the urinary bladder and relaxations of the femoral artery. As a result, the Schild slopes were not constrained to unity in these tissues.

The pA_2 values for pFHHSiD at muscarinic receptors mediating relaxations of the canine femoral artery, rat aorta and jugular vein were not significantly ($P > 0.05$) different either from each other or from pA_2 values determined at receptors mediating contractions of the guinea-pig ileum. However, the pA_2 value for pFHHSiD in the OMM was significantly ($P < 0.05$) greater than those obtained in the tissues listed. Conversely, the pA_2 values at receptors mediating contractions of the canine femoral and saphenous veins, guinea-pig trachea, urinary bladder and negative inotropic responses were significantly ($P < 0.05$) lower than those in the ileum. The lowest pA_2 value was obtained at receptors in the left atria.

When the pK_B values (obtained after imposing the unity constraint on the Schild plot) were considered, good agreement was observed between the pK_B values for receptors present in the ileum, OMM, aorta and jugular vein. The pK_B values at M_1 receptors in the femoral and saphenous veins (7.22 and 6.87) were lower than those observed at M_3 receptors, whilst the lowest pK_B value was that observed at atrial M_2 receptors. The pK_B value at receptors mediating contractions of the trachea (7.08) was lower than those observed at M_3 receptors mediating ileal contractions.

In terms of selectivity, therefore, pFHHSiD was 70 fold more selective for M_3 receptors mediating ileal contractions than for M_2 receptors mediating negative inotropic responses, and 6 fold more selective than for M_1 receptors mediating contractions of the saphenous vein. However, when all the functional M_3 pA_2 values for the isolated tissues are considered (in which the pA_2 values range from 7.1 (trachea) to

Table 1 Muscarinic receptor subtypes mediating responses in various bioassays

Bioassay	*Muscarinic receptor subtype*	*Reference*
Canine saphenous vein	M_1	O'Rourke & VanHoutte (1987)
Guinea-pig left atria	M_2	Clague *et al.* (1985)
Guinea-pig ileum	M_3	Clague *et al.* (1985)
Guinea-pig OMM	M_3	Eglen & Whiting (1988)
Guinea-pig trachea	M_3	Eglen & Whiting (1988)
Guinea-pig urinary bladder	M_3	Eglen & Whiting (1987)
Rat aorta	M_3	Eglen *et al.* (1988)
Rabbit jugular vein	?	
Canine femoral vein	?	Rubanyi & Vanhoutte (1987)
Canine femoral artery	?	Rubanyi & Vanhoutte (1987)
Human astrocytoma (1321 N1) cells	M_3	Kunsyz *et al.* (1989)
Human SH-SY5Y cells	M_1	Sera *et al.* (1988)

OMM – Oesophageal muscularis mucosae.
? – Muscarinic receptor subtype present remains to be classified.
The classification scheme employed in this table is after that described by Doods *et al.* (1987). This scheme is based upon differential antagonist affinities, estimated in pharmacological studies.

Table 2 pA_2 values and Schild plot slopes for pFHHSiD at muscarinic receptors in various isolated preparations

Species	*Preparation*	*pA_2*	*Schild slope*
Canine	Femoral vein	7.11 ± 0.11 (7.22)	1.08 ± 0.07
Canine	Femoral artery	7.90 ± 0.14	0.75 ± 0.04*
Canine	Saphenous vein	7.08 ± 0.07 (6.87)	0.92 ± 0.08
Guinea-pig	Left atria	6.01 ± 0.05 (6.17)	1.02 ± 0.10
Guinea-pig	Ileum	7.85 ± 0.08 (7.87)	1.10 ± 0.12
Guinea-pig	OMM	8.22 ± 0.03 (8.06)	0.97 ± 0.04
Guinea-pig	Trachea	7.13 ± 0.11 (7.08)	0.94 ± 0.10
Guinea-pig	Bladder	7.59 ± 0.10	0.74 ± 0.08*
Rat	Aorta	7.67 ± 0.10 (7.71)	1.02 ± 0.16
Rabbit	Jugular vein	7.89 ± 0.13 (7.66)	0.86 ± 0.11
Human	Astrocytoma (1321 N1) cells	7.64 ± 0.10 (7.73)	1.13 ± 0.06
Human	SH-SY5Y cells	7.90 ± 0.10 (7.83)	0.96 ± 0.04

OMM – Oesophageal muscularis mucosae.
Values are mean ± s.e.mean, n = 6–10.
* Significantly ($P < 0.05$) different from unity. The values in parentheses are those calculated after imposing the unity constraint (Kenakin, 1987).

8.2 (OMM)) the selectivity for M_3 versus M_2 receptors ranged from 13 to 163 fold, respectively.

Cell culture studies

In both the astrocytoma (1321 NI) and SH-SY5Y cells, pFHHSiD (at concentrations of 0.1, 0.3, 1 and 3 μM) produced dextral shifts in the carbachol concentration-response curve with no suppression of the maximal response. The pA_2 value in 1321 NI cells was similar to those values observed at M_3 receptors in the isolated smooth muscle tissues (Table 2). The pA_2 values for pirenzepine and methoctramine at muscarinic receptors in this cell line have been previously reported (Kunysz *et al.*, 1989) to be 7.3 and 6.0, respectively.

In SH-SY5Y cells, the pA_2 value for pFHHSiD was also similar to values obtained at M_3 receptors in the smooth muscle tissues (Table 2). In order to characterize the receptor mediating this response further, the pA_2 values for pirenzepine and methoctramine were 8.40 ± 0.10 (Schild slope = 1.03 ± 0.05) and 6.80 ± 0.20 (Schild slope = 0.95 ± 0.08), respectively. These values are consistent with responses mediated by stimulation of an M_1 receptor (Eglen & Whiting, 1986).

Discussion

In the present study, we have characterized the action of pFHHSiD, using responses at muscarinic receptors in isolated tissues and cell cultures. The functional data were consistent with data reported by Lambrecht *et al.* (1988). These authors showed that pFHHSiD exhibited a relatively high pA_2 value for M_3 muscarinic receptors mediating ileal smooth muscle contractions and discriminated M_2 receptors (atria) from M_1 receptors (vas deferens). However, other data obtained in the present study indicate that it may also exhibit dissociation constants (as estimated by the pA_2 value) that depend on the M_3 preparation studied.

The pA_2 values at atrial M_2 and ileal M_3 receptors were quantitatively very similar to those obtained by Lambrecht *et al.* (1988). The pA_2 value at M_1 receptors in the canine saphenous vein (7.1) was also similar to that reported by Lambrecht *et al.* (1988) at M_1 receptors in the rabbit vas deferens (6.9). A similar value was found at receptors mediating contractions of the canine femoral vein suggesting that M_1 receptors also mediate contractions in this tissue. In agreement with this postulate, both a high pA_2 value for pirenzepine (7.9), and low value for methoctramine (6.1) were also derived. Previous reports have shown that pA_2 values for pirenzepine at M_1 receptors generally range from 8.0–8.4 (see Eglen & Whiting, 1986; Mitchelson, 1988 for reviews) whereas the low value for methoctramine was indicative of a lack of M_2 receptor function.

Muscarinic receptor subtypes vary in different isolated vascular tissues, depending upon the physiological response, species and anatomical location. The porcine basilar artery and coronary artery exhibit contractile responses which are mediated by M_2 and M_3 receptors, respectively (Van Charldorp *et al.*, 1988), whereas contractile responses in the canine saphenous vein and bovine coronary artery are mediated by M_1 receptors (O'Rourke & Vanhoutte 1987; Duckles, 1988). The canine femoral vein also exhibits a contractile response to muscarinic agonists (Rubanyi & Vanhoutte, 1988) although the receptors have yet to be characterized.

The muscarinic receptor mediating release of EDRF in vascular tissue exhibits a pharmacology similar to the M_3 receptor defined on the basis of differential antagonist affinities. The pA_2 values for pirenzepine, AF-DX 116, 4-DAMP and methoctramine in the rat aorta (Choo *et al.*, 1986; Eglen *et al.*, 1988), rabbit ear artery (Hynes *et al.*, 1986) and bovine coronary artery (Duckles, 1988) were similar to those observed in ileal smooth muscle (Eglen *et al.*, 1988). pFHHSiD, in view of its reported selectivity for M_3 over M_2 and M_1 receptors, may enable a more definitive profile of these tissues to be elucidated.

The pA_2 values at receptors mediating relaxations of the canine femoral artery, rat aorta and rabbit jugular vein were similar to values obtained at ileal M_3 receptors. However, the low Schild slope observed in experiments using the femoral artery, indicate deviation from competitive antagonism. These data suggest that M_3 receptors mediate endothelial-dependent relaxations in a variety of vascular tissues in at least two species, in agreement with previous literature (see Introduction for references). The pA_2 value obtained at receptors in

the astrocytoma (1321 N1) cells was similar to the pA_2 value obtained at receptors in the isolated ileum confirming previous data that M_3 receptors mediate responses in these tissues (Kunysz *et al.*, 1989).

However, anomalous pA_2 values for pFHHSiD were found in some isolated tissues. The pA_2 value was significantly higher in the OMM, a preparation which has been previously shown to possess M_3 receptors (Eglen & Whiting, 1988). Whilst the reason for the higher value is unknown, anomalously higher values have also been reported for pirenzepine (Kamikawa *et al.*, 1985; Eglen & Whiting, 1988). The muscarinic receptor mediating contractions of the oesophageal muscularis mucosae, for example, has been shown (Kamikawa *et al.*, 1985; Eglen *et al.*, 1988) to exhibit pA_2 values for atropine, AF-DX 116, 4-DAMP, gallamine, silabenzhexol and methoctramine similar to those found at ileal and tracheal M_3 receptors. However, dissimilar pA_2 values for pirenzepine were also observed (OMM, 7.4; ileum and trachea, 6.8). The reason for this disparity was unknown, but it was considered not to be due to the presence of M_1 receptors (Eglen & Whiting, 1988).

Conversely, the pA_2 values for pFHHSiD in the trachea were lower than observed in the ileum. The trachea has been previously shown to possess M_3 receptors (Eglen & Whiting, 1988), and the reason for the relatively low value observed in this study is discussed below. The guinea-pig urinary bladder has also been shown (Eglen & Whiting, 1987) to exhibit pA_2 values for pirenzepine and 4-DAMP similar to those at ileal M_3 receptors, although extensive characterization of muscarinic 'receptors' in this tissue has only been undertaken using radioligand binding studies (Nilvebrandt & Sparf, 1988). The relatively low pA_2 value at M_3 receptors in the urinary bladder was accompanied by a Schild slope less than unity, indicating deviation from competitive antagonism. One explanation for the diminution in maxima observed with pFHHSiD may be the low effective receptor reserve for carbachol in this tissue (as judged by the low $-EC_{50}$ value) leading to conditions of hemiequilibrium. Additional experiments are clearly required to elucidate the reason for these relatively 'low' pA_2 values at previously well characterized M_3 receptors.

The pA_2 value for pFHHSiD at receptors in SH-SY5Y cells was approximately 6 fold higher than the pA_2 value obtained at M_1 receptors in the isolated tissue studies but less than the pA_2 values obtained at M_3 receptors. The reason for this disparity is unknown. Sera *et al.* (1989) have proposed, on the basis of antagonist affinities, that M_1 receptors mediate the inositol phospholipid hydrolysis response in the SH-SY5Y cell. The pA_2 values obtained in the present study for pirenzepine and methoctramine are in accord with this hypothesis. However, Lambert & Nahorski (1989) have reported, again using antagonist affinity data, that stimulation of inositol phospholipid hydrolysis is mediated by M_3 receptors in this cell line. The pA_2 value obtained in the present study for pFHHSiD, but not pirenzepine, was indicative of the presence of an M_3 receptor.

It is apparent, therefore, that the pA_2 values for pFHHSiD are critically dependent upon the preparation studied e.g. dissimilar pA_2 values were observed at M_3 receptors in the ileum (7.85) and at M_3 receptors in the trachea (7.08). Similarly different values were observed at M_1 receptors in the femoral vein (7.11) and at M_1 receptors in SH-SY5Y cells (7.90).

Two possible explanations for these data are that either the differences in pA_2 values are a reflection of further muscarinic receptor heterogeneity or that some of the experiments were undertaken under nonequilibrium conditions. We feel that the former is unlikely because (a) the disparities in pA_2 values are too small i.e. less than 10 fold, to use as evidence for heterogeneity (see Eglen & Whiting, 1986 for further discussion) and (b) that these differences between tissues has only been observed in our laboratory with this particular compound. The use of a 60 min preequilibration period and the use of an agonist resistant to degradation or uptake argues against the possibility of the antagonist not being in equilibrium. The trachea has been shown (Roffel *et al.*, 1987) to possess both M_2 and M_3 receptors, and the contribution of both receptors to the response could theoretically result in a low pA_2 value with a unity slope (Kenakin, 1987). However, the ileum also possesses a heterogeneous population of M_2 and M_3 receptors and a higher pA_2 value was observed, consistent with stimulation of M_3 receptors.

The ability of pFHHSiD to exhibit a range of pA_2 values at M_3 receptors raises questions about its suitability for characterization of muscarinic receptors in particular and questions about the use of antagonists to classify receptor subtypes in general. Leff & Martin (1988) have raised similar questions with regard to the use of antagonists to classify receptors for 5-hydroxytryptamine. The affinities of the endogenous ligand, acetylcholine, for each of the putative muscarinic receptor subtypes are very similar (A.D. Michel, unpublished observations), suggesting that the 'receptive site', for the endogenous ligand is similar between subtypes. However, the potential ability of antagonists to bind to accessary sites on the receptor molecule may confer selectivity. In this respect, however, the role of 'tissue specific' binding areas on the membrane is critical. This may lead to a variation in dissociation constants of an antagonist at the same receptor, but located in different tissues. These considerations provide a more parsimonious explanation for the differences in pA_2 values for pFHHSiD at M_3 receptors than further muscarinic receptor heterogeneity. In the majority of bioassays studied in this report, it is unknown which muscarinic receptor subtype is expressed, and where more than one subtype is expressed, which subtype is coupled to a given response. Presumably, when more expression data are available, it may be possible to separate receptor heterogeneity from the role of membrane environment on antagonist affinity.

In summary, the present study has confirmed the ability of pFHHSiD to distinguish between three subtypes of muscarinic receptor in some isolated functional tissues although anomalous pA_2 values were observed at other M_3 receptors. We conclude that pFHHSiD is of limited use in muscarinic receptor classification. Further experiments are required to determine if the disparities observed at M_3 receptors are a reflection of problems of the compound or indicative of further muscarinic receptor heterogeneity. These data emphasize the importance of estimating the putative selectivity of antagonists in a wide range of preparations.

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Differential effect of zinc on the vertebrate $GABA_A$-receptor complex

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1 γ-Aminobutyric acid (GABA) responses were recorded from rat superior cervical ganglia (SCG) in culture using the whole cell recording technique.

2 Zinc (50–300 μM) reversibly antagonized the GABA response in embryonic and young post-natal neurones, while neurones cultured from adult animals were far less sensitive and occasionally resistant to zinc blockade. Cadmium (100–300 μM) also antagonised the GABA response, while barium (100 μM–2 mM) was ineffective.

3 The differential blocking effect of zinc on cultured neurones of different ages also occurred in intact SCG tissue.

4 The GABA log dose-response curve constructed with foetal or adult cultured neurones was reduced in a non-competitive manner by zinc. This inhibition was minimally affected by the membrane potential.

5 The GABA response recorded intracellularly from guinea-pig pyriform cortical slices was enhanced by zinc (300–500 μM), which occurred concurrently with a decrease in the input conductance of the cell. The enhancement was unaffected by prior blockade of the GABA uptake carrier by 1 mM nipecotic acid. This phenomenon could be reproduced by barium (300 μM) and cadmium (300 μM).

6 We conclude that the vertebrate neuronal $GABA_A$-receptor becomes less sensitive to zinc with neural ($GABA_A$-receptor?) development, and the enhanced GABA response recorded in the CNS is a consequence of the reduction in the input conductance and not due to a direct effect on the receptor complex.

Introduction

The γ-aminobutyric acid (GABA) receptor complex in the vertebrate nervous system has recently been established as a multimeric receptor protein with an integral ion channel (Schofield *et al.*, 1988), possessing numerous binding sites for the attachment of benzodiazepines, barbiturates, convulsants and many other modulators of GABA action (Duman *et al.*, 1987; Schwartz, 1988) including divalent cations e.g., zinc (Smart & Constanti, 1982; 1983).

The current interest in zinc as a putative and novel GABA antagonist followed our original observations of a zinc-induced inhibition of the GABA-evoked Cl^- conductance recorded from crustacean muscle (Smart & Constanti, 1982). The invertebrate muscle GABA receptor was blocked by 10–100 μM zinc (Smart & Constanti, 1982; Albert *et al.*, 1986). We suggested that zinc was binding to some putative histidine groups resident near the 'mouth' of the GABA-operated ion channel and thereby stabilizing this channel in a closed conformation.

It is quite likely that the invertebrate muscle GABA receptor differs structurally from the vertebrate neuronal $GABA_A$-receptor, since the latter responds to benzodiazepines and barbiturates and the former does not. In a previous comparison of the action of zinc on vertebrate $GABA_A$-receptors two unexpected results were observed: firstly, the $GABA_A$-receptor resident on adult rat sympathetic ganglia was entirely resistant to any blockade by zinc (Smart & Constanti, 1982) and, secondly, GABA responses recorded intracellularly from CNS pyramidal neurones in the pyriform cortex displayed an overt enhancement in the presence of zinc (Smart & Constanti, 1983).

In more recent studies, the action of zinc on $GABA_A$-receptors would seem to vary with the type of preparation, being either completely ineffective on rat pyriform slices (Hori *et al.*, 1987) and *in vivo* rat cortical neurones (Wright, 1984), or producing a block of GABA responses on frog dorsal root ganglia (DRG) neurones (Yakushiji *et al.*, 1987; Akaike *et al.*, 1987), *in vivo* cat primary afferent fibres (Curtis & Gynther, 1987) and also on cultured hippocampal neurones of the mouse (Westbrook & Mayer, 1987). These studies of zinc on GABA function suggest that one possible role for the endogenous zinc in the central nervous system (CNS) (Crawford & Connor, 1972; Donaldson *et al.*, 1973) may be to interact with, or modulate the $GABA_A$-receptor. However, due to the apparent variety of effects zinc is deemed to produce on neuronal GABA responses, we have re-investigated the action of this divalent cation on GABA responses recorded from dissociated and intact sympathetic neurones and brain slices.

Our data suggest that the modulation of GABA responses by zinc critically depends not only on the type of preparation used but also on the stage of neuronal development. Preliminary accounts of this work have been previously communicated (Smart & Constanti, 1988; 1989).

Methods

Sympathetic neuronal tissue culture

Superior cervical ganglia (typically 4–16) were obtained from foetal (E17–21), young post-natal (P1–6) and adult (>P90) Sprague-Dawley rats and dissociated by use of a combination of enzymatic and mechanical methods, as previously described in detail (Smart, 1987). Minor modifications to the protocol included: coating dishes with a 20 μg ml^{-1} laminin solution (Flow Labs.) (incubated at room temperature for at least 2 h); use of 7S nerve growth factor (from mouse submaxillary glands, 50 ng ml^{-1}, Calbiochem) in the L-15 based growth medium and inhibition of Schwann and fibroblast cell growth was controlled by a 24 h incubation in 10 μM cytosine arabinoside (Sigma).

Preparation of intact sympathetic ganglia

Intact superior cervical ganglia (SCG) were isolated from urethane anaesthetized (1.5 mg kg^{-1}) Sprague-Dawley rats (200–250 g) (Adams & Brown, 1975). The desheathed ganglion was

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pinned down onto a Sylgard base and perfused with Krebs solution containing (mM): NaCl 118, KCl 4.7, $MgCl_2$ 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25 and glucose 11; bubbled with 95% O_2/5% CO_2, pH 7.4 at 20–24°C.

Brain slice preparation

Transverse (450 μm) slices of pyriform cortex were prepared from Albino guinea-pigs (250–600 g) with a Campden Vibroslice/M tissue cutter as previously described (Constanti & Sim, 1987) and superfused with oxygenated Krebs solution (23–25°C) containing (mM): NaCl 118, KCl 3, $CaCl_2$ 1.5, $NaHCO_3$ 25, $MgCl_2$ 1.2 and glucose 11; bubbled with 95% O_2/5% CO_2, pH 7.4. Occasionally, slices remained viable even after a 24 h pre-incubation; no obvious differences were detected between recordings made on these slices and those made on freshly dissected slices.

Electrophysiology

Whole-cell recording Patch electrodes (1–5 MΩ) were formed from borosilicate glass and heat-polished to a final diameter of 0.5–1.5 μm. Experiments were performed by the whole-cell recording (wcr) configuration with a List EPC7 patch-clamp amplifier. The cells were continuously superfused in the culture dish with a HEPES-Krebs solution containing (mM): NaCl 140, KCl 4.7, $MgCl_2$ 1.2, $CaCl_2$ 2.5, glucose 11 and HEPES 5; pH 7.4 at 25°C. Phosphate or sulphate salts were omitted to avoid precipitation of the divalent transition metals. The recording pipette solution contained either: (mM) KCl 30, K Asp 120, $MgCl_2$ 1, EGTA 0.5, $CaCl_2$ 0.28 and HEPES 5; buffered to pH 6.8 with 1 M NaOH (final concentration 3 mM); or: KCl 150, $MgCl_2$ 1, EGTA 0.5, $CaCl_2$ 0.28 and HEPES 5; buffered to pH 6.8 with 1 M NaOH. Data were recorded on a Brush-Gould 2400S ink-jet pen recorder. GABA was applied to the neurones either via bath application or by pressure injection from a patch pipette filled with 50 μM GABA, positioned approximately 20–50 μm from the cell soma. Ejection pressures were typically 30–150 kPa for 10–100 ms at 0.1 Hz. Healthy neurones had typical resting potentials in the range of −45 to −75 mV and spike amplitudes >100 mV. All drugs were obtained from Sigma or BDH Ltd.

Intracellular recording Intracellular recordings were performed on pyramidal cells from olfactory cortical layers II–III (Martinez *et al.*, 1987), or from intact sympathetic ganglia by a Dagan 8100 preamplifier (Constanti & Galvan, 1983). Intracellular electrodes were filled with 4 M potassium acetate (60–80 MΩ). GABA was applied either via bath superfusion or by ionophoresis, using 1 M GABA acidified with 1 M HCl to pH 3–4. The ionophoretic electrode was positioned as close as possible to the intracellular electrode and ionophoretic pulses applied at 1–2 min intervals with positive ejection currents (5–200 nA) and a retaining current of −10 nA. The position of the GABA pipette was adjusted until a 3–15 s GABA application (10–30 nA) evoked consistent, brief depolarizations at the resting membrane potential (typically −80 mV for olfactory neurones). Higher ejection currents for more prolonged periods were avoided to minimize any shifts in the GABA equilibrium potential or overt GABA receptor desensitization (Kriegstein & Connors, 1986). Data were recorded on a Brush-Gould 2400 chart recorder. Healthy cells used in this study had resting potentials of −75 to −85 mV and spike amplitudes >90 mV for CNS neurones, and −45 to −55 mV resting potential and spike amplitudes >70 mV for ganglionic neurones.

Results

Zinc inhibits GABA-induced membrane currents on foetal and young post-natal cultured superior cervical ganglia neurones

The bath application of GABA (1–20 μM) to foetal rat cultured SCG neurones routinely induced an inward membrane current and an increase in the membrane conductance recorded under whole-cell voltage clamp (Figure 1a). The inward current was also associated with an increase in membrane current noise (Figure 1a). Generally, the GABA response was

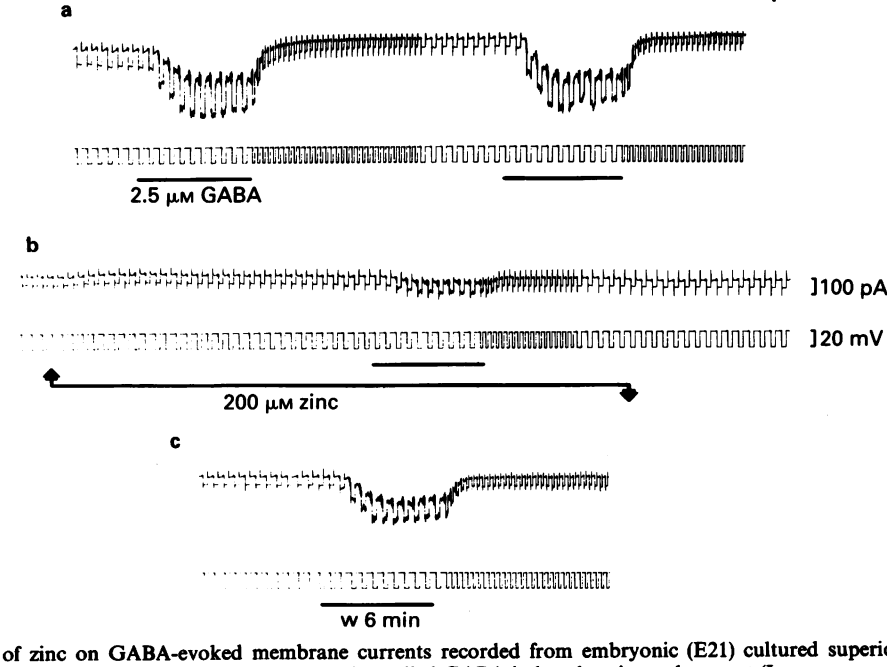


Figure 1 Effect of zinc on GABA-evoked membrane currents recorded from embryonic (E21) cultured superior cervical ganglia (SCG) neurones under whole-cell voltage clamp. (a) Bath-applied GABA induced an inward current (I_{GABA}; upper trace) at a holding potential of −55 mV with an associated conductance increase observed following voltage commands to −75 mV (300 ms, 0.1 Hz; lower trace). An increased membrane current noise was also evident. (b) Zinc (200 μM) was applied for 10 min and induced a small outward current and dramatically inhibited I_{GABA} which was reversible after a 6 min wash (w 6 min) (c). All drugs were applied for the duration of the solid lines in this and subsequent figures. The chart speed was increased every 10 s for the duration of each voltage command pulse.

After recovery from initial bath-applied GABA doses, the cell input conductance was frequently reduced (a), but usually after only one dose of GABA it attained a steady state and was not further reduced following recovery from subsequent GABA applications (c).

well maintained during the perfusion of GABA (1–2 min), exhibiting only a slight propensity for fade or desensitization. Following the washout of GABA, the input conductance of the cell was typically depressed and occasionally would never recover to the pre-GABA level (Figure 1a). GABA responses after bath application were quite reproducible exhibiting only a slow 'run-down' with time after formation of the whole-cell recording mode (cf. Stelzer *et al.*, 1988; Figure 1a and c). The application of zinc (100–300 μM) to cultured sympathetic neurones produced a small outward current and a small reduction in the input conductance. Additionally, the inward GABA current and conductance increase were also markedly reduced (Figure 1b). In 100 μM zinc, GABA responses were inhibited by approximately 57 ± 1.7% (mean ± s.e.mean, $n = 25$ cells, cultured from either E21 or P1–5 age rats). The GABA response quickly recovered on zinc washout (Figure 1c). Zinc was always effective in blocking the GABA responses recorded from all the foetal and young postnatal (P + 1–5) cultured SCG neurones used in this study ($n = 30$).

GABA was also routinely applied via pressure ejection from a patch pipette juxtaposed to within 50 μm of a whole-cell voltage-clamped neurone. This mode of drug application allowed many more GABA responses to be recorded from a single cell with virtually no 'run-down' over 2–3 h recording. Reproducible GABA-induced inward currents (evoked at 0.1 Hz) peaked rapidly in less than 100 ms, concomitant with an increased membrane current noise, and decayed to the control resting current level usually within 1–3 s. In many cells, higher frequency emission of GABA (up to 4 Hz) did not promote any obvious reduction in the peak of the GABA responses (desensitization) which eventually summated to

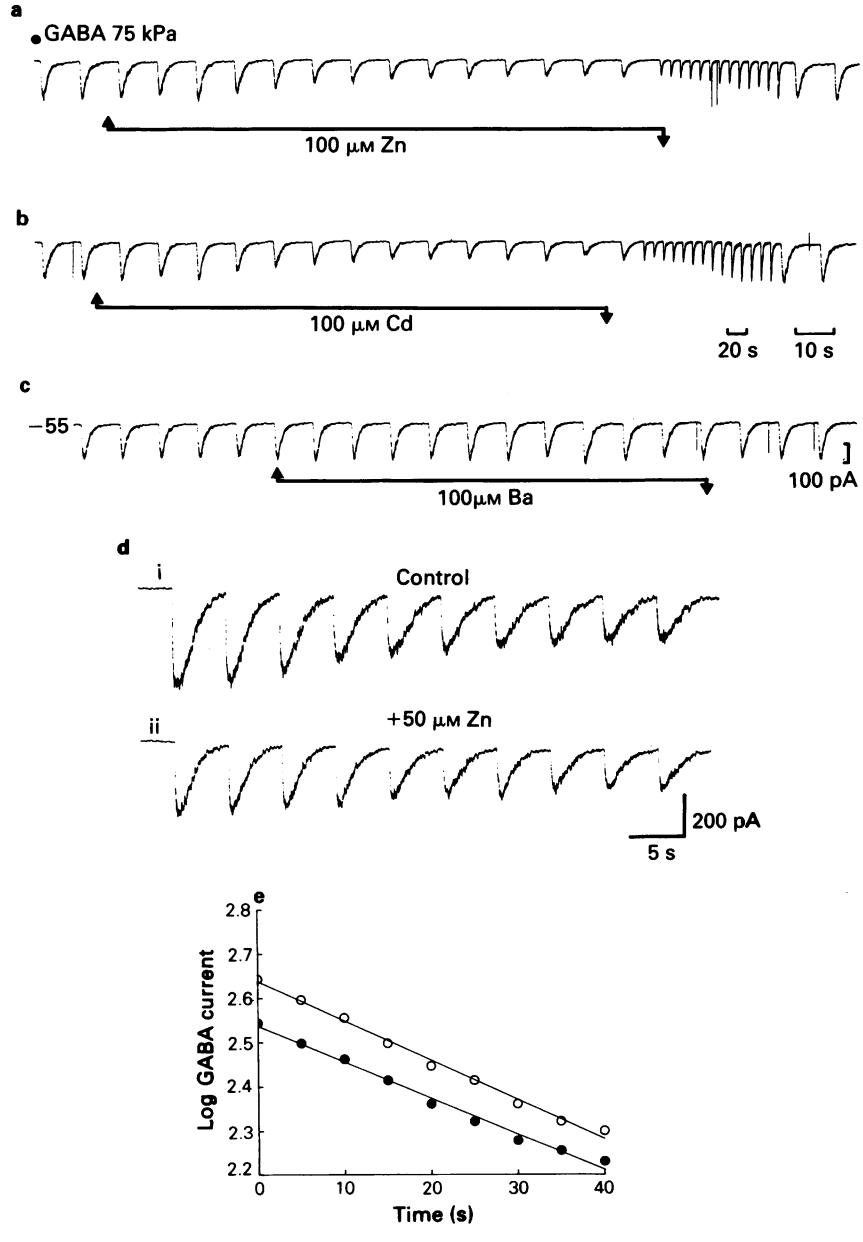


Figure 2 Effect of group IIa and IIb divalent cations on the GABA-induced inward current (I_{GABA}) in embryonic cultured superior cervical ganglia (SCG) neurones under whole-cell recording mode at −55 mV holding potential. GABA was pressure applied (75 kPa, 30 ms, 0.1 Hz) from an adjacent patch pipette 50–100 μm from the cell. Group IIb cations, zinc (Zn, a) and cadmium (Cd, b) were equi-effective and reversible GABA antagonists, whereas barium (Ba), a group IIa cation, was ineffective (c). The chart recorder was slowed during the recoveries from zinc and cadmium. Divalent cations were applied for approximately 2 min. (d) Zinc did not enhance GABA desensitization on SCG neurones. Desensitization of the GABA response was induced by repetitive pressure ejections of GABA (77.2 kPa, 50 ms, 0.2 Hz) onto an SCG neurone voltage clamped at −55 mV. Sequential GABA responses in control solution were slowly reduced with time (d (i)); in the presence of 50 μM zinc (d (ii)), this slow decay was unaffected, as observed by plotting the logarithm of GABA current amplitudes against time from the start of the pressure applications (e). In (e), (○) control; (●) + 50 μM zinc.

form a smooth current. Figure 2a illustrates that the antagonism of GABA responses by zinc was not limited to just bath-applied GABA, since responses to GABA applied by brief pressure were also readily antagonized by zinc (100–300 μM) causing a reversible reduction of 51% in this cell.

To test whether the inhibition of GABA responses was a unique feature of zinc, we also studied the effect of cadmium, another transition divalent metal ion also belonging to group IIb. This ion, at similar concentrations, was just as effective as zinc in promoting a reversible inhibition of the GABA-evoked membrane current (Figure 2b; 55% inhibition; $n = 3$). However, this inhibitory action was not a general feature of all divalent cations since the group IIa divalent ion, barium, did not inhibit the GABA response over a wide concentration range (100 μM–2 mM; $n = 3$) (Figure 2c).

GABA responses recorded from cultured sympathetic neurones showed only a slight propensity to desensitize or fade (cf. Figure 1a). Whether zinc was inhibiting the GABA response by enhancing desensitization was studied by measuring the decay rate of GABA responses evoked by repetitive pressure applications of GABA. The decay rate in normal Krebs was a slow process (112.5 ± 6.7 s; $n = 3$; Figure 2d(i)) and may monitor only part of the desensitizing or fading phenomenon. It was unlikely to reflect a shift in the GABA reversal potential, since the conductance change also declined simultaneously (cf. Figure 1a). Zinc 50 μM produced a reduction in the evoked GABA currents (Figure 2d), but clearly did not affect the decay time constant (122.7 ± 5.5 s; $n = 3$; Figure 2e).

This inhibition of GABA responses by zinc also occurred on foetal rat sensory ganglia cultured under identical conditions. Dissociated cultures of rat dorsal root ganglia (from the thoracic and lumbar spinal regions; age E21) yielded two main cell populations based on cell diameter. GABA responses were readily observed following pressure ejection, although the sensitivity to GABA (measured as the amount of inward current and conductance recorded under voltage clamp) seemed considerably lower on these sensory ganglionic neurones compared to the sympathetic cultures of an equivalent age. Bath application of 50 or 100 μM zinc inhibited the GABA responses by 29.3 ± 6.7% and 60.6 ± 5.2% ($n = 5$ cells) respectively on both the major neuronal cell types.

The reduction of GABA responses by zinc on sympathetic neurones was most probably due to a direct effect on the GABA receptor/channel and not apparently due to a shift in the GABA reversal potential (E_{GABA}), which remained unaltered (Figure 3a and b). Also, the GABA chord conductance was inhibited by zinc in an apparently voltage-independent manner (Figure 3c). The lack of any fade in the GABA response recorded in many neurones also indicated that the increased transmembrane chloride ion flux induced by GABA (Adams & Brown, 1975) probably did not cause any substantial shift in E_{GABA} under wcr conditions (where usually $[Cl^-]_o = [Cl^-]_i = 152$ mM providing an $E_{GABA} = 0$ mV; for potassium aspartate intracellular solutions, $[Cl^-]_i = 32.5$ mM yielding an $E_{GABA} = -40$ mV).

Currently, the blockade of GABA responses by zinc has been observed mostly in cell cultures or enzymatically-treated cells (Akaike *et al.*, 1987; Westbrook & Mayer, 1987). Whether this block could be a phenomenon generated solely in dissociated cell culture, or by enzyme treatment (Inoue & Akaike, 1987) was investigated by utilizing young manually desheathed post-natal intact rat SCG (P + 5 – 6). The potency of zinc as a GABA antagonist was now assessed on a preparation possessing an integral cytoarchitecture including both pre- and postganglionic nerve trunks.

Intracellular recording from these ganglia and ionophoretic application of GABA revealed a monophasic depolarization and conductance increase which was clearly dose-dependent and readily reversible. The application of 100 μM zinc caused a small inhibition at high GABA ionophoretic doses, but on increasing the zinc concentration to 300 μM, a more overt non-competitive inhibition of the GABA dose-conductance relation was apparent, similar to that observed in cultured neurones (*vide infra*). Previously, in intact adult rat SCG, we showed that bath-applied GABA responses were completely unaffected by zinc (100–500 μM; Smart & Constanti, 1982).

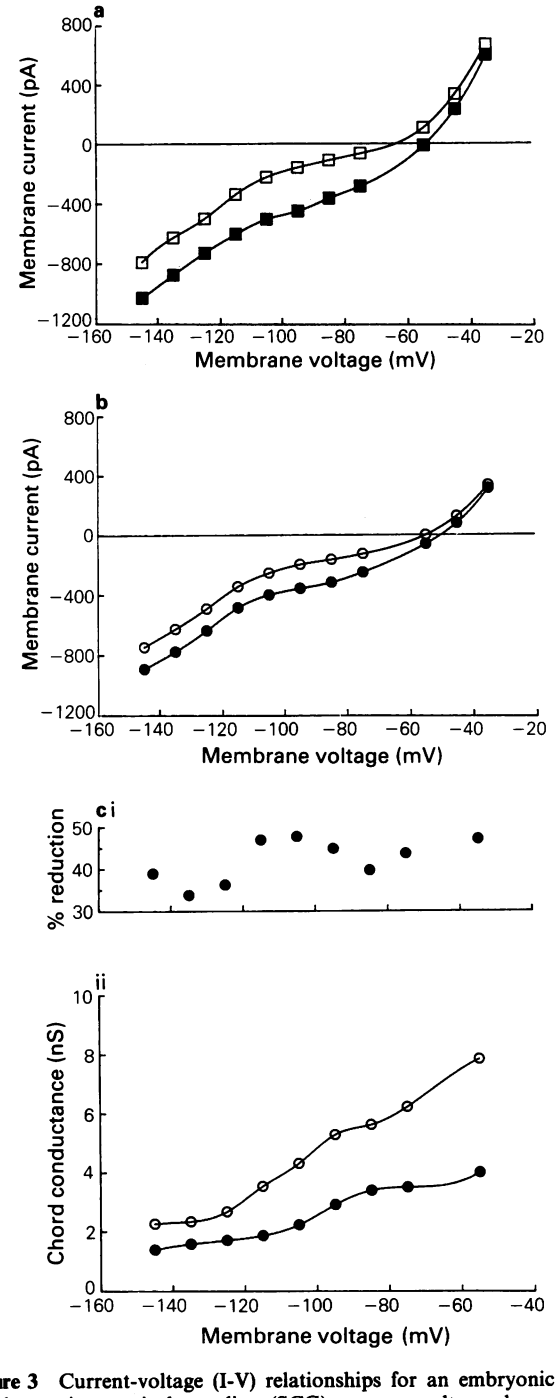


Figure 3 Current-voltage (I-V) relationships for an embryonic cultured superior cervical ganglion (SCG) neurone voltage clamped at −55 mV. (a) Control I-V relation was constructed in the absence (□) and presence (■) of 5 μM GABA. The GABA-evoked current, at different command potentials, was obtained by subtraction of the two curves and plotted as the chord conductance in (c). (b) In the same cell following recovery from the initial dose of GABA, two further I-V relationships were measured in the presence of 100 μM zinc (○) and also 100 μM zinc + 5 μM GABA (●). (c) (ii) The GABA chord conductance in the absence (○) and presence (●) of 100 μM zinc was plotted against voltage. The GABA reversal potential was −37 mV. The effect of zinc was essentially devoid of any voltage sensitivity over this potential range, reducing the GABA chord conductance by 48% at −55 mV and 40% at −135 mV membrane potential ((i) shows a plot of the % reduction in GABA chord conductance against membrane potential). Note the reduction in control GABA conductance at hyperpolarized potentials.

The present results with foetal cultures and the young intact SCG, prompted us to reinvestigate the action of zinc on intact adult rat ganglia and also on adult SCG cultures obtained from animals of a similar age (> P + 90).

Zinc is less effective as a GABA antagonist in adult sympathetic neurones

Bath application of 50 μM GABA to an adult SCG neurone (2–3 months old), recorded under current clamp, resulted in a membrane depolarization and conductance increase (Figure 4a). Larger doses of GABA were now required to observe any response, due to the presence of avid GABA uptake mechanisms in this tissue (Bowery *et al.*, 1979). Zinc (200–500 μM) slightly hyperpolarized the cell, similar to the effects observed on foetal cultured neurones, and also increased the resting input resistance. However, GABA responses were unaffected by zinc, as previously found (Smart & Constanti, 1982) (Figure 4b and c). The apparent lack of effect of zinc was not due to poor penetration into the desheathed ganglion, since spontaneous transmitter release increased in zinc as observed by the appearance of 'membrane potential noise' (Figure 5c). Also, zinc induced the appearance of anode break spikes following the cessation of the hyperpolarizing electrotonic potential in the presence of GABA (Figure 5c; cf. Smart & Constanti, 1982).

The intact mature preparations were clearly less sensitive to zinc than either the intact young post-natal SCG or the cultures of foetal neurones. This reduced sensitivity of GABA responses to zinc blockade also applied to mature neurones cultured from 'equivalent-age' adult animals (age > P + 90).

Pressure-ejection of GABA onto cultured adult neurones, produced a rapid inward current followed by a 'noisy' decay back to the holding current analogous to the GABA response recorded in foetal neurones. By using similar pressure ejection parameters for GABA to those used on foetal neurones, bath application of zinc now produced a much smaller reduction in the GABA response (Figure 5b) (32.5% reduction in this cell). In contrast, foetal SCG neurones cultured for a similar period of time (15 DIV; Figure 5a(i)) to the adult neurones (20 DIV; Figure 5b) exhibited GABA responses that were more substantially blocked by identical concentrations of zinc (67.5%) (Figure 5a).

The reduced sensitivity of adult neuronal GABA responses to zinc was occasionally manifest as a complete resistance to zinc blockade ($n = 5$ cells) (Figure 6), comparable to our observations on the intact adult SCG. Figure 6a shows GABA responses obtained on adult cultured neurones following pressure ejection of GABA. Zinc (100 μM) did not inhibit the GABA response even when the concentration was increased to 300 μM (Figure 6b), which in foetal SCG neurones will elicit an 80% reduction in the GABA response. However, 300 μM zinc did induce a small increase in neuronal excitability, signalled by the presence of >1 spike associated with each GABA current (arrow, Figure 6b). The input conductance of the neurone was only slightly decreased by 300 μM zinc, which may have been the cause of a small apparent enhancement in the GABA response (16.8 ± 2.1%) observed occasionally and only when the GABA response was not blocked by zinc (Figure 6b) ($n = 5$ cells). Interestingly, barium (500 μM–2 mM) caused a larger reduction in the input conductance of these adult cells and the GABA responses were also frequently enhanced by 5–10% (data not shown). These small and infrequent enhancements of GABA action by zinc or barium on cultured ganglion neurones were routinely observed more clearly on intact cortical brain slice GABA responses and may have a similar underlying mechanism (see Figure 11).

The apparently 'zinc resistant', adult ganglionic GABA receptor was a *bona-fide* member of the $GABA_A$-receptor subtype family, since GABA responses were readily blocked entirely and reversibly by 10 μM (+)-bicuculline methochloride (Figure 6c). These data suggest that the GABA receptor in the rat SCG may undergo subtle developmental changes affecting function, which can partly be resolved by the action of zinc on the GABA-induced Cl^- current. This postulated developmental modification was highlighted further by comparing dose-response curves obtained for GABA on adult and foetal cultured SCG neurones in the presence and absence of zinc.

Foetal cultured SCG neurones were exposed to pressure ejected GABA and a dose pressure-response curve was constructed by varying the ejection pressure. The GABA log dose-response curve was depressed by 100 μM zinc in a non-competitive manner, exhibiting a small increase in the degree of blockade at higher agonist doses (Figure 7a). At low agonist doses the percentage reduction in the GABA response was 50% and this increased to 65% at the top of the curve.

In contrast, on adult cultured SCG neurones, a plot of the peak GABA-induced conductance change against log GABA concentration revealed that zinc was now considerably less effective, although a non-competitive mode of action was still appropriate (Figure 7b). The inhibitory effect of zinc also showed a slight dependence on agonist concentration, reducing the GABA response by 27% at low GABA doses (10 μM) and by 33% at higher concentrations (40 μM).

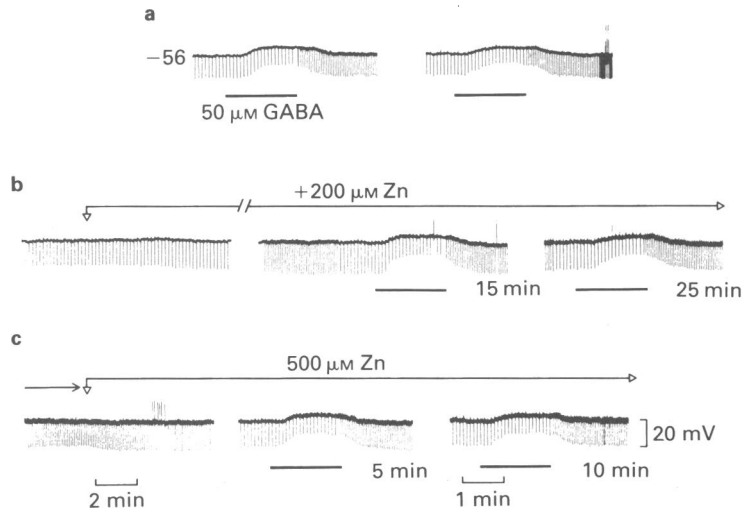


Figure 4 The GABA response is insensitive to zinc in intact adult rat superior cervical ganglia (SCG). (a) Intracellular recording of current clamp responses to bath applied GABA which consistently evoked a small depolarization (upward deflection) and conductance increase (reduced hyperpolarizing electrotonic potential amplitude evoked by −0.16 nA, 300 ms, 0.2 Hz current pulses). (b) Bath application of 200 μM zinc caused a small hyperpolarization and reduction in membrane conductance, but no inhibition of the GABA response after 15–25 min. (c) Increasing the zinc concentration to 500 μM still did not produce any GABA-inhibition, but caused a further small hyperpolarization and the appearance of some spontaneous action potentials together with an increased baseline 'noise'.

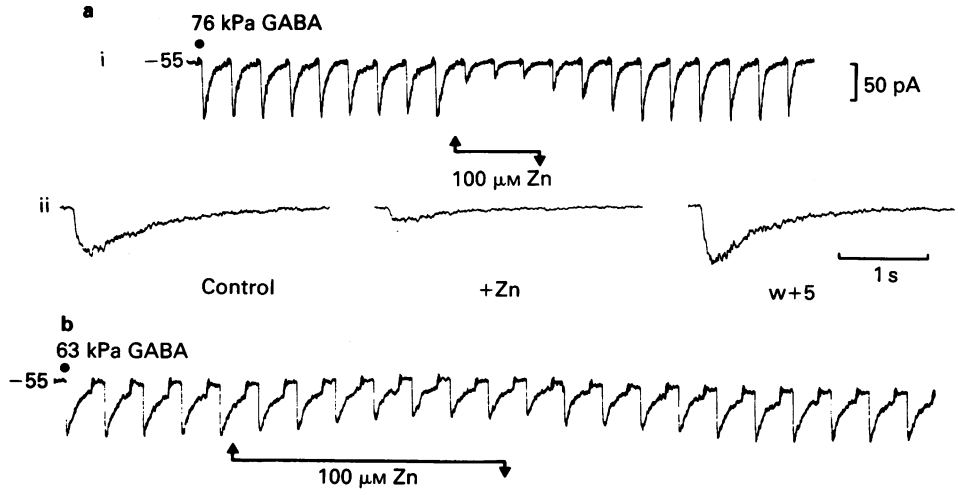


Figure 5 GABA responses are differentially sensitive to inhibition by zinc in rat cultured superior cervical ganglia (SCG) neurones obtained from foetal or adult animals. GABA was applied by pressure application (76 kPa; 50 ms, 1 pulse $30\,s^{-1}$) to embryonic cultured neurones (a(i)) at −55 mV under whole-cell voltage clamp. Bath application of zinc induced a rapid and reversible inhibition of the inward GABA-evoked membrane currents, shown on an expanded time scale in (a(ii)). By performing a similar protocol with neurones cultured from adult rats (P + 90) (b) and with similar ejection pressures (63 kPa, 30 ms, 1 pulse $30\,s^{-1}$) for GABA, it was revealed that zinc was much less potent as a GABA antagonist.

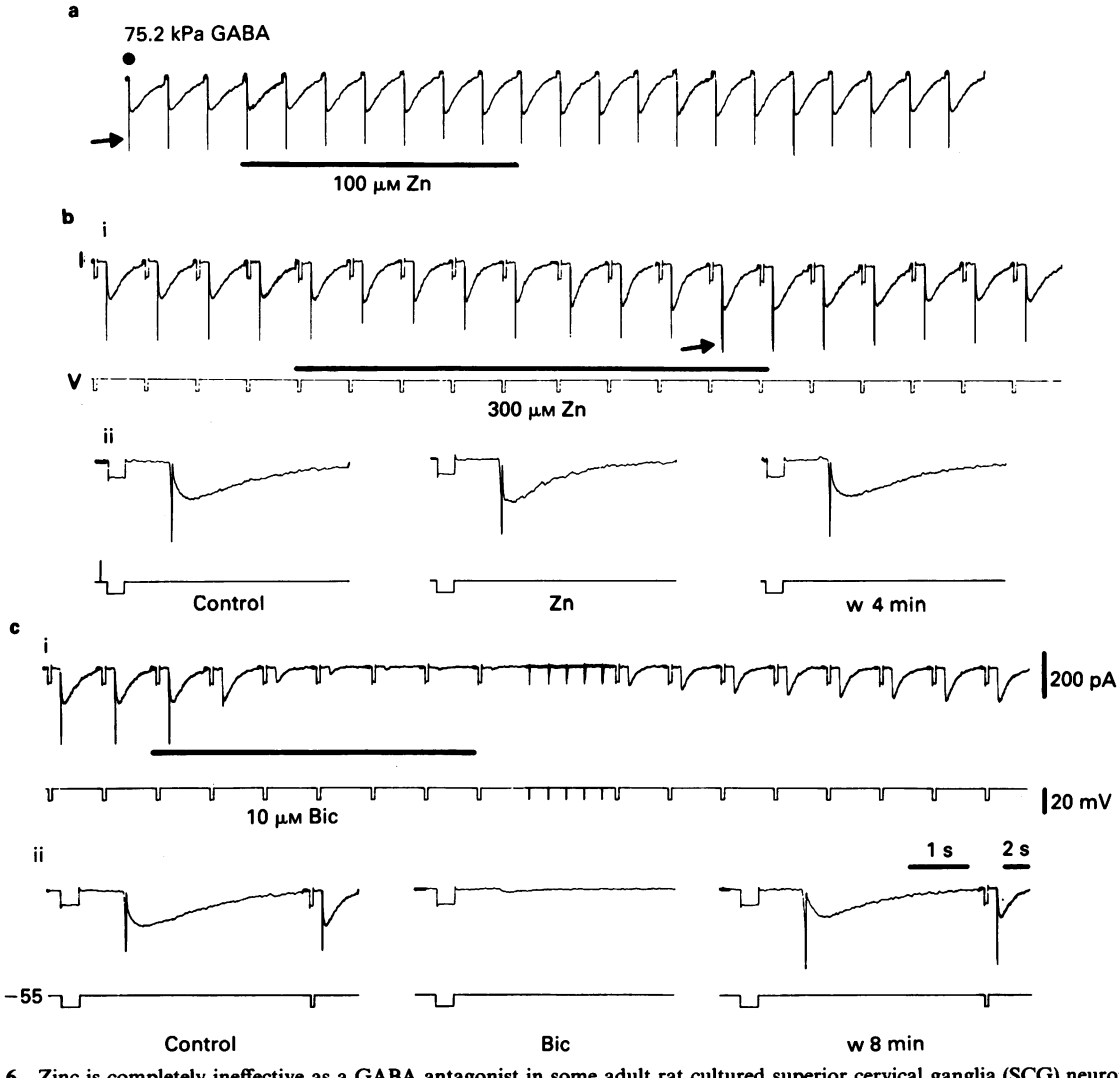


Figure 6 Zinc is completely ineffective as a GABA antagonist in some adult rat cultured superior cervical ganglia (SCG) neurones. (a, b) Whole-cell voltage clamp records of the GABA-induced inward current (I_{GABA}) recorded following pressure ejection of 50 μM GABA (75.2 kPa, 50 ms, 1 pulse $30\,s^{-1}$). The downward deflection beyond the inward current represents an uncontrolled, truncated sodium current (arrow) due to the cell firing an action potential in response to the GABA-induced current. Membrane conductance was monitored by applying hyperpolarizing voltage commands from −55 mV holding potential (−10 mV, 300 ms, 0.03 Hz). GABA responses were completely unaffected by bath application of 100–300 μM zinc which eventually caused the appearance of multiple uncontrolled spikes (b(i) arrow). (b(ii)) Shows selected records at higher time resolution. In the same cell after washout of zinc, I_{GABA} was completely abolished by 10 μM (+)-bicuculline (Bic) (c(i)), but it recovered on washout after 8 min; shown on expanded time scale in (c(ii)).

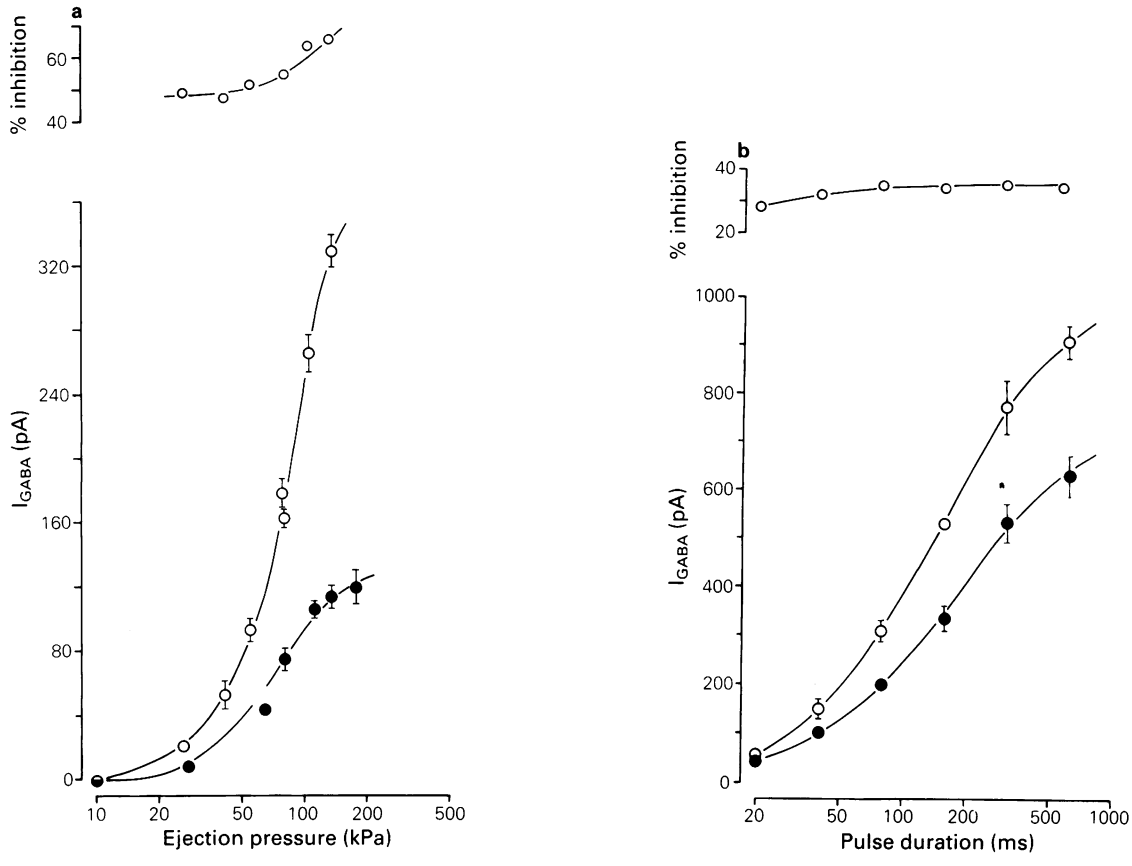


Figure 7 GABA dose-response curves recorded from foetal (a) and adult (b) rat cultured superior cervical ganglia (SCG) neurones by whole-cell voltage clamp. GABA was applied by pressure ejection, varying either the pressure or pulse duration to achieve a dose-response relationship, and the resulting GABA-induced inward current (I_{GABA}) measured. In control Krebs, the GABA log dose-response curves were sigmoidal. Bath application of 100 μM zinc resulted in a non-competitive depression in both curves but clearly zinc was more effective on the foetal cells (a). Insets show the percentage reduction in the control GABA response by zinc. The points in (a) are mean values and vertical lines s.e.mean determined from 5 successive exposures to the same pressure application of GABA. In (b) mean values were taken from 3–8 separate determinations. The GABA response was calculated as the mean peak inward current, from at least three successive responses, following the attainment of a steady-state response each time after alteration of either the ejection pressure or pulse duration. The ejection pressures and pulse durations were chosen randomly to avoid any possible cumulative effects of GABA caused by merely incrementing the effective GABA concentration. In (a) and (b), (○) control responses; (●) responses in the presence of zinc 100 μM.

The insensitivity of the adult ganglionic GABA receptor to zinc implies that other adult GABA$_A$-receptors, present in CNS tissue, should also be unaffected by zinc. However, our previous study (Smart & Constanti, 1983) revealed an enhancement of GABA responses in the presence of this cation. Does this dual action of zinc suggest the existence of more than one subtype of GABA$_A$-receptor?

Zinc enhances the GABA response recorded from adult brain slices

Olfactory cortical neurones responded to bath-applications of GABA (1–4 mM; 1–2 min) with a slow depolarization and associated conductance increase (Brown & Scholfield, 1979). In general agreement with our earlier findings (Smart & Constanti, 1983), these responses were clearly enhanced (but never blocked) after a 10–20 min pre-incubation with 300–500 μM zinc. In addition, zinc also caused a 5–10 mV membrane depolarization associated with a dramatic decrease (up to 50%) in the resting input conductance and an increased incidence of spontaneous neuronal firing, which persisted during the GABA response.

The enhancement of GABA responses by zinc was probably not mediated by an inhibition of GABA uptake carriers (Brown *et al.* 1980), since the effect was clearly observed even in the presence of 1 mM nipecotic acid, a GABA uptake inhibitor (Figure 8) (Johnston *et al.*, 1976). In the combined presence of zinc and nipecotic acid, GABA now evoked distinctly larger depolarizations and apparent conductance increases relative to control, accompanied by characteristic repetitive neuronal firing. Recovery from the effects of zinc was typically incomplete, despite prolonged washing (up to 1 h).

To determine the time course of the zinc-induced enhancement, GABA was focally-applied to single neurones by microionophoresis. GABA produced dose-dependent depolarizations at the resting membrane potential (−80 mV), accompanied by large increases in input conductance ($n = 9$ cells). The peak of the GABA response was attained within 5–10 s of ionophoresis, and recovery was complete within a few seconds of terminating the current pulse. When the membrane potential was depolarized to −70 mV, GABA elicited a biphasic response, consisting of an early, small hyperpolarization followed by a larger depolarizing component (Figure 9). A clear increase in the amplitude of the late GABA depolarization and peak conductance change relative to control was observed after 15–30 min in zinc (Figure 9). In addition, some reduction of the early hyperpolarizing GABA response was also apparent, but this may have been masked by the enhanced, and now much larger, depolarizing component. Zinc also induced a progressive fall (47%) in the resting input conductance which was commensurate with the GABA response enhancement (Figure 9d). Concurrently, the cell also began to fire characteristic, spontaneous action potentials. The shape of these spikes was similar to the shape of anti-

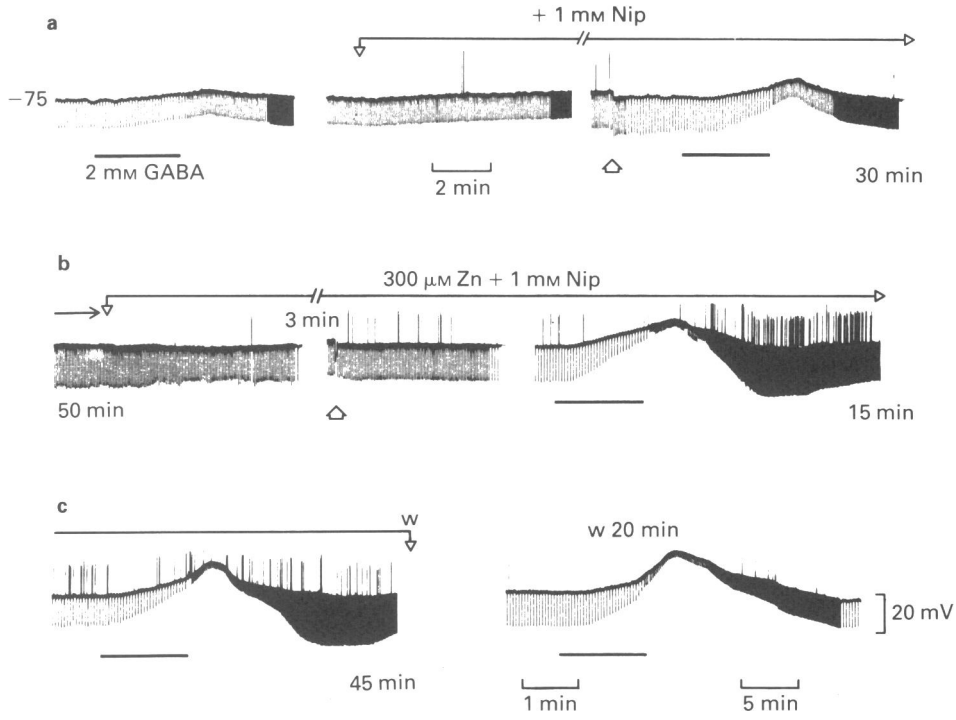


Figure 8 Current clamp recording from an olfactory cortical neurone (resting potential −75 mV) showing the enhancement of GABA responses by zinc in the presence of a GABA uptake inhibitor. Hyperpolarizing current pulses (−0.38 nA, 200 ms) were applied throughout at 0.33 Hz and upward deflections are spontaneous action potentials (truncated). (a) A depolarizing response to bath-applied GABA obtained in control solution and after 30 min in nipecotic acid (Nip). (b and c) After 50 min, zinc and nipecotic acid were applied in combination. GABA, applied after 15 or 45 min, now elicited notably larger responses. Little recovery of the GABA response was obtained after a 20 min washout. Open arrows show where the membrane potential was readjusted back to −75 mV.

dromic action potentials evoked in this preparation (Scholfield, 1978), suggesting that they may be generated at a site distal to the soma (uninfluenced by GABA) and retrogradely conducted back towards the somatic recording site. All these effects of zinc were typically slow to reverse on return to normal Krebs solution, requiring >1 h washing for recovery.

Whether this enhancing action of zinc was due to a large shift in the GABA reversal potential was investigated by applying the same ionophoretic dose of GABA at various membrane holding potentials, in the absence or presence of zinc (Figure 10a). The control GABA response, which was depolarizing at −80 mV, became biphasic at −70 mV, whilst at −50 mV, both early and late components were clearly hyperpolarizing. A plot of the GABA response amplitude against membrane potential (Figure 10b) revealed the reversal potential of the early hyperpolarization to be −76 mV, while the later depolarizing component reversed at −60 mV. After 20 min in 300 μM zinc, there was little change in the reversal potential of the early hyperpolarization. However, the reversal level of the late component was shifted by +10 mV. At potentials negative to −60 mV, the GABA-induced input conductance increases were not being exaggerated by an outward rectification of the resting membrane. Indeed, shifting the membrane potential from −80 to −60 mV (either in control or in zinc solution) revealed only a small (15%) but typical fall in cell input conductance. Interestingly, a sharp increase in the resting conductance, observed between −60 and −50 mV, was apparently inhibited by zinc, suggesting that a voltage-dependent conductance mechanism operating over this voltage range had been blocked.

The temporal correlation between the zinc-induced reduction in the input conductance and the enhancement of the GABA response prompted us to test whether these effects of zinc could be reproduced by other divalent cations, e.g. barium and cadmium. Barium (300 μM; $n = 3$) depolarized the membrane by 25 mV and increased the resting input resistance by 160% (cf. Constanti & Galvan, 1983; Figure 11). After readjustment of the membrane potential to −70 mV, the re-application of GABA now produced enhanced depolarizations and peak changes in input conductance. This apparent enhancement was maintained for as long as barium was present, and was only slowly reversible after a prolonged washout. Some increase in spontaneous (antidromic ?) firing was also observed in barium (Figure 11a), particularly during the washout period, but not so intense as that seen with zinc. There was also a 5–10 mV positive shift in the apparent reversal level of the late depolarizing GABA component (cf. effect of zinc) and the time course of the GABA enhancement was commensurate with the reduction in resting input conductance (Figure 11b). This suggested that the apparent ability of zinc and barium to enhance the GABA response may result from the overall change in neuronal electrical properties following the reduction of the resting membrane conductance.

In contrast, cadmium (300 μM; $n = 3$) showed a dual effect on the cortical GABA response, causing an initial (<5 min) enhancement with a small fall in input conductance. However, after 20 min the responses to subsequent GABA applications were consistently reduced in amplitude. Unfortunately, the interpretation of this result was clouded by the development of an irreversible increase in the input conductance in the presence of cadmium.

Discussion

The original reason for this study was, initially, to reaffirm our observation that zinc enhanced cortical GABA responses while not affecting sympathetic ganglionic GABA responses and to attempt to answer the question of why $GABA_A$ receptors located within different regions of the vertebrate nervous system respond differentially to zinc. We believe the answer may be two fold, relying firstly on a possible differentiation of the $GABA_A$-receptor protein with neuronal development, and secondly, on the spatial distribution of GABA receptors on neurones with vast dendritic networks, particularly in the CNS.

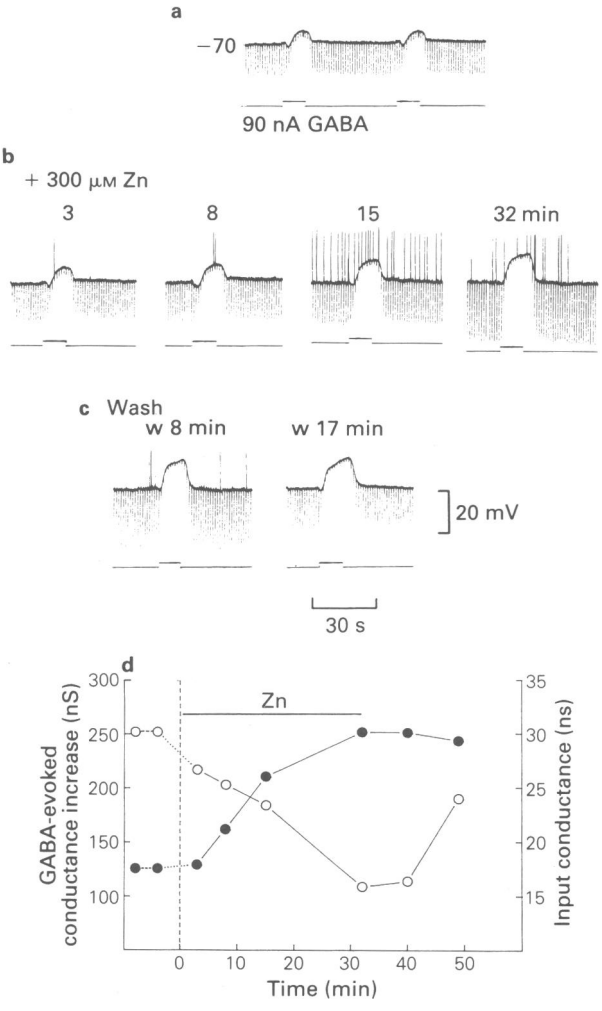


Figure 9 Biphasic responses to ionophoretically-applied GABA (90 nA, 10 s) recorded from an olfactory cortical neurone (resting potential maintained at −70 mV throughout, by direct current injection). Brief (80 ms) hyperpolarizing current pulses (−0.47 nA) were applied at 1 Hz. The control GABA response was very reproducible (a) and after 3, 8, 15 or 32 min superfusion with zinc exhibited a progressive enhancement. An increase in input resistance and spontaneous cell firing were also induced by zinc. (c) Partial recovery of membrane properties and GABA responses after zinc washout. (d) Plot of peak GABA-evoked conductance increase (left ordinate, ●) and resting input conductance (right ordinate, ○) against time. Zinc was applied at time zero and washed out after 32 min. The onset of the GABA enhancement was closely mirrored by the fall in input conductance.

Inhibition of GABA responses by zinc: a role for GABA$_A$ receptor differentiation?

On the basis of our original observations, GABA responses recorded from rat SCG were assumed to be resistant to the actions of zinc. However, the present data from dissociated cultures of foetal ganglionic neurones clearly resolved an inhibition by zinc of the GABA response. We analysed this apparent anomaly by choosing to study cultured and intact ganglionic neurones from rats of different ages, to decide if the zinc inhibitory action was dependent on neuronal age or merely a susceptibility developed only by cultured neurones. The data indicated that zinc is an effective antagonist of foetal or young post-natal GABA responses whether recorded from intact or cultured neurones, but as the neurones mature, the GABA response becomes apparently less susceptible to zinc inhibition and eventually may become resistant. The latter is also independent of whether or not the neurones are cultured or resident in an intact preparation. This suggested that the GABA$_A$-receptor may undergo a subtle modification with time, rendering the receptor less susceptible to zinc inhibition, even though it apparently still possesses a sensitivity to bicuculline and barbiturates (unpublished observations).

Alternatively, zinc may be resolving a different population of GABA receptors which are expressed preferentially at a later stage in neuronal development. At present, these two possibilities are indistinguishable from our experimental evidence.

The discovery of heterogeneity in the GABA$_A$-receptor subunits (Levitan *et al.*, 1988; Wisden *et al.*, 1988; Pritchett *et al.*, 1989), particularly the α subunit, with regard to the age of the animal and distribution within the CNS, suggests an intriguing explanation for zinc inhibition of GABA responses. The α_3 subunit seems prominent in the CNS of young calves, whereas α_1 and α_2 do not show much variation with age (Levitan *et al.*, 1988). If a similar situation occurs with other species of neural tissue (cf. Pritchett *et al.*, 1989; Fuchs & Sieghart, 1989; Lolait *et al.*, 1989), then we might predict that a GABA receptor of subunit composition $\alpha_3\beta$ would be more sensitive to zinc compared to $\alpha_1\beta$ or $\alpha_2\beta$ which should be relatively insensitive to zinc. This does not discount the possibility that heterogeneity in other subunits, e.g., β or γ may also be important in conferring a sensitivity on the GABA$_A$-receptor to zinc block.

Previous studies have observed many different effects of zinc on GABA responses, including a competitive inhibition on frog dorsal root ganglion neurones (Akaike *et al.*, 1987). This competitive-type inhibition was not observed in the present experiments where the zinc block was insurmountable when the agonist concentration was raised. These mature amphibian neurones clearly respond differently to zinc compared to mature mammalian ganglia. In foetal mouse hippocampal neurones, zinc produced a non-competitive blockade of GABA responses (Westbrook & Mayer, 1987) at similar concentrations to those used in the present study. The block was also apparently voltage-independent over the range +40 to −120 mV, which would support our conclusion from current-voltage plots, that zinc is a voltage-independent blocker of the GABA$_A$-receptor complex on sympathetic neurones (cf. Figure 3b).

The non-competitive depression of the sympathetic GABA response by zinc, observed in our study, is likely to be a genuine effect on the receptor complex (preliminary observations with fluctuation analysis, suggest that zinc reduces the probability of opening (P_0) for the GABA channel, rather than the single channel conductance) and not due to complexation of GABA by zinc for two reasons: firstly, the dose-response curves do not match those expected following a theoretical consideration of various complexation models. These predict a far greater depression at low agonist doses and little if any at higher agonist concentrations (Smart & Constanti, 1982). Secondly, the minimal inhibition of GABA responses in some adult neuronal tissues and the apparent enhancement in pyriform cortex also argue against any role for complexation masquerading as a genuine inhibition. One other study on vertebrate neurones has also observed a block by zinc of GABA responses, monitored as a reduction in the GABA-induced depolarization of cat primary afferent nerve terminals (Curtis & Gynther, 1987). Interestingly, other divalent cations including cobalt, nickel and cadmium, reproduced the effect of zinc on the spinal afferent. However, in the same preparation, the GABA-induced depression in the firing rate of spinal interneurones was completely unaffected by these cations (zinc was apparently untested; Curtis & Gynther, 1987). Apart from these studies, zinc has been shown to have either no effect on GABA responses or to induce an enhancement. Hori *et al.* (1987) observed little effect of zinc on ionophoretic GABA responses recorded from pyriform cortex slices. However, their incubation time for zinc was quite short (10 min) and our present study, also in pyriform cortex slices, indicates that an incubation time of at least 10–20 min is required before the apparent enhancement effect of zinc is observed. Wright (1984)

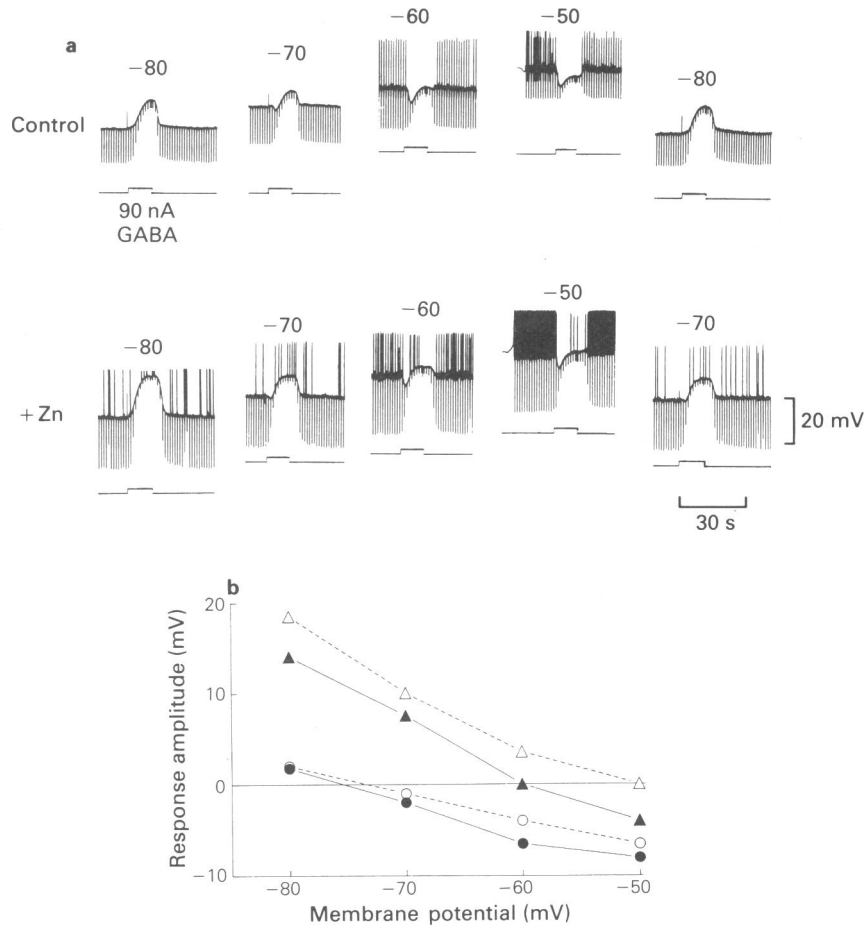


Figure 10 Effect of zinc on the reversal level of GABA responses. (a) Control (upper panel) ionophoretically-evoked GABA responses (90 nA, 10 s) recorded from an olfactory neurone held at different membrane potentials (−80 to −50 mV). Negative current pulses (−0.47 nA, 80 ms, 1 Hz) were applied throughout, to monitor membrane resistance. The biphasic character of the response was revealed at −70 mV, while at −50 mV, both early and late phases were clearly hyperpolarizing. (Lower panel) corresponding GABA responses obtained after 20 min in 300 μM zinc. (b) Plot of GABA response amplitude against membrane potential for same cell as in (a). Responses were measured at latencies of 3 (●) and 10 s (▲) in control solution, and 2 (○) and 10 s (△) after 22 min in zinc. The implied reversal level of the late depolarization was shifted from −60 to −50 mV in the presence of zinc.

also found that zinc did not affect GABA responses in the rat cortex *in vivo*, although zinc was applied ionophoretically for only 1 min.

Enhancement of GABA responses by zinc: a role for dendritic leak conductances?

We previously proposed that the enhancement of GABA responses in cortical neurones by zinc was due to a specific modulatory action of zinc at the postsynaptic $GABA_A$-receptor (Smart & Constanti, 1983). However, in the present study, the enhancement was reproduced by barium (and to some extent by cadmium) which requires a different, more general explanation. All of these divalent cations depolarized and reduced the resting input conductance. The latter effect persisted even when the membrane potential was manually 'clamped', although the identity of the affected ionic component is unresolved. The reduction of the leak conductance by divalent cations, coupled with the biphasic nature of the GABA response (see below) could underlie the apparent GABA enhancement, particularly since the delay in development of a clear enhancement (sometimes up to 30 min for zinc) was always temporally correlated with the change in resting conductance.

Biphasic (Cl^- dependent) responses to GABA have been recorded on a wide variety of central neurones (Alger & Nicoll, 1982; Scharfman & Sarvey, 1987; Thomson, 1988; Connors *et al.*, 1988). The early fast hyperpolarizing response probably reflects a somatic action of GABA, while the later depolarizing component reflects activation of dendritic receptors. The marked difference in the reversal level of the two response types is then thought to reflect an actively maintained difference in transmembrane Cl^- gradient in the dendrites, compared with the soma (Misgeld *et al.*, 1986; Thompson *et al.*, 1988). The reversal levels determined for these biphasic components (−76 and −60 mV, respectively) are very similar to those measured for comparable chloride-mediated GABA responses in hippocampal cells (Newberry & Nicoll, 1985; Misgeld *et al.*, 1986). Zinc and barium produced a consistent positive shift in the apparent reversal level of the late depolarization, which became the dominant component of the response. Some increase in the amplitude of the potential changes induced by GABA (particularly dendritic) might be expected simply from the zinc-induced decrease in the resting conductance (Ginsborg, 1967). However, the enhanced conductance increases measured during GABA responses, could only be explained if an extra component of conductance was now being added to the total input conductance measured in the soma. It is probable that the ionophoretic GABA pipette, positioned close to the intracellular (somatic) recording electrode, was releasing GABA onto somatic and proximal dendritic regions (bath-applied GABA would presumably access all sensitive regions simultaneously). If zinc or barium were inactivating a component of the resting conductance located on dendritic membranes, then this could allow a

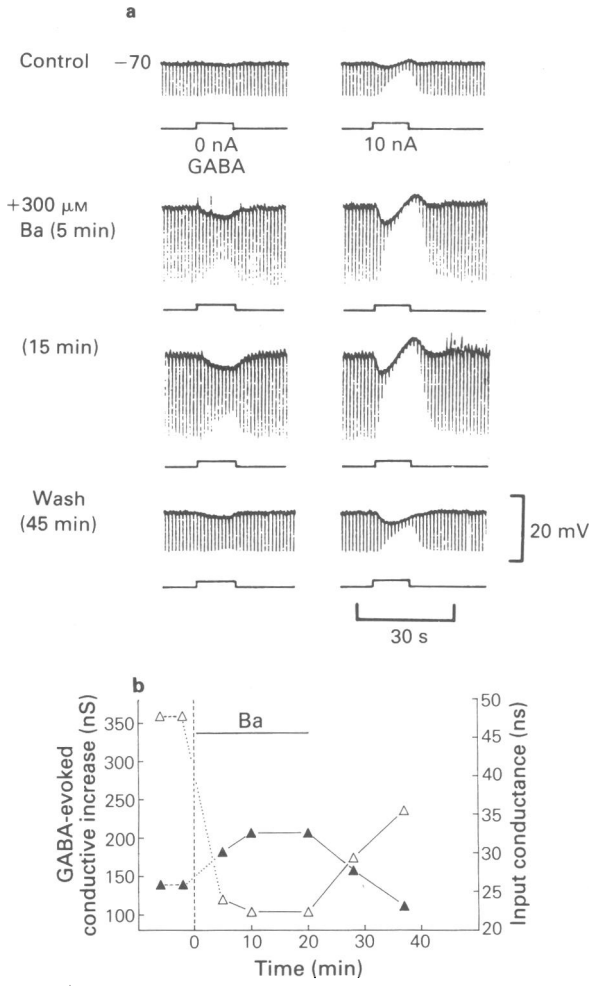


Figure 11 Enhancement of GABA responses by barium (Ba). (a) Two ionophoretic responses to GABA (0 nA = removal of retaining current, and 10 nA, 10 s) were applied to an olfactory neurone maintained at −70 mV throughout. Upper records, control responses, then responses elicited after 5 or 15 min in barium solution; lower records were obtained 45 min after barium washout. Barium induced a dramatic increase in cell input resistance and a clear (reversible) enhancement of GABA effects (injected current pulses = −0.45 nA, 150 ms, 1 Hz). (b) Peak GABA-evoked conductance increase (left ordinate, ▲) and resting conductance (right ordinate, △) plotted against time. Barium (300 μM) was applied for 20 min from time zero. The GABA response enhancement and change in input conductance induced by barium followed a similar time course.

greater proportion of the dendritic GABA response to be resolved back in the soma. A blocking effect of barium on resting dendritic K^+ conductances has been demonstrated by Redman *et al.* (1987) in sympathetic ganglia. Interestingly, Newberry & Nicoll (1985) also observed an enhancement of ionophoretic (somatic) GABA responses on hippocampal CA1 cells in the presence of barium (1 mM), although the underlying GABA conductance was not measured.

The initial enhancing effect of cadmium on the adult cortical GABA response could also be explained by an action on the resting input conductance. However, at later times, membrane conductance increased, presumably diminishing the contribution of dendritic GABA conductances to the total response, which would then be manifest as an inhibition.

A physiological role for zinc?

Zinc is known to be preferentially concentrated in specific neuronal structures including the hippocampus and cortex (Crawford & Connor, 1972; Donaldson *et al.*, 1973; Friedman & Price, 1984). Zinc can also be taken up into neuronal tissue (Wolf *et al.*, 1984; Wensink *et al.*, 1988) and high concentrations have been observed in nerve terminals, particularly in the hippocampal mossy fibre terminations (Ibata & Otsuka, 1969; Perez-Clausell & Danscher, 1985) and also within terminal boutons in many other regions of the telencephalon (Perez-Clausell & Danscher, 1985). Electrical or chemical stimulation of the mossy fibres has revealed the presence of a calcium-dependent 'releasable' pool of zinc (Assaf & Chung, 1984; Howell *et al.*, 1984; Charlton *et al.*, 1985) supporting the suggestion that zinc may be released during 'normal' neurotransmission playing a physiological/neuromodulatory role.

Our data indicate that if zinc does interact with the $GABA_A$-receptor *in vivo*, then this is more likely to occur in young 'immature' neurones. Recent studies suggest that neurotransmitters including GABA and glutamate, may influence the growth and development of nerve fibres (Mattson, 1988), but it remains to be seen if zinc and GABA can indeed modulate neuronal development and synaptic plasticity.

In conclusion, it is suggested that the vertebrate $GABA_A$-receptor may undergo a subtle modification with time, rendering the receptor less sensitive to zinc. In addition, agents which block dendritic leak channels may actually enhance the recorded ligand-gated conductances when these conductances are spatially spread from the soma to the dendrites.

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Effects on renal sympathetic axons in dog of acute 6-hydroxydopamine treatment in combination with selective neuronal uptake inhibitors

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1 In anaesthetized dogs, we have investigated the effect on renal responses to sympathetic nerve stimulation of acute treatment with the catecholaminergic neurotoxin 6-hydroxydopamine ($2\,mg\,kg^{-1}$ i.v.), administered alone or after blockade of neuronal catecholamine uptake pathways for noradrenaline (NA) or dopamine with desmethylimipramine or benztropine, respectively.

2 Under control conditions, renal nerve stimulation caused renal vasoconstriction, reduced glomerular filtration and sodium and water excretion and caused net efflux of NA and dopamine into the renal venous plasma. Two h after administration of 6-hydroxydopamine alone, there was abolition of both functional responses and catecholamine efflux during nerve stimulation.

3 In animals pretreated with desmethylimipramine ($1\,mg\,kg^{-1}$), 6-hydroxydopamine had no significant effect on functional responses to renal nerve stimulation and nerve-evoked efflux of NA was only moderately reduced. Efflux of dopamine was still markedly reduced by 6-hydroxydopamine, but more variably than occurred without desmethylimipramine treatment.

4 In animals pretreated with benztropine ($0.2\,mg\,kg^{-1}$), nerve-evoked efflux of dopamine, but not that of NA, was protected against reduction by 6-hydroxydopamine. A higher dose of benztropine ($1\,mg\,kg^{-1}$) protected efflux of both NA and dopamine against 6-hydroxydopamine.

5 We conclude that acute treatment with a low dose of 6-hydroxydopamine is an effective method of inactivating peripheral sympathetic nerves. The differential effects of desmethylimipramine and benztropine in preserving nerve-evoked efflux of NA and dopamine after 6-hydroxydopamine support the view that these catecholamines originate predominantly from different intrarenal axons. However, neither uptake blocker appears to be completely specific in its actions.

Introduction

Dopamine is a potent renal vasodilator in man and animals (Goldberg, 1972) and the correlation of urinary dopamine production with renal sodium excretion has led to suggestions that this catecholamine is an endogenous natriuretic factor (see Lee, 1987; Bell, 1987).

In 1973, Bell & Lang showed that electrical stimulation of specific sites within the forebrain of the anaesthetized dog activated a vasodilator pathway to the kidney, the effects of which were antagonised selectively by peripheral administration of the dopamine antagonist haloperidol. Since that time a substantial body of biochemical and morphological data has substantiated the presence of dopaminergic sympathetic axons in the renal nerves of the dog (e.g Bell *et al.*, 1978; Dinerstein *et al.*, 1983; Muller *et al.*, 1984; Bradley & Hjemdahl, 1984; Ferguson *et al.*, 1988; also see Bell, 1988 for summary). Ultrastructural studies of the distribution of dopaminergic axons within the kidney (Ferguson *et al.*, 1988) and the effects of dopamine receptor activation (Felder *et al.*, 1988) indicate that neurogenic dopamine is likely to influence both glomerular filtration and tubular sodium handling. On the other hand, attempts to activate the dopaminergic nerves selectively with a variety of reflex modalities have so far been unsuccessful (Hjemdahl *et al.*, 1988) and, as the renal tubular epithelium has the capacity to synthesize dopamine from filtered L-dihydroxyphenylalanine (L-DOPA) (Baines & Chan, 1980), it has been suggested that the dopamine involved in natriuresis is of non-neuronal origin (see Lee, 1988).

In order to resolve this situation, it would be desirable to prevent neurogenic release of intrarenal catecholamines pharmacologically during acute experiments on renal function. We have therefore investigated the effects of acute animal treatment with low doses of the catecholamine neurotoxin 6-hydroxydopamine (6-OHDA). Furthermore, as this neurotoxin gains entry to axons only via stereospecific uptake pathways (Kostrzewa & Jacobowitz, 1974) and these pathways differ between noradrenergic and dopaminergic axons (Horn, 1979), we have explored the possibility of selectively damaging either noradrenergic or dopaminergic axons by administering the neurotoxin in the presence of selective uptake inhibitors. The effects of these treatments on responses to electrical stimulation of the renal sympathetic nerves have been assessed in terms of both renal function and catecholamine efflux into renal venous blood.

Methods

Adult mongrel dogs of either sex (body mass $13 \pm 0.7\,kg$, $n = 24$) were anaesthetized with α-chloralose (initially $70\,mg\,kg^{-1}$ i.v.) following induction with sodium thiopentone and artificially ventilated under positive pressure, with a tidal volume nomogram published by Harvard Instruments.

Surgical preparation

Blood pressure and heart rate were recorded via a catheter inserted into a branch of the right femoral artery. A second arterial catheter was introduced into the base of the aorta via the left femoral artery for withdrawal of arterial blood samples. For sampling of renal venous blood, a catheter bearing a J-shaped tip was passed up the inferior vena cava from the right femoral vein and positioned in the left renal vein under direct observation through a small midline laparotomy.

Following the insertion of all catheters, the left kidney was exposed via a retroperitoneal flank incision and the left ureter was cannulated for urine collection. All visible nerves associated with the left renal artery were cut and the distal ends

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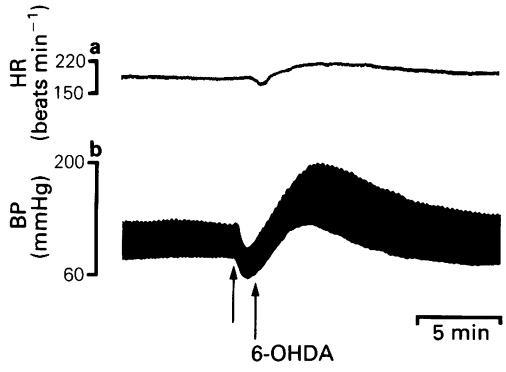


Figure 1 Recordings of (a) heart rate (HR) and (b) blood pressure (BP) in a chloralose-anaesthetized dog. At the first arrow, α- and β-adrenoceptor blockade was instituted by intravenous administration of phentolamine (0.5 mg kg^{-1}) and propranolol (0.1 mg kg^{-1}). At the second arrow, 6-hydroxydopamine (6-OHDA, 2 mg kg^{-1}) was administered by slow intravenous injection.

were passed through a pair of silver loop stimulating electrodes. The stimulation site was insulated and immobilized by immersion in a 1:1 mixture of vaseline and paraffin wax (m.p. 45°C). Biphasic square wave stimulating pulses of 1 ms duration were delivered at supramaximal voltage at 5 Hz for periods of 5 min.

Experimental protocol

At the completion of surgery, all dogs received a saline load of 5 ml kg^{-1} i.v. and a bolus of 50 mg kg^{-1} inulin (25 mg ml^{-1}). A constant infusion of 0.75 mg kg^{-1} inulin at the same concentration was then begun. A stabilization period of at least 1 h was then allowed and the criterion for commencement of the experiment was a stable urine production rate of at least 0.05 ml min^{-1} from the left kidney. Experimental urine collection periods of 5–10 min were used and resting blood samples were taken at the end of the urine collection periods and no more than 5 min before commencement of the next period of renal nerve stimulation. During nerve stimulation, blood samples were taken during the 4th min of stimulation. All blood samples were 8 ml in volume and were withdrawn at constant rates of 1 ml s^{-1} for arterial and 0.2 ml s^{-1} for venous blood. Consecutive periods of stimulation were separated by at least 25 min.

After collection of control resting and stimulation samples, some animals were treated with desmethylimipramine (1 mg kg^{-1}) (Geigy) or benztropine (0.2 or 1 mg kg^{-1}) (Merck, Sharp & Dohme) 30 min before the next collection period. All animals were then administered 6-OHDA bromide (Sigma), freshly dissolved in 10–20 ml normal saline containing 1 mg ml^{-1} ascorbic acid, as a slow (2–4 min) i.v. injection. Because 6-OHDA in these concentrations exerted considerable direct effects on cardiovascular adrenoceptors, all animals were premedicated immediately beforehand with phentolamine (0.5 mg kg^{-1}) and propranolol (0.2 mg kg^{-1}). After a period of between 1 and 2 h had been allowed for 6-OHDA to act, further responses to stimulation were assessed. In preliminary experiments with two dogs, we ascertained that nerve-evoked catecholamine efflux and renal vasoconstriction were not affected 2 h after treatment with phentolamine and propranolol alone.

Assays

Blood samples were placed on ice in tubes containing 900 μg EDTA and 600 μg reduced glutathione. Dihydroxybenzylamine was added to aliquots of plasma as an internal standard (16 ng ml^{-1} sample) and the catecholamines were extracted by adsorption onto alumina under alkaline conditions and re-elution into 200 μl 0.1 M perchloric acid, as described previously (Petrovic *et al.*, 1986). They were then separated by reverse phase liquid chromatography (LiChrosorb, Merck) and assayed by oxidative electrochemistry. The results were analysed by a computerised integrator (Shimadzu CR 1B), corrected for extractive losses and expressed as mass of catecholamine free base. Recoveries were between 40% and 70% and the minimum amounts of catecholamine that could be detected corresponded to plasma levels of 5–10 pg ml^{-1}. Nerve-evoked efflux of catecholamines from the kidney was calculated in terms of the net change in venous-arterial plasma concentrations of amine between resting conditions and the relevant stimulation period. As has been justified previously (Petrovic *et al.*, 1988), no corrections were made for possible extraction losses of arterial amines during passage through the kidney.

Inulin concentrations in aliquots of plasma (200 μl) and urine (10 μl) were assayed by the anthrone reaction (Fuhr *et al.*, 1955). Clearance of inulin was taken as glomerular filtration rate (GFR) and renal arteriovenous concentration difference were used to calculate renal plasma flow. Plasma and urine concentrations were measured by flame photometry. Statistical differences of population means were calculated by the two-tailed paired Student's t test and the Wilcoxon matched-pairs signed-rank test.

Results

For all dogs used, initial resting blood pressure was 130 ± 6 mmHg and heart rate was 130 ± 11 beats min^{-1}. Resting renal plasma flow was 5.5 ± 0.9 ml min^{-1} kg^{-1}, GFR 1.40 ± 0.12 ml min^{-1} kg^{-1}, urine flow 0.01 ± 0.003 ml min^{-1} kg^{-1} and urinary sodium excretion 0.37 ± 0.08 mmol min^{-1} kg^{-1}. Absolute resting plasma levels of noradrenaline (NA) and dopamine were 1.056 ± 0.167 ng ml^{-1} and 0.063 ± 0.020 ng ml^{-1} respectively for arterial blood and 0.555 ± 0.090 ng ml^{-1} and 0.094 ± 0.027 ng ml^{-1} for renal venous blood. Arterial and venous values were not signifi-

Table 1 Renal function data in anaesthetized dogs at rest and during renal nerve stimulation, before and 2 h after administration of 6-hydroxydopamine (6-OHDA, 2 mg kg^{-1})

	Control		*6-OHDA*	
	Rest	*Stimulation*	*Rest*	*Stimulation*
GFR (ml min^{-1} kg^{-1})	1.52 ± 0.12	0.25 ± 0.06***	1.67 ± 0.39	1.44 ± 0.47
Renal plasma flow (ml min^{-1} kg^{-1})	4.64 ± 0.94	1.54 ± 0.27**	5.59 ± 1.88	5.95 ± 2.38
Urine flow (ml min^{-1} kg^{-1})	0.011 ± 0.003	0.002 ± 0.001*	0.012 ± 0.007	0.005 ± 0.001
Sodium excretion (mmol min^{-1} kg^{-1})	0.55 ± 0.12	0.08 ± 0.01*	0.79 ± 0.29	0.58 ± 0.23

Data shown are means ± s.e.mean (n = 5). * $P < 0.05$, ** $P < 0.02$, *** $P < 0.01$, different from corresponding values at rest by paired Student's t test.

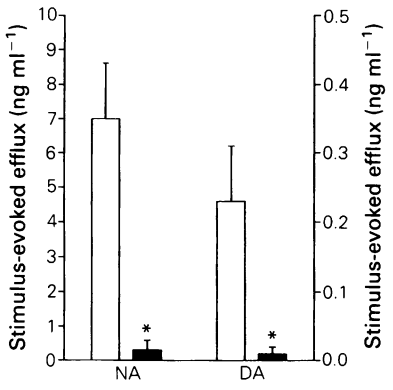


Figure 2 Stimulus-evoked efflux from the kidney of noradrenaline (NA) and dopamine (DA) under control conditions (open columns) and 2 h after administration of 2 mg kg^{-1} 6-hydroxydopamine (solid columns). Asterisks indicate significant reductions ($P < 0.05$, Student's t test) in efflux following drug treatment ($n = 5$).

cantly different for either NA or dopamine, in agreement with previous findings that there is no net efflux of catecholamine from the acutely denervated kidney (Petrovic *et al.*, 1988).

Effects of renal nerve stimulation

Renal nerve stimulation had no consistent effect on blood pressure or heart rate but, for all dogs used, it reduced renal plasma flow by 77% to a mean of 1.13 ml min^{-1} kg^{-1}, GFR by 86% to 0.18 ± 0.03 ml min^{-1} kg^{-1}, urine flow by 80% to 0.002 ± 0.000 ml min^{-1} kg^{-1} and sodium excretion by 77% to 0.14 ± 0.06 mmol min^{-1} kg^{-1}. During nerve stimulation, arterial catecholamine levels did not change significantly but renal venous levels of both NA and dopamine rose substantially. Calculation of differences at rest and during stimulation indicated that stimulus-evoked efflux of NA was 5.39 ± 0.85 ng ml^{-1} and stimulus-evoked efflux of dopamine was 0.28 ± 0.08 ng ml^{-1}, giving a dopamine:NA ratio for stimulus-evoked efflux of 0.05. Mean stimulus-evoked outputs of catecholamines, calculated as the product of amine efflux and renal plasma flow, were 102 ± 30 ng min^{-1} NA and 4.0 ± 1.4 ng min^{-1} dopamine, data that are similar to those obtained in previous studies (Bradley & Hjemdahl, 1984; Petrovic *et al.*, 1988).

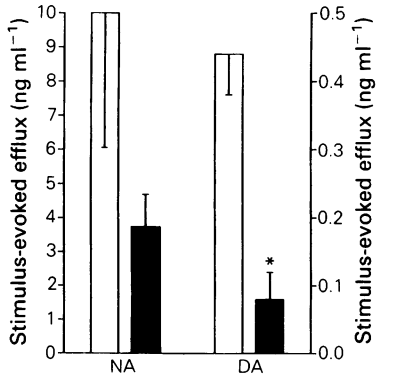


Figure 3 Stimulus-evoked efflux from the kidney of noradrenaline (NA) and dopamine (DA) in animals pretreated with desmethylimipramine 1 mg kg^{-1} (open columns) and 2 h after subsequent administration of 2 mg kg^{-1} 6-hydroxydopamine (6-OHDA) (solid columns). The asterisk denotes significant reduction of efflux ($P < 0.05$, Student's t test) following 6-OHDA treatment ($n = 6$).

Effects of 6-OHDA treatment

In 6 animals, 6-OHDA was administered alone in a dose of 2 mg kg^{-1}. Even in the presence of adrenoceptor antagonists, this was followed by a substantial rise in heart rate and blood pressure which returned to resting values over about 15 min (Figure 1). Two h after 6-OHDA, resting blood pressure was 122 ± 17 mmHg, heart rate was 163 ± 33 beats min^{-1} and GFR, renal plasma flow and urine production were all unchanged from the control values in the same animals (Table 1). However, renal nerve stimulation now produced no fall in renal blood flow or renal sodium or water excretion (Table 1). Also, there was no stimulus-evoked rise in blood catecholamines (Figure 2) and calculated renal catecholamine production fell from 205 ± 71 to 96 ± 34 ng min^{-1} for NA and from 4.8 ± 2.0 to −3.1 ± 2.8 ng min^{-1} for dopamine. In 2 of these animals, the effects of 6-OHDA were assessed after one as well as after 2 h. With the shorter time interval, nerve stimulation-evoked changes in renal function and catecholamine output were less than under control conditions, but were still appreciable. All further experiments therefore utilized a 2 h treatment period.

Effect of desmethylimipramine treatment on the response to 6-OHDA

Seven animals were pretreated with desmethylimipramine (DMI) 1 mg kg^{-1}, 30 min before 6-OHDA administration. DMI alone had no significant effect on resting cardiovascular or renal functions, but enhanced the renal vasoconstrictor response to nerve stimulation (Table 2). In addition, it enhanced the stimulus-evoked release of NA, from 2.56 ± 1.08 ng ml^{-1} to 10.00 ± 3.94 ng ml^{-1} ($P < 0.05$). Since stimulus-evoked dopamine efflux after DMI (0.44 ± 0.06 ng ml^{-1}) was not significantly different from that under control conditions (0.30 ± 0.18 ng ml^{-1}), the elevation of NA efflux was due to increased overflow as well as to the concentrating effect of reduced blood flow.

Pretreatment with DMI caused virtually complete protection of functional neurogenic renal responses against

Table 2 Renal function data in anaesthetized dogs before and after administration of 6-hydroxydopamine (6-OHDA, 2 mg kg^{-1}) in the presence of desmethylimipramine (1 mg kg^{-1})

	Control		*6-OHDA*	
	Rest	*Stimulation*	*Rest*	*Stimulation*
GFR (ml min^{-1} kg^{-1})	1.39 ± 0.31	0.11 ± 0.04**	1.02 ± 0.27	0.41 ± 0.23*
Renal plasma flow (ml min^{-1} kg^{-1})	4.07 ± 1.33	0.48 ± 0.24*	2.65 ± 0.76	1.43 ± 0.56*
Urine flow (ml min^{-1} kg^{-1})	0.008 ± 0.002	0.001 ± 0.000*	0.007 ± 0.002	0.004 ± 0.001*
Sodium excretion (mmol min^{-1} kg^{-1})	0.69 ± 0.33	0.14 ± 0.11	0.53 ± 0.28	0.33 ± 0.19*

Data shown are mean ± s.e.mean ($n = 7$). * $P < 0.05$, ** $P < 0.01$, different from corresponding values at rest by paired Student's t test.

6-OHDA, as 2h after 6-OHDA administration renal nerve stimulation caused falls in renal plasma flow, GFR and urine production that were similar in magnitude to those seen under control conditions in the same animals (Table 2). Stimulus-evoked NA efflux was still reduced from pre-6-OHDA values, to $3.74 \pm 0.95\,ng\,ml^{-1}$, but this degree of reduction (58%) was significantly less than that after 6-OHDA alone ($P < 0.01$) and not significantly different from the efflux obtained before DMI administration (Figure 3). Furthermore, stimulus-evoked NA production was not reduced at all (138 ± 24 cf. $81 \pm 16\,ng\,min^{-1}$). By contrast with the preservation of NA overflow, dopamine efflux was still attenuated considerably by 6-OHDA after DMI pretreatment when expressed either in terms of venous dopamine levels (Figure 2) or in terms of dopamine production (1.7 ± 3.2 cf. $5.6 \pm 2.2\,ng\,min^{-1}$, $P < 0.05$). However, the effect of 6-OHDA on dopamine efflux after DMI treatment varied more between animals than was the case with 6-OHDA treatment alone.

Effects of benztropine on responses to 6-OHDA

The effects of treatment with the dopamine uptake blocking agent benztropine 30 min before 6-OHDA was examined in detail at 2 dose levels, 0.2 and $1\,mg\,kg^{-1}$. Neither of these doses of benztropine had any significant effects on resting cardiovascular or renal function, or on nerve-evoked changes in renal function or catecholamine efflux from the kidney (Table 3).

With both 0.2 and $1\,mg\,kg^{-1}$ benztropine pretreatment, nerve stimulation 2h after 6-OHDA had no consistent effect on renal blood flow or urine production, similar to the situation after 6-OHDA alone (Table 3). In 2 of 5 animals that received $0.2\,mg\,kg^{-1}$ and 3 of 6 animals that received $1\,mg\,kg^{-1}$ benztropine, nerve stimulation was accompanied by increases in renal plasma flow of about 20%. However, these changes were not accompanied by measurable increases of GFR or of water or sodium excretion. After pretreatment with $0.2\,mg\,kg^{-1}$ benztropine, stimulus-evoked production of dopamine was no longer affected significantly by 6-OHDA (4.7 ± 2.1 cf. $8.4 \pm 1.2\,ng\,min^{-1}$, $P > 0.20$), although the absolute level of dopamine in renal venous effluent was still reduced from control (Figure 4a), probably due in part to the absence of vasoconstriction. Nerve-evoked NA efflux was reduced substantially by 6-OHDA when assessed as either peak venous levels attained (Figure 4a) or as NA production (52 ± 14 cf. $141 \pm 20\,ng\,min^{-1}$, $P < 0.05$).

After pretreatment with $1\,mg\,kg^{-1}$ benztropine, stimulus-evoked production of either catecholamine was not reduced by 6-OHDA (NA 119 ± 50 cf. $119 \pm 49\,ng\,min^{-1}$, dopamine 3.9 ± 1.0 cf. $2.6 \pm 1.5\,ng\,min^{-1}$). However, comparison of absolute levels of each substance in renal venous blood showed that, while NA efflux was reduced by 77%, from $5.79 \pm 2.4\,ng\,ml^{-1}$ to $1.39 \pm 0.59\,ng\,ml^{-1}$ ($P < 0.05$), dopamine efflux was not significantly altered from that during nerve stimulation in the control period ($0.074 \pm 0.030\,ng\,ml^{-1}$ cf. $0.124 \pm 0.074\,ng\,ml^{-1}$ before 6-OHDA) (Figure 4b).

In each of an additional 2 dogs pretreated with $3\,mg\,kg^{-1}$ benztropine, neurogenic vasoconstrictor responses and near-normal stimulus-evoked overflow of both NA and dopamine persisted after 6-OHDA treatment.

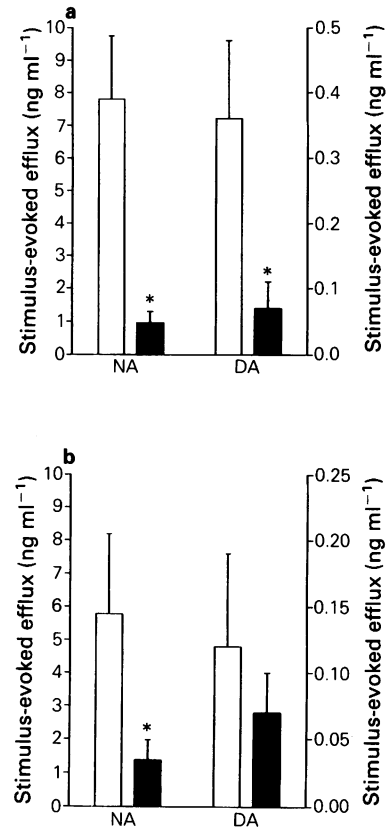


Figure 4 Stimulus-evoked efflux from the kidney of noradrenaline (NA) and dopamine (DA) in animals pretreated with benztropine (a) 0.2 or (b) $1\,mg\,kg^{-1}$ (open columns) and 2h after subsequent administration of $2\,mg\,kg^{-1}$ 6-hydroxydopamine (6-OHDA) (solid columns). Asterisks indicate significant reductions ($P < 0.05$, Student's t test) in efflux following 6-OHDA treatment ($n = 5$ for each dose level of benztropine).

Discussion

Stimulation of the renal sympathetic nerves at 5 Hz reduced renal plasma flow, glomerular filtration rate and sodium and

Table 3 Renal function data in anaesthetized dogs before and after 6-hydroxydopamine (6-OHDA, $2\,mg\,kg^{-1}$) in the presence of benztropine (0.2 or $1.0\,mg\,kg^{-1}$)

	Dose benztropine ($mg\,kg^{-1}$)	*Control*		*6-OHDA*	
		Rest	*Stimulation*	*Rest*	*Stimulation*
GFR	0.2	1.46 ± 0.42	0.14 ± 0.04†*	1.90 ± 0.70	1.47 ± 0.24
($ml\,min^{-1}\,kg^{-1}$)	1.0	1.38 ± 0.30	0.34 ± 0.05†**	1.37 ± 0.25	1.28 ± 0.35
Renal plasma flow	0.2	5.65 ± 1.7	1.87 ± 0.48†	6.93 ± 2.17	5.59 ± 1.07
($ml\,min^{-1}\,kg^{-1}$)	1.0	5.08 ± 1.24	1.58 ± 0.31†*	5.02 ± 1.94	7.91 ± 3.68
Urine flow	0.2	0.01 ± 0.004	0.002 ± 0.001†	0.007 ± 0.001	0.009 ± 0.002
($ml\,min^{-1}\,kg^{-1}$)	1.0	0.02 ± 0.01	0.006 ± 0.003†	0.006 ± 0.001	0.006 ± 0.001
Sodium excretion	0.2	0.27 ± 0.06	0.06 ± 0.01†*	0.41 ± 0.06	0.39 ± 0.05
($mmol\,min^{-1}\,kg^{-1}$)	1.0	0.82 ± 0.32	0.21 ± 0.05†	0.72 ± 0.45	0.65 ± 0.38

Data shown are means ± s.e.mean ($n = 5$).
† $P < 0.05$, different from corresponding values at rest by Wilcoxon test.
* $P < 0.05$, different from corresponding values at rest by paired Student's t test.

water excretion, in line with the documented effects on renal function of noradrenergic nervous activation (Di Bona, 1982; Kopp *et al.*, 1983). These effects were accompanied by a net overflow into the renal venous bloodstream of NA and dopamine. Nerve-evoked efflux of dopamine from the dog kidney is well documented (Kopp *et al.*, 1983; Bradley & Hjemdahl, 1984; Petrovic *et al.*, 1988) and the amounts detected, which are 5–10% of the total catecholamine released, are considerably greater than could be explained on the basis of release only from noradrenergic nerves (Kahan *et al.*, 1984; Petrovic *et al.*, 1988). The release of dopamine has therefore been interpreted by the above workers as evidence for the presence of renal dopaminergic nerves. However, an alternative explanation (see Soares-da-Silva, 1988) is that dopamine could be released by electrical activity from vesicular storage sites in preterminal noradrenergic axons.

Animal treatment with high doses of 6-OHDA ($30\,mg\,kg^{-1}$ or more) is a routine method of destroying catecholaminergic axon terminals, both in the periphery (e.g. Laverty *et al.*, 1965; Malmfors & Sachs, 1968; Tranzer & Thoenen, 1968) and in the central nervous system (e.g. Uretsky & Iversen, 1969; Laverty & Taylor, 1970). This action is secondary to displacement of endogenous NA and appears to be due to intra-axonal oxidation of 6-OHDA, with consequent production of free radicals and mitochondrial damage (Kostrzewa & Jacobowitz, 1974; Silvka & Cohen, 1985).

Recently, Soares-da-Silva and his co-workers have shown that chronic treatment with a much lower dose of 6-OHDA ($2\,mg\,kg^{-1}$) is also effective in depleting endogenous NA from sympathetic terminals but does not produce structural damage (Esplugues *et al.*, 1985; Soares-da-Silva & Davidson, 1985). In the present study, we found that acute administration of the same low dose of 6-OHDA abolished functional responses to renal nerve stimulation, and the associated efflux of catecholamines, within 2 h. At this time, resting cardiovascular and renal functions and resting plasma catecholamine levels were all normal. This protocol of 6-OHDA treatment therefore appears to be a selective way of inactivating sympathetic neuroeffector transmission in acutely prepared animals. It has considerable potential advantages over the use of postjunctional adrenoceptor antagonists, which usually block neurotransmission only incompletely (see Bell, 1985) and also over the adrenergic neurone blocking drug guanethidine, which elevates plasma catecholamines for some hours (C. Bell, unpublished observations). Furthermore, it offers an alternative to surgical denervation in acute experiments on either anaesthetized or conscious animals.

Entry of 6-OHDA into catecholaminergic axons relies on active transport by the membrane processes that are normally responsible for re-uptake of endogenous amine (Kostrzewa & Jacobowitz, 1974). This was confirmed by our observation that, following pretreatment with the $Uptake_1$ inhibitor DMI, 6-OHDA had no attenuating effect on vasoconstrictor or antinatriuretic responses to renal nerve stimulation, nor on nerve-evoked renal production of NA. The interaction of DMI and 6-OHDA on nerve-evoked efflux of dopamine was less consistent. In several animals the effect of 6-OHDA on dopamine appeared to be reduced in the presence of DMI, while in others dopamine release was totally abolished, similar to the effect of 6-OHDA alone. However, statistical analysis demonstrated significant protection of NA but not of dopamine production, suggesting that the two amines are released from distinct, 6-OHDA-sensitive stores.

The dopamine uptake inhibitor benztropine by itself had no significant effect on either renal function or on catecholamine overflow in the denervated kidney, either at rest or during nerve stimulation, and neither $0.2\,mg\,kg^{-1}$ nor $1\,mg\,kg^{-1}$ benztropine pretreatment consistently protected nerve-evoked functional renal responses against 6-OHDA. With the lower dose of benztropine ($0.2\,mg\,kg^{-1}$), there was also no protection of nerve-evoked NA overflow but, in contrast, substantial efflux of dopamine persisted after 6-OHDA. This interaction, like that of DMI and 6-OHDA, indicates that dopamine and NA originate from separate neuronal stores. Furthermore, the fact that benztropine protected dopamine but not NA release against 6-OHDA suggests that access to the dopamine storage sites relies on dopamine uptake ($Uptake_{DA}$) rather than $Uptake_1$ (Koe, 1976). Although it is not known whether $Uptake_{DA}$ is restricted absolutely to the membranes of dopaminergic neurones, this result seems most likely to be due to dopamine liberation from specific dopaminergic axons rather than from a dopamine pool able to be released from preterminal noradrenergic axons.

As exogenous dopamine exerts a natriuretic effect by renal vascular dilatation and by inhibition of tubular sodium reabsorption, neurogenic release of dopamine after the noradrenergic renal nerves had been inactivated might produce similar effects. In fact, renal nerve stimulation after benztropine plus 6-OHDA pretreatment increased renal plasma flow in several animals, but not in others. One explanation of this inconsistency would be that benztropine partially protected both noradrenergic and dopaminergic nerves. In general, $Uptake_{DA}$ antagonists have been found to exert some $Uptake_1$ blocking activity at doses that inhibit dopamine uptake (Horn *et al.*, 1971; Koe, 1976; Farnebo *et al.*, 1979; Garattini & Mennini, 1988) and we found some protection against 6-OHDA after pretreatment with $1\,mg\,kg^{-1}$ benztropine. In addition, a slightly higher dose ($3\,mg\,kg^{-1}$) appeared, on the basis of functional responses, to inhibit $Uptake_1$ substantially. We have previously shown that $10\,mg\,kg^{-1}$ benztropine alone elevates nerve-evoked renal efflux of both NA and dopamine (Petrovic & Bell, 1985).

Alternatively, our experimental conditions may not have been optimal for demonstration of dopamine-mediated natriuretic responses. For instance, variation in chronic volume status of the animals before experimentation might have altered responsiveness to dopamine, either directly or by effects on circulating factors such as atrial natriuretic peptide.

In summary, we have shown that acute treatment of anaesthetized dogs with a low dose of 6-OHDA is an effective method for producing functional sympathectomy. The relative changes in 6-OHDA-induced falls in NA and dopamine overflow following animal pretreatment with DMI or benztropine provide further evidence for release of the two catecholamines from separate populations of intrarenal nerves. However, neither DMI nor benztropine appeared to be completely selective in their effects on uptake pathways.

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Adrenergic-cholinergic interactions in left atria: a study using K^+ channel agonists, antagonist and pertussis toxin

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1 The role of activation of potassium conductance in the antagonism by the muscarinic agonist carbachol of positive inotropic responses to α- and β-adrenoceptor stimulation was studied in electrically driven left atrial strips of the rabbit.

2 The potassium channel antagonist, 4-aminopyridine, attenuated the direct negative inotropic response to carbachol and the reversal by carbachol of positive inotropic responses to the α-adrenoceptor agonist phenylephrine (in the presence of timolol). The inhibitory effect of carbachol on positive inotropic responses to the β-adrenoceptor agonist isoprenaline was much less affected by 4-aminopyridine.

3 Pretreatment of rabbits with pertussis toxin also attenuated the direct negative inotropic response to carbachol and the inhibitory effect of carbachol on positive inotropic responses to phenylephrine.

4 Neither carbachol nor phenylephrine, alone or in combination, had any effect on left atrial adenosine 3':5'-cyclic monophosphate (cyclic AMP) levels.

5 The potassium channel agonist, pinacidil, exerted a dose-dependent negative inotropic response in rabbit left atria and reversed positive inotropic responses to phenylephrine and isoprenaline. In the dose-range tested, pinacidil had a greater inhibitory effect on positive inotropic responses to phenylephrine than on positive inotropic responses to isoprenaline.

6 Pretreatment of left atria with pinacidil or cromakalim, another potassium channel agonist, antagonized positive inotropic responses to phenylephrine but not to isoprenaline.

7 These results suggest that activation of potassium conductance plays an important role in the inhibition by carbachol of positive inotropic responses of rabbit left atria to phenylephrine but not to isoprenaline.

Introduction

The functional and biochemical consequences of muscarinic receptor activation in the heart are complex, and appear to vary with the species and the region of the heart being investigated (Loffelholtz & Pappano, 1985). In left atria, stimulation of muscarinic receptors results in a marked, cyclic nucleotide-independent, direct negative inotropic effect. In addition, we have recently demonstrated that the muscarinic agonist carbachol is able to antagonize or reverse positive inotropic responses of rabbit left atria to α- and β-adrenoceptor agonists, as well as to the adenylate cyclase activator, forskolin and the phosphodiesterase inhibitor, isobutylmethyl xanthine (IBMX) (MacLeod, 1986; 1987; MacLeod & Diamond, 1986; Ray and MacLeod, unpublished observations).

The direct negative inotropic response of mammalian left atria to muscarinic receptor stimulation is believed to be due primarily to the ability of muscarinic agonists to activate an outward potassium current (TenEick *et al.*, 1976; Cerbai *et al.*, 1988). Activation of this current shortens the duration of the atrial action potential, thus indirectly reducing the influx of calcium during excitation. Recently it has been demonstrated that muscarinic receptors in left atrium are linked directly to potassium channels by means of a pertussis toxin-sensitive guanine nucleotide-binding protein (G-protein) (Pffafinger *et al.*, 1985). In contrast, the mechanism of interaction of muscarinic agonists with positive inotropic agents in left atria has not been completely elucidated. Muscarinic receptors are known to be coupled to adenylate cyclase by a pertussis toxin-sensitive G-protein, and muscarinic agonists have been demonstrated to reduce the increase in adenosine 3':5'-cyclic monophosphate (cyclic AMP) produced in response to β-adrenoceptor stimulation (Endoh *et al.*, 1985; Sorota *et al.*, 1985; MacLeod, 1986). However, in left atrium, the decrease in tension produced by muscarinic agonists in the presence of β-adrenoceptor agonists is greater than can be explained by the reduction in cyclic AMP levels alone (Brown *et al.*, 1980; Endoh *et al.*, 1985; MacLeod, 1986). In addition, the muscarinic antagonism of positive inotropic responses to α-adrenoceptor stimulation occurs via a cyclic AMP-independent process (MacLeod 1986), while the antagonism by carbachol of positive inotropic responses to forskolin and IBMX is not accompanied by lowering of the associated increases in cyclic AMP (MacLeod & Diamond, 1986; Ray & MacLeod, unpublished observations). Recently, it was postulated that carbachol may act by antagonizing calcium influx increased by α- and β- adrenoceptor agonists (MacLeod 1987), but the mechanism of this effect remains unknown.

The purpose of the work presented here was to investigate the hypothesis that muscarinic activation of potassium conductance contributes to the functional antagonism by carbachol of positive inotropic responses of rabbit left atria to the α-adrenoceptor agonist, phenylephrine, and the β-adrenoceptor agonist, isoprenaline. Two different approaches were used in this investigation. First, an attempt was made to interfere with the ability of carbachol to activate the potassium current, using a potassium channel antagonist, 4-aminopyridine and an uncoupler of muscarinic receptors from potassium channels, pertussis toxin. The effects of 4-aminopyridine on negative inotropic responses to carbachol alone and in the presence of isoprenaline and phenylephrine were compared. In addition, the influence of pretreatment of rabbits with pertussis toxin on direct negative inotropic responses to carbachol and on the reversal by carbachol of positive inotropic responses to phenylephrine was determined. Secondly, the effects of the potassium channel agonists, pinacidil and cromakalim, on positive inotropic responses of left atria to isoprenaline and to phenylephrine were investigated.

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We reasoned that if muscarinic activation of potassium conductance plays a role in the functional interaction of carbachol with phenylephrine or isoprenaline, then pinacidil and cromakalim should also antagonize positive inotropic responses to these agonists.

Methods

Isolated tissue preparation

Rabbits of either sex were housed individually and had free access to food and water. For experiments involving pertussis toxin, animals were injected with pertussis toxin (1.75 $\mu g\,kg^{-1}$) or normal saline through the ear vein, 48 h before the experiments. On the day of experiment, animals were killed by stunning and exsanguination. Hearts were rapidly removed and placed in modified Chenoweth-Koelle solution (composition, mM: NaCl 120, KCl 5.7, $CaCl_2$ 2.2, $MgCl_2$ 0.9, $NaHCO_3$ 25 and glucose 10) at room temperature, aerated with 95% O_2–5% CO_2. Left atria were removed from the heart, and were cut into 4–5 strips. One end of each atrial strip was attached to a bipolar platinum electrode which was placed in a 20 ml isolated tissue bath containing modified Chenoweth-Koelle solution maintained at 35°C and oxygenated with 95% O_2–5% CO_2. The other end of each muscle preparation was attached by a cotton thread to a Grass FT.03 force displacement transducer. Tissues were stimulated to contract at a frequency of 1.0 Hz with pulses of 5 ms duration at a voltage two times threshold. Responses were recorded on a Grass polygraph (model 7E). All preparations were initially placed under a resting tension of 0.5 g, and the resting tension was adjusted throughout the 60 min equilibration period to give the optimal basal developed tension.

Cumulative dose-response curves to carbachol alone or in the presence of 100 nM isoprenaline or 100 μM phenylephrine were obtained in atria under various conditions. All experiments involving phenylephrine were conducted in tissues pretreated with 1 μM timolol to block the β-adrenoceptor-mediated component of its response. To determine the influence of 4-aminopyridine on responses to carbachol, tissues were randomly assigned to receive either 4-aminopyridine (50 or 500 μM) or vehicle for 10 min, before a dose-response curve to carbachol alone or in the presence of isoprenaline or phenylephrine was obtained.

Cumulative dose-response curves to isoprenaline and phenylephrine (plus timolol) were also determined in the absence and presence of pinacidil and cromakalim. Tissues were pretreated with either vehicle (dimethylsulphoxide), cromakalim or pinacidil for 10 min before dose-response curves to isoprenaline or phenylephrine were obtained. To investigate further the effects of pinacidil, cumulative dose-response curves to this agonist were obtained in untreated tissues and in tissues pretreated with isoprenaline (100 nM) or phenylephrine (100 μM).

Cyclic nucleotide determination

The effects of phenylephrine and carbachol, alone and in combination, on cyclic AMP levels were determined. Tissues were divided into four groups: untreated preparations; preparations treated with 100 μM phenylephrine (in the presence of timolol) for 6 min; preparations treated with 30 μM carbachol alone for 3 min; and preparations treated with 100 μM phenylephrine for 6 min, with 30 μM carbachol being added for the final 3 min. Tissues were frozen with clamps cooled in liquid nitrogen and stored at −80°C until assayed. Cyclic AMP was assayed by use of radioimmunoassay kits as previously described (MacLeod, 1986).

Drugs

Isoprenaline hydrochloride, phenylephrine hydrochloride, timolol maleate and 4-aminopyridine were obtained from Sigma Chemical Co., St. Louis, U.S.A. Carbamylcholine chloride was obtained from Aldrich Chemical Co., Wisconsin, U.S.A. and pertussis toxin from List Biological Laboratories Ltd, California, U.S.A. Pinacidil and cromakalim were generous gifts from Eli-Lilly and Co., Indianapolis, U.S.A., and Beecham Pharmaceuticals, Surrey, U.K., respectively. Radioimmunoassay kits for cyclic AMP were obtained from New England Nuclear, Massachusetts, U.S.A. Unless otherwise specified, drugs were dissolved in water and further dilutions were made in modified Chenoweth-Koelle solution. The stock solution of isoprenaline also contained ascorbic acid (1 $mg\,ml^{-1}$) in order to minimise oxidation of isoprenaline. In some experiments pinacidil was dissolved in 0.1 N hydrochloric acid, as reported by Yanagisawa *et al.*, (1988), to obtain a stock solution and further dilutions were made in modified Chenoweth-Koelle solution.

Statistics

Data were analysed for significant differences by one way analysis of variance followed by Neumann-Keul's range test. Significant differences were considered to exist when $P < 0.05$.

Results

Effects of 4-aminopyridine

In preliminary experiments it was observed that 4-aminopyridine exerted a dose-dependent positive inotropic effect in rabbit atrium over a dose range of 50 μM to 1 mM. The effects of 50 and 500 μM 4-aminopyridine on negative inotropic responses to carbachol in the absence and presence of positive inotropic agents were determined.

The tension of left atrial strips in the presence of 50 μM 4-aminopyridine was not significantly different from that in its absence. In comparison, 500 μM 4-aminopyridine produced a much greater increase in contractile force (Figure 1a). Pretreatment of the left atrial strips with 50 μM 4-aminopyridine for 10 min caused a small rightward shift of the carbachol dose-response curve and depressed the maximum response to carbachol when this was expressed as a percentage of the tension before the addition of carbachol (Table 1). Increasing the dose of 4-aminopyridine to 500 μM produced a greater shift in the carbachol dose-response curve (Figure 1a,

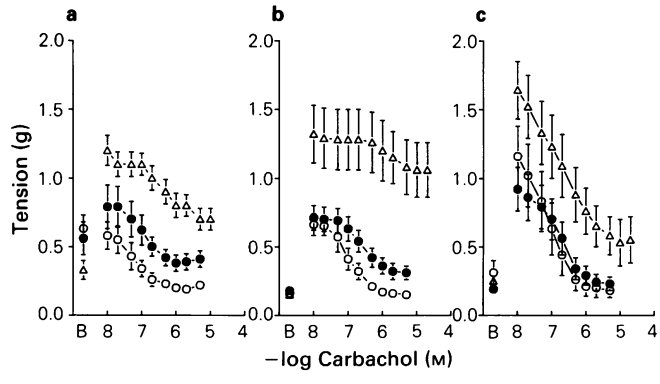


Figure 1 Effect of 4-aminopyridine treatment on negative inotropic responses to carbachol in the absence (a) and presence of phenylephrine (b) and isoprenaline (c) in isolated left atrial strips of rabbit. Left atrial strips were treated with normal saline (○), or 50 μM (●) or 500 μM (△) 4-aminopyridine either alone (a) or in combination with 100 μM phenylephrine plus 1 μM timolol (b) or 100 nM isoprenaline (c) prior to obtaining cumulative dose-response curves to carbachol. Tissues were treated with 4-aminopyridine for 10 min with phenylephrine or isoprenaline being added for the final 3 min. The contractile response is expressed as the tension in grams (g). On the abscissa scale, B represents the left atrial tension before the addition of drugs. Each curve represents mean of 5–10 experiments; s.e.mean shown by vertical bars.

Table 1 Effects of 4-aminopyridine on pD_2 values and maximum negative inotropic responses to carbachol in the presence and absence of positive inotropic agents

	Control†		*4-Aminopyridine*†			
			50 μM		500 μM	
Treatment	pD_2	*Tension* (% initial)	pD_2	*Tension*	pD_2	*Tension*
Carbachol	7.26 ± 0.03	31.0 ± 3.5	6.94 ± 0.05	50.7 ± 6.0	6.36 ± 0.20*	57.2 ± 8.8
Carbachol + phenylephrine (100 μM)	6.80 ± 0.17	23.4 ± 3.2	6.48 ± 0.07	40.1 ± 5.8*	5.86 ± 0.24*	79.5 ± 5.0*
Carbachol + isoprenaline (100 nM)	7.25 ± 0.20	12.6 ± 2.5	7.07 ± 0.18	21.8 ± 3.1	6.73 ± 0.31	26.3 ± 5.7*

* Significantly different from the control response within the same group.
† Data represent mean ± s.e.mean of 5–10 experiments.

Table 1), although the overall percentage tension remaining in the presence of a maximum concentration of carbachol was not further increased (Table 1). However, in three out of nine left atrial strips, 500 μM 4-aminopyridine shifted the carbachol dose-response curve to the right without having any inhibitory effect on the maximum negative inotropic response to carbachol. In these tissues, the tension remaining in the presence of the maximum concentration of carbachol was 26.1 ± 2.5% (mean ± s.e.mean) of the initial tension. In the remaining six tissues, the inhibitory effect of 500 μM 4-aminopyridine on the negative inotropic response to carbachol was much greater than that of 50 μM 4-aminopyridine. In this group of tissues, following treatment with 500 μM 4-aminopyridine the tension remaining in the presence of the maximum concentration of carbachol was 72.7 ± 6.3% of the initial tension.

Phenylephrine (100 μM) plus timolol produced a positive inotropic effect in atrial strips, which was completely reversed by carbachol (Figure 1b). The left atrial tension in the presence of 50 μM 4-aminopyridine plus phenylephrine was not different from that obtained with phenylephrine alone. However, in the presence of the combination of 50 μM 4-aminopyridine and phenylephrine, the carbachol dose-response curve was shifted slightly to the right and the maximum negative inotropic response to carbachol was significantly attenuated (Figure 1b, Table 1). The positive inotropic effect of 500 μM 4-aminopyridine was additive to that of phenylephrine, and in the presence of the combination of phenylephrine plus 500 μM 4-aminopyridine, the carbachol dose-response curve was shifted further to the right and the maximum inhibitory effect of carbachol was further reduced (Figure 1b, Table 1).

Carbachol also antagonized the positive inotropic response to 100 nM isoprenaline in a dose-dependent manner (Figure 1c). The tension in the presence of the combination of 10 nM isoprenaline and 50 μM 4-aminopyridine was not significantly different from that in the presence of isoprenaline alone, and this concentration of 4-aminopyridine had very little effect on either the carbachol pD_2 value, or the maximum inhibitory effect of carbachol in the presence of isoprenaline (Figure 1c, Table 1). The combination of 500 μM 4-aminopyridine and 100 nM isoprenaline produced a further increase in tension (Figure 1c). Despite the high initial tension, there was only a small reduction in the magnitude of the maximum negative inotropic response to carbachol in the presence of isoprenaline plus 500 μM 4-aminopyridine (Figure 1c, Table 1). No significant change in the potency of carbachol in the presence of isoprenaline and either concentration of 4-aminopyridine was detected (Table 1).

Responses to pinacidil in the absence and presence of phenylephrine and isoprenaline

Pinacidil exerted a dose-dependent negative inotropic effect in rabbit left atrial strips (Figure 2a). Pinacidil was much less potent than carbachol and the maximum negative inotropic response to pinacidil was not attained in the dose-range tested in the present study. Pinacidil also reversed positive inotropic responses to 100 μM phenylephrine plus timolol and to 100 nM isoprenaline (Figure 2b). However, in the dose-range tested, pinacidil was more effective in overcoming the positive inotropic response to phenylephrine than that to isoprenaline. The tension remaining in the presence of 1 mM pinacidil was 15.9 ± 4.0% of the phenylephrine-induced increase in tension, and 43.6 ± 6.1% of the isoprenaline-induced increase in tension.

Effects of pinacidil and cromakalim on positive inotropic responses to phenylephrine and isoprenaline

Both pinacidil and cromakalim were dissolved in DMSO for this series of experiments. Tissues were treated with either DMSO alone or DMSO containing different concentrations of pinacidil or cromakalim for 10 min prior to obtaining dose-response curves to phenylephrine or isoprenaline. DMSO itself reduced the basal tension of left atrial strips, and no significant difference in the initial tension, prior to obtaining the phenylephrine or isoprenaline dose-response curves, was observed when tissues were treated with either DMSO alone or with pinacidil or cromakalim (data not shown). Phenylephrine produced a dose-dependent positive inotropic effect which was not affected by DMSO. Pretreatment of tissues

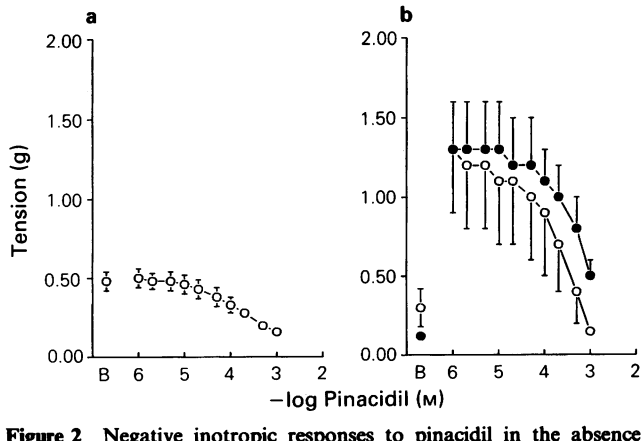


Figure 2 Negative inotropic responses to pinacidil in the absence and in the presence of phenylephrine and isoprenaline in isolated left atrial strips of rabbit. Cumulative dose-response curves to pinacidil were obtained either in the absence (a) or in the presence (b) of 100 μM phenylephrine plus 1 μM timolol (○) or 100 nM isoprenaline (●). Tissues were exposed to phenylephrine or isoprenaline for 3 min prior to obtaining pinacidil dose-response curves. The contractile response is expressed as the tension in grams (g). On the abscissa scale, B represents the left atrial tension before the addition of drugs. Each curve represents the mean of 5–12 experiments; s.e.mean shown by vertical bars.

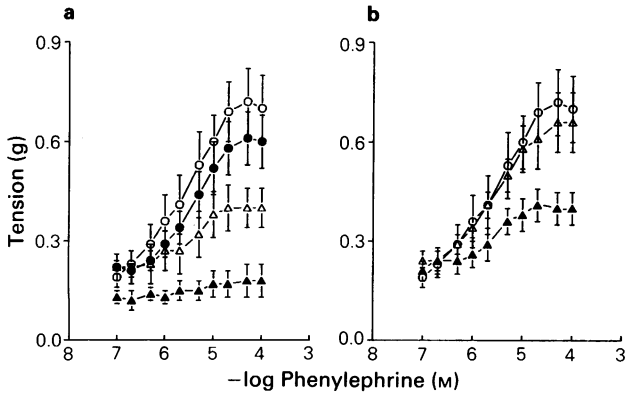


Figure 3 The effect of pinacidil or cromakalim on positive inotropic responses of left atrial strips to phenylephrine. Cumulative dose-response curves to phenylephrine were obtained after treating left atrial strips with dimethylsulphoxide (○) or 100 μM (●), 300 μM (△) or 1 mM (▲) pinacidil (a) or cromakalim (b) for 10 min. The contractile response to phenylephrine is expressed as the tension in grams (g). Each curve represents the mean of 5–10 experiments; s.e.mean shown by vertical bars.

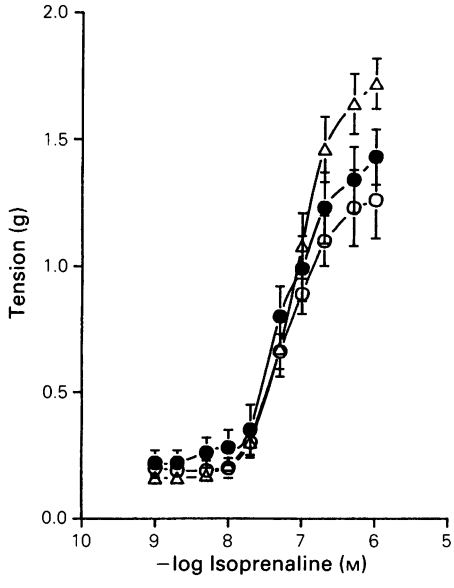


Figure 4 The effect of pinacidil or cromakalim on positive inotropic responses of left atrial strips to isoprenaline. Cumulative dose-response curves to isoprenaline were obtained after treating the left atrial strips with dimethylsulphoxide (○), 1 mM pinacidil (△) or 1 mM cromakalim (●) for 10 min. The contractile response to isoprenaline is expressed as attained tension in grams (g). Each curve represents mean of 5–7 experiments; s.e.mean shown by vertical bars.

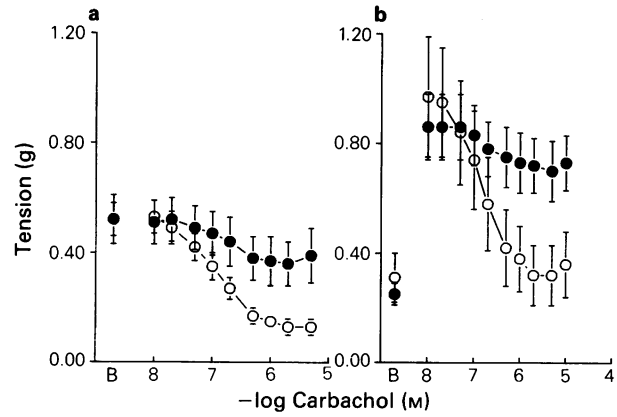


Figure 5 The effect of pretreatment of rabbits with pertussis toxin on negative inotropic responses to carbachol in the absence (a) and presence (b) of phenylephrine in isolated left atrial strips. Cumulative dose-response curves to carbachol were obtained in left atrial strips from rabbits pretreated with normal saline (○) or 1.75 μg kg^{-1} pertussis toxin (●) 48 h prior to the experiment. Tissues were exposed to normal saline (a) or 100 μM phenylephrine plus timolol (b) for 3 min before obtaining the dose-response curves to carbachol. The contractile response to carbachol is expressed as the tension in grams (g). On the abscissa scale, B represents the left atrial tension before the addition of drug. Each curve represents the mean of 5–8 experiments; s.e.mean shown by vertical bars.

with pinacidil antagonized the maximum positive inotropic response to phenylephrine in a dose-dependent manner, without affecting the phenylephrine pD_2 value (Figure 3a, Table 2). The maximum inhibitory effect was observed in the presence of 1 mM pinacidil, which completely abolished the positive inotropic response to phenylephrine. Although cromakalim also attenuated the positive inotropic response to phenylephrine, in the dose-range tested, cromakalim was found to be less effective than pinacidil (Figure 3b, Table 2).

Table 3 Effect of phenylephrine and carbachol, alone and in combination, on adenosine 3′:5′-cyclic monophosphate (cyclic AMP) levels in isolated left atrial strips of rabbit

Treatment	*Cyclic AMP* (pmol g^{-1} wet wt.)†
Control	938.4 ± 205.9
Phenylephrine (100 μM)	803.0 ± 127.8
Carbachol (30 μM)	1022.5 ± 145.0
Phenylephrine (100 μM) + Carbachol (30 μM)	1085.0 ± 104.6

† Mean ± s.e.mean of 4 experiments.

Table 2 Effect of pinacidil and cromakalim on maximum positive inotropic responses and pD_2 values to phenylephrine and isoprenaline

	Phenylephrine		*Isoprenaline*	
Treatment	*Tension* (g)†	*pD_2*	*Tension* (g)†	*pD_2*
Control	0.72 ± 0.09	5.45 ± 0.08	1.26 ± 0.11	6.97 ± 0.19
Pinacidil				
100 μM	0.61 ± 0.07	5.31 ± 0.12	—	—
300 μM	0.42 ± 0.05	5.21 ± 0.23	—	—
1 mM	0.18 ± 0.05*	ND	1.72 ± 0.10*	7.08 ± 0.07
Cromakalim				
300 μM	0.65 ± 0.10	5.60 ± 0.16	—	—
1 mM	0.40 ± 0.05	5.68 ± 0.12	1.41 ± 0.11	7.14 ± 0.23

* Significantly different from the control response.
† Data represent mean ± s.e.mean of 5–10 experiments.
ND Not determined.

Like pinacidil, cromakalim did not have any effect on the phenylephrine pD_2 value. The magnitude of the maximum positive inotropic response of left atria to isoprenaline was slightly less than double that to phenylephrine (Figure 4, Table 2). Neither pinacidil (1 mM) nor cromakalim (1 mM) antagonized the contractile response to isoprenaline (Figure 4). In fact, pinacidil enhanced the maximum positive inotropic response to this agonist (Table 2).

Effect of pertussis toxin pretreatment on responses to carbachol in the presence and absence of phenylephrine

Pretreatment of rabbits with pertussis toxin (1.75 $\mu g\,kg^{-1}$) did not have any significant effect on the basal developed tension of left atrial strips, or on positive inotropic responses to 100 μM phenylephrine plus timolol (data not shown). However, both the direct negative inotropic response to carbachol, and the reversal by carbachol of positive inotropic responses to phenylephrine, were attenuated by pertussis toxin treatment (Figure 5a,b). The tension remaining in the presence of a maximum concentration of carbachol alone was 24.0 $\pm$ 5.8% in atria from control rabbits, and 69.0 $\pm$ 9.4% in atria from pertussis toxin-treated rabbits. Similarly, the tension remaining in the presence of the maximum concentration of carbachol when combined with phenylephrine was 29.8 $\pm$ 5.0% in control atrial strips and 88.2 $\pm$ 9.9% in atria from rabbits pretreated with pertussis toxin. Neither carbachol nor phenylephrine, alone or in combination, significantly altered left atrial cyclic AMP levels (Table 3).

Discussion

The results of the present investigation support the hypothesis that the activation of potassium conductance contributes to the antagonism by carbachol of positive inotropic responses to α-adrenoceptor agonists, but is less important in the antagonism by carbachol of positive inotropic responses to β-adrenoceptor agonists, in rabbit left atria.

In the present study 4-aminopyridine and pertussis toxin were used to interfere with the ability of carbachol to activate the outward potassium current. Evidence from electrophysiological studies suggests that in cardiac tissue, 4-aminopyridine can prolong the action potential duration by inhibiting the outward current responsible for repolarization of the membrane (Van Bogaert *et al.*, 1982, Gilmour *et al.*, 1986) which is carried by potassium. 4-Aminopyridine has also been reported to antagonize noncompetitively the direct negative inotropic and action potential shortening effects of muscarinic receptor stimulation in guinea-pig atria (Freeman, 1979). This was suggested to result from antagonism by 4-aminopyridine of muscarinic receptor-mediated increases in potassium current. In the present study, 4-aminopyridine exerted a dose-dependent positive inotropic response in agreement with previous work (Yanagisawa & Taira, 1979; Wollmer *et al.*, 1981). 4-Aminopyridine also non-competitively antagonized the direct negative inotropic response to carbachol. However, in three out of nine atrial strips from different animals, the higher concentration of 4-aminopyridine (500 μM) did not reduce the maximum negative inotropic response to carbachol, although it shifted the carbachol dose-response curve to the right. In the remaining six atrial strips, the inhibitory effect of 500 μM 4-aminopyridine on maximum negative inotropic responses to carbachol was much greater than that of 50 μM 4-aminopyridine. It is possible that in the three resistant atrial strips, 4-aminopyridine was not able to antagonize adequately the carbachol-induced activation of potassium conductance. A similar observation was made by Furukawa *et al.*, (1985) while studying the interaction of acetylcholine and 4-aminopyridine in dog atrium. Alternatively, it is also possible that activation of potassium conductance may not completely explain the direct negative inotropic response to carbachol in the left atrium and that some other mechanism may contribute to the negative inotropic response to muscarinic receptor stimulation.

The cyclic AMP-independent direct negative inotropic response to carbachol was attenuated in left atria from pertussis toxin pretreated rabbits. It has been reported that in the presence of pertussis toxin, muscarinic agonists lose their ability to activate the outward potassium current (Pffafinger *et al.*, 1985), promote rubidium efflux (Martin *et al.*, 1985) and exert a direct negative inotropic response (Endoh *et al.*, 1985; Sorota *et al.*, 1985). The attenuation of the direct negative inotropic response to carbachol by pertussis toxin found in the present investigation is consistent with the result of the others which suggest that muscarinic receptors are uncoupled from potassium channels in the presence of pertussis toxin.

Carbachol completely reversed the positive inotropic response of rabbit left atrial strips to the α-adrenoceptor agonist phenylephrine, although neither phenylephrine plus timolol, nor carbachol, alone or in combination, altered cyclic AMP levels, in agreement with a previous report from this laboratory (MacLeod, 1986). The antagonism by 4-aminopyridine and pertussis toxin of the negative inotropic response to carbachol in the presence of phenylephrine suggests that muscarinic-receptor-induced activation of potassium conductance may explain the reversal by carbachol of positive inotropic responses to phenylephrine. The mechanism by which α-adrenoceptor agonists increase the contractile force of the heart is not completely understood. However, recently it has been reported that α-adrenoceptor agonists block an outward current responsible for repolarization of the membrane, which in turn may prolong action potential duration (Handa *et al.*, 1982; Apkon & Nerbonne, 1988) and promote the influx of calcium. Carbachol may antagonize this process non-competitively, by opening potassium channels, which in turn will shorten action potential duration and reduce the influx of calcium.

Both pertussis toxin pretreatment and 4-aminopyridine were found to exert a greater attenuating effect on the response to carbachol in the presence of phenylephrine than on the direct negative inotropic response to carbachol. There are two possible explanations for this observation. First, activation of potassium conductance by carbachol may be sufficient to explain the inhibitory effect of carbachol on the positive inotropic response to phenylephrine, but not the direct negative inotropic response to carbachol, as suggested above. Alternatively, the greater inhibitory effect of pertussis toxin and 4-aminopyridine on the response to carbachol in the presence of phenylephrine may be due to an additive effect between pertussis toxin or 4-aminopyridine and phenylephrine, resulting in greater antagonism of the carbachol-stimulated potassium current.

Carbachol also antagonized the positive inotropic response of left atrial strips to isoprenaline in a dose-dependent manner, but the response to carbachol in the presence of isoprenaline was only slightly inhibited by 4-aminopyridine. Previous results from this laboratory (MacLeod, 1986) and others (Brown *et al.*, 1980; Endoh *et al.*, 1985) have shown that muscarinic agonists also antagonize isoprenaline-induced increases in cyclic AMP levels in left atria. The results of the present study suggest that because antagonism by carbachol of isoprenaline-stimulated cyclic AMP generation contributes to the functional interaction between isoprenaline and carbachol, carbachol retains its inhibitory effect on the positive inotropic response to isoprenaline even in the presence of potassium channel blockade.

Pinacidil produced a dose-dependent negative inotropic response in rabbit left atrial strips, although it was less potent than has been found in dog atrium (Yanagisawa *et al.*, 1988) and in cat right ventricular papillary muscle (Steinberg *et al.*, 1988). The direct negative inotropic response to pinacidil is believed to be related to its ability to promote the efflux of potassium, which in turn may shorten the action potential duration and reduce the influx of calcium (Steinberg *et al.*,

1988). The explanation for the lower sensitivity of rabbit atrium to pinacidil is not known. It is possible that there are species differences in the sensitivity to pinacidil. Moreover, experiments in both dog atrium and cat papillary muscle were conducted at a temperature of 37°C, whereas our experiments were carried out at 35°C. Recently, Sanguinetti *et al.*, (1988) have reported that cromakalim loses its ability to shorten the action potential duration in ferret papillary muscle as the temperature is lowered.

Pinacidil also reversed positive inotropic responses of rabbit left atria to isoprenaline and phenylephrine. However, in the dose-range tested, pinacidil had a much greater inhibitory effect on the positive inotropic response to phenylephrine than on the positive inotropic response to isoprenaline. In addition, pretreatment of left atrial strips with either pinacidil or cromakalim non competitively antagonized the positive inotropic response to phenylephrine, but not to isoprenaline. These results also suggest that activation of potassium conductance may be sufficient to antagonize the positive inotropic response to phenylephrine but not to isoprenaline. The lack of inhibitory effect of pinacidil and cromakalim on positive inotropic responses to isoprenaline is in agreement with the results of Endoh and co-workers (Endoh & Iijima, 1983; Iijima *et al.*, 1987). They found that in dog atrium, carbachol, but not the potassium channel agonist, nicorandil, was able to reverse the contractile and electrophysiological effects of isoprenaline, and concluded that carbachol was able to overcome the effects of isoprenaline because it antagonized isoprenaline-induced increases in cyclic AMP.

In summary, the results of the present investigation suggest that activation of potassium conductance in left atria by carbachol, pinacidil or cromakalim results in antagonism of positive inotropic responses to α-adrenoceptor stimulation. However, positive inotropic responses to β-adrenoceptor stimulation appear to be more resistant to inhibition by K^+ channel activation.

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The influence of 5-hydroxytryptamine agonists and antagonists on identified sympathetic preganglionic neurones in the rat, *in vivo*

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1 5-Hydroxytryptamine (5-HT) was applied by microiontophoresis in the vicinity of identified sympathetic preganglionic neurones in the upper thoracic spinal cord of the rat, *in vivo*.

2 Sympathetic preganglionic neurones responded in one of three ways to 5-HT: by (a) excitation (76%), (b) inhibition (4%) or (c) in a biphasic manner (5%).

3 The excitatory responses evoked by 5-HT were mimicked by 5-carboxamidotryptamine (5-CT) and α-methyl-5-hydroxytryptamine (α-Me-5-HT). The inhibitory and biphasic responses evoked by 5-HT were mimicked by 2-methyl-5-hydroxytryptamine (2-Me-5-HT). The observed responses evoked by 5-HT and selective agonists may be different on the same cell. In several instances a single neurone excited by one agonist was inhibited by another agonist.

4 The 5-HT_2-receptor antagonists, ketanserin and LY 53857, failed to abolish selectively the excitatory responses evoked by 5-HT and α-Me-5-HT, when applied by microiontophoresis. The antagonists non-selectively reduced the excitatory responses evoked by 5-HT, 5-CT, α-Me-5-HT, D,L-homocysteic acid (DLH) and noradrenaline (NA). A reduction in synaptically evoked activity was also observed.

5 The 5-HT_3-receptor antagonist, ICS 205-930, failed to abolish the inhibitory responses evoked by 5-HT.

6 It was concluded that the excitatory responses evoked by 5-HT are mediated by a receptor that is neither 5-HT_2 or 5-HT_3, but shows similarities to the 5-HT_1-like receptor profile. The inhibitory actions of 5-HT are mimicked by 2-Me-5-HT, but the receptor is not 5-HT_3, or 5-HT_1-like or 5-HT_2.

Introduction

Neuroanatomical studies have shown the existence of discrete groups of 5-hydroxytryptamine (5-HT)-containing neurones in the brainstem with extensive projections to the spinal cord (Dahlstrom & Fuxe, 1965; Loewy & McKellar, 1981), where many make direct synaptic contact with dendrites and somata of sympathetic preganglionic neurones (SPN, Bacon & Smith, 1988). Electrical or chemical stimuli applied to these brainstem regions produced either excitation or inhibition of sympathetic activity, and it is suggested that both effects may be mediated by the release of 5-HT at terminals in the spinal cord. This dual action may be achieved via two receptor subtypes located at synapses somewhere in the sympathetic spinal circuitry (Coote, 1988 for review). In a recent attempt to mimic the action of this innervation, 5-HT was given intrathecally at the T_9, T_{10} level of the spinal cord whilst recording postganglionic sympathetic activity in a renal nerve of the rat. A sympatho-excitatory effect mediated via 5-HT_1-like receptors and a sympatho-inhibitory response mediated via 5-HT_2-like receptors was revealed (Yusof & Coote, 1988). Such an organisation could explain the results of earlier studies of cat SPN in which 5-HT, applied directly in their vicinity by iontophoresis, excited the majority but had an inhibitory effect on a significant minority of cells (Coote *et al.*, 1981; Kadzielawa, 1983). However, McCall (1983) using the cat came to the opposite conclusion, that the excitatory effect of 5-HT on SPN was mediated via 5-HT_2 receptors since it could be blocked by metergoline or methysergide, even though these are relatively unspecific antagonists.

There has been no study of this sort on rat SPN, where most of the studies of the neuroanatomy and pharmacology of 5-HT neurones have been conducted. Therefore, the present investigation was designed to determine the action of 5-HT and its agonists and antagonists, when applied directly in the vicinity of antidromically identified single SPN in the upper thoracic spinal cord of the anaesthetized rat.

A brief account of part of this work has been published (Coote & Lewis, 1989a).

Methods

Experiments were carried out on male Sprague-Dawley rats anaesthetized with α-chloralose (35 mg kg^{-1}) and urethane (700 mg kg^{-1}), given intravenously after initial halothane induction. Blood pressure was monitored with a polyethylene cannula filled with heparinised 0.9% NaCl, placed retrogradely in the right femoral artery and connected to a transducer. An electrocardiogram (ECG) was recorded via leads connected to the right forelimb and left hindlimb, amplified (Neurolog NL103) via high impedance probe (Neurolog NL100), filtered (Neurolog NL125) and displayed on an oscilloscope (Tektronix RM565). Instantaneous heart rate was obtained from the R-R interval, using an interface unit (Neurolog NL515) and rate meter (Neurolog NL253), and displayed on a chart recorder. In some experiments a bilateral pneumothorax was performed to stabilize the recording conditions, respiration being maintained by positive pressure artificial ventilation (Harvard Instruments). Temperature of the animals was maintained at 37°C by means of a thermostatically controlled heating blanket.

The animal's head was placed in a head holder and the vertebral column firmly fixed by three metal clamps. Conventional methods were used for recording and identifying SPN in the T_2 segment. The cells were antidromically activated by stimulation of the left cervical sympathetic nerve as described elsewhere (Gilbey *et al.*, 1982). Action potentials of SPN were recorded through one barrel containing 4 M NaCl (2 MΩ) of a seven-barrel glass micropipette assembly connected to a high impedance input stage (Neurolog NL100AK), amplified (Neurolog NL104), Filtered (Neurolog NL125) and monitored

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on an oscilloscope (Gould 1425). Single unit activity was detected with a spike discriminator (Neurolog NL200), counted with a pulse integrator (Neurolog NL601) and a period generator (Neurolog NL304), and displayed on a latch counter (Neurolog NL606) and graphically on a rectilinear chart recorder (Rikadenki R-02). All data were stored on magnetic tape (Racal, Store 4).

For microiontophoresis, 5 drug-containing barrels of the micropipette assembly were connected via a silver wire to a microiontophoresis unit (Grayden Electronics), and one barrel containing 1 M NaCl was connected to an automatic current compensation module of this unit. The following drugs were dissolved in distilled water and adjusted to the required pH using either 0.1 M HCl or 0.1 M NaOH: L-glutamate 0.5 M (monosodium salt, Sigma, pH 8.0), D,L-homocysteic acid 0.1 M (DLH, Sigma, pH 8.0), 5-hydroxytryptamine creatinine sulphate 0.01 M (Sigma, pH 4.5), noradrenaline bitartrate 0.1 M (Sigma pH 4.5), 5-carboxamidotryptamine maleate 0.01 M (5-CT, Glaxo, pH 4.5), α-methyl-5-hydroxytryptamine maleate 0.01 M (α-me-5-HT, Glaxo, pH 4.5), 2-methyl-5-hydroxytryptamine maleate 0.01 M (2-me-5-HT, Glaxo, pH 4.5), ketanserin bitartrate 0.01 M (Janssen, pH 4.5), 4-isopropyl-7-methyl-9(2-hydroxy-1-methylpropoxycarbanyl)4,6,6a,7,8,9,10,10a-octahydroindole (4,3 Fg) quinoline maleate 0.01 M (LY 53857, Eli Lilly, pH 4.5), (3 α-tropanyl)-1-H-indole-3-carboxylic acid ester 0.01 M (ICS 205-930 HCl, Sandoz, pH 4.5).

Retaining currents (5-30 nA) were applied to the drug containing barrels between ejection periods. Compounds were ejected using positive currents (5-100 nA), apart from glutamate or DLH, which were ejected with negative currents.

5-Hydroxytryptamine receptor antagonists and analogues

To study the action of antagonists of 5-HT or its agonists, the following protocol was adopted. Three steady state cycles of responses of SPN to 5-HT and of one or more control agonists were achieved by rigorously ensuring that ejection and retention currents and their duration were the same throughout the tests. In addition, before a testing procedure, ejection currents for each of the agonists was adjusted so as to give responses of SPN that were of approximately equal magnitude. Drugs used in addition to 5-HT were, noradrenaline (NA), 5-CT, α-Me-5-HT and 2-Me-5-HT, as well as excitatory amino acids (EAA).

Results

Sympathetic preganglionic neurones (SPN) in T_2 segment of the spinal cord were identified as previously described (Gilbey *et al.*, 1982). The majority (68%) of SPN were not spontaneously active, and apart from the excitatory amino acids (EAA), no drug used in this study was able to excite silent SPN even though ejection currents up to 100 nA were applied. Therefore, the following results were obtained from both spontaneously active and previously silent SPN in which a stable firing rate was induced by continuous application of an excitatory amino acid, either glutamate or DLH. Comparisons of the action of each of the agonists used in this study showed that there was no significant difference in their effect on spontaneously active SPN and EAA-driven SPN.

5-Hydroxytryptamine

5-HT, 0-80 nA, applied in the vicinity of 118 SPN for between 15–60 s, caused 90 SPN rapidly to increase their firing rate during the application of the ejection current, reaching a maximum after the removal of this current (Figure 1). Neural activity then decreased gradually back to baseline levels over a period of 9 min. The maximum firing rate and the duration of SPN discharge increased with increasing ejection currents.

In a small number of SPN the application of 5-HT, as well as increasing the firing rate, induced a period of burst firing

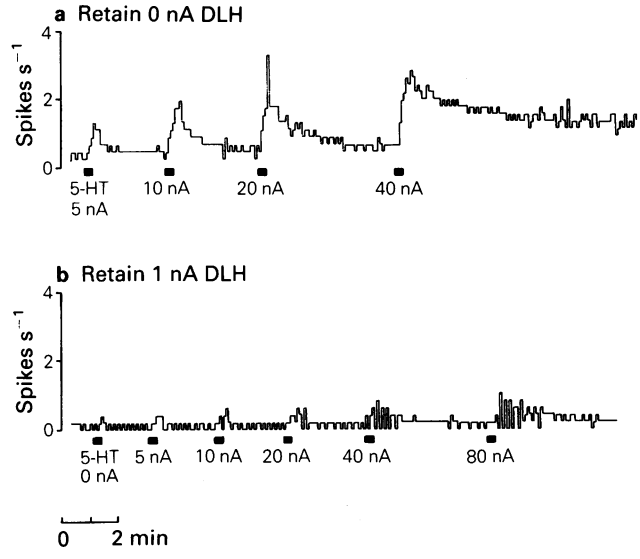


Figure 1 Continuous frequency-time histograms showing the excitatory responses evoked by increasing iontophoretic currents of 5-hydroxytryptamine (5-HT) on an identified, sympathetic preganglionic neurone. The bars indicate ejection periods, with the numerals below indicating ejection currents in nanoamperes (nA). The activity of the neurones depicted in (a) is the result of leakage of D,L-homocysteic acid (DLH) in the vicinity of the neurone when no retaining current is applied to the pipette barrel. This activity is diminished on increasing the retaining current to 1 nA as shown in (b). With the higher DLH retaining current (1 nA) (b), the neurone fired in a phasic manner in response to iontophoretic 5-HT.

which ceased when the firing rate returned to control levels (Figure 1b). This pattern of activity was characterized by a burst of up to six action potentials with a maximum frequency of 8 Hz, the cell then stopped firing for some 8 s before repeating the burst of activity.

In five of the 118 SPN, application of 5-HT caused a decrease in their activity. This started during the application of the ejection current, reaching the nadir before its removal (Figure 6). The firing rate then gradually returned to baseline levels over a period of 2 min. The magnitude of these inhibitions of activity increased with increasing ejection current.

In six of the 118 SPN, 5-HT application elicited a biphasic response. This consisted of an initial decrease in firing rate which reached a maximum during the application of the ejection current. It then returned to baseline values over the next minute before increasing above this level for a further minute, and then declining over the following 4 min (Figure 2). The size of the initial inhibition and following excitation was directly related to the size of the ejection current.

No biphasic response comprising an initial excitation followed by an inhibition was observed.

5-HT_1-like receptor agonist

5-CT, 5–80 nA, applied for between 15–60 s, increased the firing rate of 44 out of 52 SPN tested (Figure 3a), in a manner

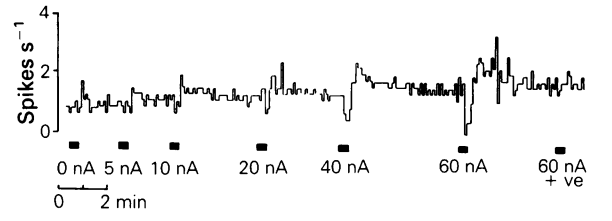


Figure 2 Continuous frequency-time histogram showing the biphasic responses evoked by increasing iontophoretic currents of 5-hydroxytryptamine (5-HT) on a spontaneously active, identified sympathetic preganglionic neurone. The neurone was unresponsive to 60 nA positive current (+ve).

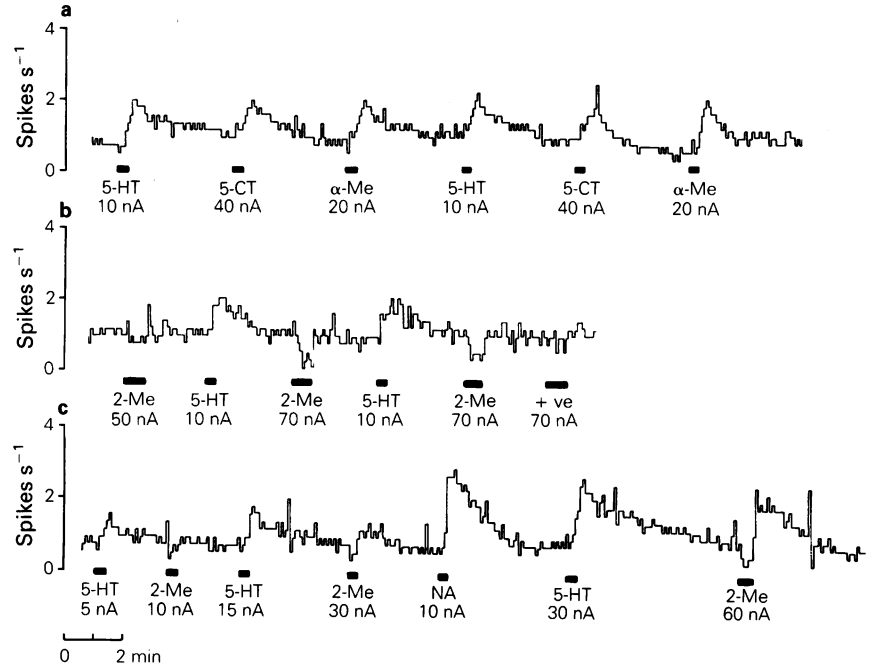


Figure 3 Continuous frequency-time histograms showing the effects of 5-hydroxytryptamine agonists on three different identified sympathetic preganglionic neurones. (a) The excitatory effects of 5-hydroxytryptamine (5-HT), 5-carboxamidotryptamine (5-CT) and α-methyl-5-hydroxytryptamine (α-Me) on a non-spontaneously active neurone. No retention or ejection current was applied to the D,L-homocysteic acid (DLH) containing barrel. (b) The excitatory effect of 5-HT and the inhibitory effect of 2-methyl-5-hydroxytryptamine (2-Me) on a non-spontaneously active neurone. The neurone was 'driven' by continuous application of 3 nA DLH. The neurone was unresponsive to the iontophoretic application of 70 nA positive current (+ve). (c) The excitatory responses evoked by 5-HT and noradrenaline (NA) and the biphasic responses evoked by 2-Me-5-HT on a spontaneously active neurone.

similar to that observed with the application of 5-HT. In one SPN, 5-CT caused an inhibition and had no effect on the remaining seven cells.

The actions of both 5-HT and 5-CT on 44 neurones were compared. Both agonists were excitatory on 32 cells, and had no effect on three. 5-CT evoked excitatory responses in a further six cells, in which 5-HT had no effect on four, was inhibitory on one, and evoked a biphasic response in the remaining cell. Two cells were unresponsive to 5-CT; one being excited by 5-HT and the other being inhibited. The remaining cell was inhibited by 5-CT and excited by 5-HT.

5-HT$_2$ receptor agonist

α-Me-5-HT, 10–90 nA, applied for between 15–60 s, increased the firing rate of 18 out of 27 SPN (Figure 3a) and had no effect on the remaining nine SPN. Both the magnitude of the increased discharge, and its duration, were directly related to the magnitude of the ejection current.

The actions of α-Me-5-HT and 5-HT on the same cells were compared for 24 SPN. Both agonists increased the firing rate of 11 SPN, and had no effect on seven neurones. α-Me-5-HT was excitatory on four cells, of which 5-HT evoked an inhibitory response in two, a biphasic response in one and had no effect on the remaining neurone. α-Me-5-HT had no effect on the remaining two cells, whereas 5-HT was excitatory on one and inhibitory on the other.

5-HT$_3$ receptor agonist

2-Me-5-HT, 10–90 nA, applied for 15–60 s, decreased the firing rate of 10 out of 26 SPN (Figure 3b), caused a biphasic response (inhibition followed by excitation) in seven SPN (Figure 3c) and had no effect on the remaining nine.

The inhibitory response was characterized by an onset during the application of the ejection current, reaching a maximum immediately after its removal. The activity of the SPN then gradually returned to baseline levels over a period of 2 min. The biphasic response was characterized by an initial rapid inhibition immediately followed by an excitatory phase, the activity then declining to baseline values over a period of 4 min. The magnitude of these effects of 2-Me-5-HT increased with increasing ejection currents.

A comparison of the actions of both 2-Me-5-HT and 5-HT on the same cell for 26 SPN showed that of the 23 SPN excited by 5-HT, 10 were inhibited by 2-Me-5-HT, six responded in a biphasic manner and seven were unaffected by the agonist. Two cells were unresponsive to 5-HT, one of which responded in a biphasic manner to 2-Me-5-HT. The remaining cell was inhibited by 5-HT but showed no response to 2-Me-5-HT.

Comparison of 5-HT receptor agonists on the same SPN

5-CT and α-Me-5-HT were both applied to eight identified SPN. Both agonists excited seven SPN, and had no effect on one. 5-CT and 2-Me-5-HT were both applied to three SPN. 5-CT excited all three, whilst 2-Me-5-HT produced a biphasic response in one and had no effect on two. α-Me-5-HT and 2-Me-5-HT were both applied to the same four SPN. α-Me-5-HT increased the activity of all four cells, whereas 2-Me-5-HT had a biphasic effect on one and no effect on three cells.

5-HT receptor antagonists

In seven rats, the 5-HT$_2$ receptor antagonist ketanserin failed to antagonize selectively the excitatory action of 5-HT on eight SPN, or of α-Me-5-HT on two SPN. Instead, it reduced the responses evoked by 5-HT and α-Me-5-HT, and in addition, the excitatory responses evoked by NA, 5-CT and DLH were diminished. A typical experiment is illustrated in Figure 4. Here, continuous ejection of ketanserin, with a current of 5 nA, reduced the response to NA (5 nA) and 5-HT (30 nA) and also abolished activity of the SPN, which was driven by continuous application of DLH (10 nA). All responses were reduced for almost 90 min after removal of the ketanserin ejection current. Similar non-selective effects of ketanserin were observed when tested against the excitatory response of SPN to α-Me-5-HT.

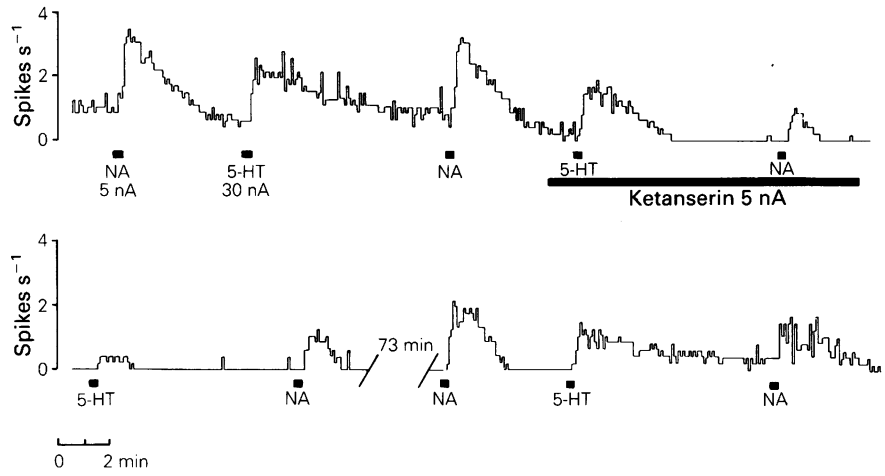


Figure 4 Continuous frequency-time histogram showing the effect of ketanserin on the excitatory responses evoked by 5-hydroxytryptamine (5-HT) and noradrenaline (NA) on a non-spontaneously active, identified sympathetic preganglionic neurone. The neurone was 'driven' by continuous iontophoretic ejection of 10 nA D,L-homocysteic acid (DLH). Ketanserin reduced the responses evoked by both agonists and caused a reduction in the DLH-evoked basal firing rate. Partial recovery of the agonist-induced responses and full recovery of the baseline firing rate was observed approximately 90 min after removal of the antagonist ejection current.

In two other experiments, ketanserin was also shown to induce a non-selective decrease in the 5-HT-induced excitatory response of spontaneously active SPN. In one example, in which the ketanserin retaining current was reduced from 20 nA to 10 nA, an increase in firing rate to NA (10 nA) was more profoundly depressed than that to 5-HT (25 nA). The firing rate gradually returned on increasing the retention current to 20 nA.

In two rats, the actions of the 5-HT_2 receptor antagonist, LY 53857, on responses evoked by 5-HT, NA, and either 5-CT or α-Me-5-HT, were studied on two DLH-driven SPN. In both experiments, there was a virtual abolition of the responses of SPN to all the agonists, including the activity induced by DLH. In one of the experiments, partial recovery of the responses to NA, but not to 5-HT or its agonists, was observed up to 40 min after removal of the LY 53857 ejection current, after which the cell was lost. In the second experiment, there was no recovery of the response to any agonist including DLH, even though the neurone could still be antidromically activated some 65 min after removal of the ejection current.

In an attempt to overcome the reduction in baseline activity of SPN induced by continuous application of the 5-HT receptor antagonists, ketanserin (5–45 nA) was ejected in pulses before and during the ejection of 5-HT. In one typical example shown in Figure 5, ketanserin (5 nA) ejection was started 30 s before and continued during the application of the test drug, either 5-HT (30 nA) or NA (10 nA). Both antagonist and agonist ejection currents were removed simultaneously. Five control responses of equal magnitude were first obtained before the start of the ejections of antagonist. Ketanserin, applied in this way, caused a dose- (current) dependent reduction in the response of the neurone to both 5-HT and NA which persisted up to 60 min after the final application of ketanserin. In addition, in spite of the short discontinuous ejection of ketanserin, the spontaneous activity of the SPN decreased to zero and has still not recovered 60 min after the last application of the antagonist. Interestingly, and unusually, both 5-HT and NA were able to excite this SPN despite the absence of ongoing spontaneous activity at the end of the test procedure. During the test period, with the higher ketanserin ejection currents, there was a reduction in the height of the agonist evoked spike.

Since 2-Me-5-HT had a predominantly inhibitory effect on SPN, the possibility that the 5-HT-induced reductions in SPN

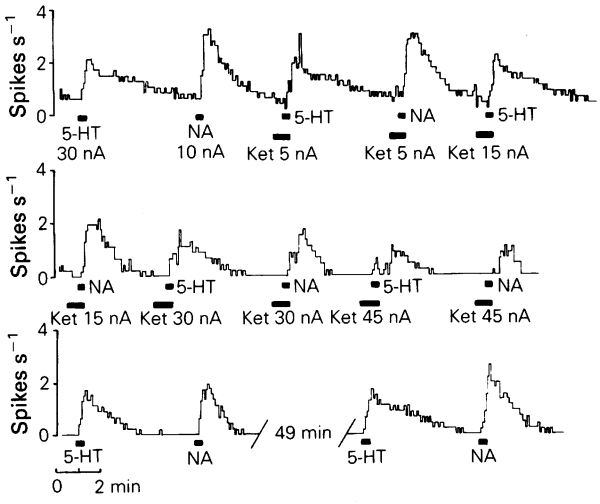


Figure 5 Continuous frequency-time histogram showing the effect of the pulsatile iontophoretic ejection of ketanserin (Ket) on the excitatory responses evoked by 5-hydroxytryptamine (5-HT) and noradrenaline (NA) on an identified spontaneously active sympathetic preganglionic neurone. The antagonist was applied 30 s before and during the agonist ejection period. With higher ejection currents, ketanserin reduced the responses evoked by both 5-HT and NA. A gradual abolition of the basal firing rate was also observed. Partial recovery of the agonist-induced responses, but not the baseline firing rate had occurred 75 min after the last antagonist ejection period.

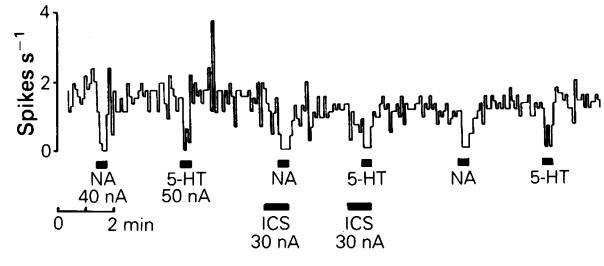


Figure 6 Continuous frequency-time histogram showing the effect of ICS 205-930 on the inhibitory responses evoked by 5-hydroxytryptamine (5-HT) and noradrenaline (NA) on identified, non-spontaneously active sympathetic preganglionic neurone. The neurone was 'driven' by continuous application of 6 nA D,L-homocysteic acid (DLH). The antagonist was applied 30 s before and during the agonist ejection period. ICS 205-930 had no effect on the inhibitory responses evoked by 5-HT and NA, but caused a gradual reduction in the DLH-induced firing rate. Recovery of the baseline firing rate occurred gradually after the end of the last antagonist ejection period.

firing rate were due to an action at a 5-HT_3 receptor was tested by use of the highly selective 5-HT_3 receptor antagonist, ICS 205-930.

In the experiment shown in Figure 6, three reproducible cycles of inhibitory responses were obtained to 5-HT (50 nA) and NA (40 nA) on a DLH driven SPN. ICS 205-930, ejected 30 s before and during application of either 5-HT or NA, had no effect on the inhibitory responses to each of these agonists, even though its repeated application did cause a small reduction in the baseline level of activity.

Discussion

The principal observation made in this study was that 5-HT, when applied in the vicinity of identified sympathetic preganglionic neurones, could either excite or inhibit these neurones. These actions of 5-HT could either be direct or indirect. The results are broadly comparable with similar, but more limited studies, in the cat (Coote *et al.*, 1981; Kadzielawa, 1983).

By using selective 5-HT agonists, we sought to determine the receptor subtypes which may be mediating these differing effects of 5-HT. The excitatory actions of 5-HT resembled those of 5-CT and α-Me-5-HT, the former being a selective 5-HT_1-like agonist whereas the latter is a 5-HT_2 receptor agonist which also has some actions at 5-HT_1-like receptors (Bradley *et al.*, 1986). The excitatory responses evoked by α-Me-5-HT were surprising in view of previous studies in this laboratory (Yusof & Coote, 1988) which showed that α-Me-5-HT evoked dose-dependent, ketanserin-sensitive, inhibitions of pre- and postganglionic sympathetic nerve activity when applied intrathecally in the rat. In contrast, in the present iontophoretic experiments, ketanserin and LY 53837 failed to antagonize selectively the excitatory responses evoked by α-Me-5-HT and 5-HT in SPN. Limiting the concentration of the antagonists in the vicinty of SPN, by applying them for brief periods or just by reducing the retaining current, did not reveal a selective blockade. Instead, both antagonists either had no effect or produced a marked reduction in all excitatory responses. In addition to diminishing the responses evoked by 5-HT and α-Me-5-HT, the excitatory responses evoked by 5-CT, NA and EAAs were also reduced, as was the ongoing synaptic activity. These neuronal depressant actions were unlikely to be due to current or local anaesthetic actions since neuronal spike amplitudes were unaltered; the effect came on gradually and far outlasted the period of antagonist application; there was no spike broadening; and no increase in recording noise levels was observed. Another possibility is that the antagonists were acting either pre- or postsynaptically to block a tonic 5-HT-mediated excitatory drive to these neurones (see review, Coote 1988). Depressant actions of ketanserin have been observed on cortical neurones (Lakoski & Aghajanian, 1985), raphe neurones (Davies *et al.*, 1988a,b) and spinal motoneurones (Roberts *et al.*, 1988), as have the depressant effects of methysergide, metergoline and cianserin on SPN (Kadzielawa, 1983; McCall, 1983). It is unlikely that all these different groups of neurones receive a tonic 5-HT_2 receptor-mediated excitatory input, especially as in the case of motoneurones, 5-HT itself evokes excitatory responses most likely mediated by 5-HT_1-like receptors (Roberts *et al.*, 1988).

Catecholamines have been shown to have an excitatory action on SPN (Coote & Lewis, 1989b; Shi *et al.*, 1988), and therefore ketanserin may be acting by blocking α_1-adrenoceptors on these neurones (Leysen *et al.*, 1981). Support for this idea comes from the observation that high iontophoretic ejection currents of the α_1-adrenoceptor antagonist, thymoxamine, reduced the spontaneous firing rate of cat SPN (Kadzielawa, 1983), however LY 53857 has been shown to have a very low affinity for α_1-adrenoceptors (Cohen *et al.*, 1985). Therefore, the depressant effects observed with higher ejection currents of both 5-HT and α-adrenoceptor antagonists on SPN, and 5-HT antagonists on other groups of neurones, are probably due to the build up of excessive antagonist concentrations, leading to non-specific neuronal depression by an as yet undetermined mechanism. Support for this comes from the observation that excitatory actions of EAAs are also depressed.

In view of the inability of ketanserin or LY 53857 to reduce selectivity, or abolish, the excitatory responses evoked by α-Me-5-HT and 5-HT on SPN, it seems unlikely that these responses are mediated by the 5-HT_2 receptor subtype. The present study has shown that 5-CT evoked an excitatory response when applied by iontophoresis in the vicinity of SPN, indicating that the excitatory effects of 5-HT may be mediated via the 5-HT_1-like receptor subtype. Support for this viewpoint comes from a study of Yusof & Coote (1988), where 5-CT was shown to have dose-dependent sympatho-excitatory actions when it was administered intrathecally to the rat thoracic spinal cord, an action unaffected by 5-HT_2 or 5-HT_3 receptor antagonists. In addition, α-Me-5-HT has a weak affinity for 5-HT_1-like receptors, and so its excitatory action could be explained by its effect on these receptors on SPN.

However, studies on rat spinal motoneurones have shown that whilst 5-HT, 5-CT and α-Me-5-HT evoke depolarizations of these neurones (Connell & Wallis, 1988; Roberts *et al.*, 1988), the receptor subtypes mediating these effects being neither 5-HT_2 or 5-HT_3, the receptor profile does not clearly resemble that of the 5-HT_1-like receptor as defined by Bradley *et al.* (1986). In the experiments of Connell & Wallis (1988), ketanserin was shown to have a weak antagonist action of a 5-HT-evoked depolarization, but only when administered at high concentrations (0.8×10^{-7}–1×10^{-6} M), hence the effect was unlikely to have been due to an action at 5-HT_2 receptor sites. Therefore, in view of the similarities between the above studies and the results obtained in the present study, whilst the excitatory responses evoked by 5-HT in SPN are neither mediated by the 5-HT_2 or 5-HT_3 receptor subtypes, it would be unwise to define them rigidly as being mediated by the 5-HT_1-like receptor subtype.

2-Me-5-HT was shown to induce inhibitory responses in SPN. These responses were totally different from the excitatory responses evoked by 5-CT and α-Me-5-HT. Since 2-Me-5-HT has only a weak affinity for the 5-HT_1-like and 5-HT_2 receptor subtypes, and 5-CT and α-Me-5-HT have low affinity for 5-HT_3 receptors (Humphrey, 1984; Richardson *et al.*, 1985), it may be that these inhibitions are mediated via the 5-HT_3 receptor subtype. However, the 5-HT-induced inhibitions were not antagonized by the highly selective 5-HT_3 receptor antagonist, ICS 205-930. Therefore, it appears that 2-Me-5-HT and 5-HT can evoke inhibitory responses in SPN which are neither mediated by the 5-HT_1-like, 5-HT_2 or 5-HT_3 receptor subtypes. A similar observation was made in studies on neurones in the guinea-pig submucous plexus, where 5-HT and 2-Me-5-HT were shown to evoke slow depolarizations of these neurones via a 5-HT receptor which did not belong to the 5-HT_1-like, or the 5-HT_2 or the 5-HT_3 receptor subtypes (Surprenant & Crist, 1988).

5-HT has previously been shown to evoke long duration, 5-HT_2 receptor-mediated, inhibitions of pre- and postganglionic sympathetic nerve activity when administered intrathecally (Yusof & Coote, 1988). In contrast, the present iontophoretic studies have shown that 5-HT evokes short duration inhibitory responses which are mimicked by 2-Me-5-HT. This discrepancy may arise as the result of the route of administration of the agonist. When 5-HT is administered iontophoretically, it must, by the nature of the technique, be acting at a site near the cell soma. In contrast, when the agonist is administered intrathecally, it may be acting at a more distant spinal site, possibly via an interneurone. Therefore, the 5-HT-evoked inhibitions observed with the two different techniques may be mediated by different mechanisms and receptor subtypes.

The idea that 5-HT-mediated inhibitions of SPN may be via interneurones has previously been postulated (Gilbey *et al.*, 1981; Yusof & Coote, 1988). Such an interneurone could act by the release of glycine, a possibility not investigated in

the present experiments, but an idea for which there is increasing evidence. 5-HT, when applied microiontophoretically, induced inhibitions of SPN which had fast onsets and short duration. These characteristics would be expected of a glycine mediated response, this agonist being shown to mediate fast inhibitory postsynaptic potentials (i.p.s.ps) in rat SPN *in vitro* (Dun & Mo, 1989). Similar fast i.p.s.ps, which are blocked by strychnine, can be induced synaptically during the application of substance P (Dun & Mo, 1988) and adrenaline (Miyazaki *et al.*, 1989). Therefore, 2-Me-5-HT and 5-HT may be acting presynaptically on a glycine interneurone to cause its release. However, whilst the 5-HT-mediated inhibitions observed during intrathecal administration may also be via an interneurone, a different mechanism or receptor may exist. The observed long duration inhibitions evoked by 5-HT and α-Me-5-HT seem inconsistent with the idea that glycine is the mediator of fast i.p.s.ps in rat SPN, and is the transmitter being released by the interneurone in this instance.

Therefore, there is now a basis for a receptor-mediated pattern of response produced by descending 5-HT pathways. We would suggest that excitatory inputs are direct and mediated by a receptor that is neither 5-HT_2 nor 5-HT_3, but shows similarities to the 5-HT_1-like receptor profile. The inhibitory inputs are indirect, being mediated both at a site distant from SPN via 5-HT_2 receptors and a second site which is presynaptic, possibly a glycine interneurone. The actions of 5-HT on this latter receptor are mimicked by 2-Me-5-HT, but the receptor is not 5-HT_1-like, or 5-HT_2 or 5-HT_3.

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Influence of two β-adrenoceptor antagonists, propranolol and pindolol, on cold adaptation in the rat

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1 Adult male rats were treated with propranolol (2.0 mg kg^{-1} day^{-1} i.p.), pindolol (0.2 mg kg^{-1} day^{-1} i.p.) or 0.9% NaCl day^{-1} i.p. and exposed to +4°C for 42 days, or treated with 0.9% NaCl day^{-1} i.p. and kept at +23°C for 42 days. They were weighed once a week, when a 24 h urine sample was also collected and colon temperature measured.

2 Urinary noradrenaline (NA), adrenaline (Ad) and dopamine were analysed by high-performance liquid chromatography with an electrochemical detector. After the acclimatization period the interscapular brown adipose tissue was excised and weighed and the activity of the oxidative enzymes succinate dehydrogenase and cytochrome oxidase measured.

3 The pindolol-treated and propranolol-treated rats gained weight during the cold-acclimatization period. The amount of interscapular brown adipose tissue increased in the cold, but the increase was lowest in the pindolol-treated group. No changes were seen in the other brown adipose tissue parameters in cold-exposed animals. The excretion of catecholamines followed the same pattern in all three cold-exposed groups, with an initial rise in noradrenaline and adrenaline excretion and a slight rise in dopamine excretion.

4 The results suggest possible connections between β-adrenoceptor antagonists, weight gain and cold acclimatization. Pindolol had a slight inhibitory effect on cold-induced brown adipose tissue hypertrophy in rats.

Introduction

Cold-acclimatized animals produce heat effectively in the brown adipose tissue (BAT), which is the primary tissue for non-shivering thermogenesis (Himms-Hagen, 1984). Heat production in the BAT is mediated mainly via the sympathetic nervous system, the most important hormone affecting this tissue being noradrenaline (NA), which exerts its effects by binding to β-adrenoceptors (Himms-Hagen, 1984). BAT is of importance not only in small mammals, but probably also in man. Newborn babies have about 30–40 g BAT per kg body weight (Benito, 1985), and it has been found even in adults in autopsy material (Hassi, 1977; Huttunen *et al.*, 1981) and at surgery (Lean *et al.*, 1986). In addition to its possible role in survival in cold environments, brown adipose tissue is probably of some significance in the mechanism of obesity (Rothwell & Stock, 1986). In the field of forensic medicine it has attracted attention because of certain aspects concerning hypothermia-related fatalities. Hypothermia victims often have a history of some kind of medication (Hirvonen, 1979; Paton, 1983; Kortelainen, 1987) and therefore the effects of certain widely used drugs, such as β-adrenoceptor antagonists, are of interest with regard to non-shivering thermogenesis and survival in the cold.

β-Adrenoceptor antagonists are frequently used in the treatment of hypertension and ischaemic heart disease, and these drugs have been shown to have acute effects on thermoregulation, e.g. hypothermia after systemic administration (Hissa *et al.*, 1974; Hirvonen *et al.*, 1978) and after injection into the lateral cerebral ventricle (Lin *et al.*, 1981). Propranolol has been observed to inhibit non-shivering thermogenesis in mammals (Koraromi, 1977; Soppela *et al.*, 1986).

We set out here to study the effects of two β-adrenoceptor antagonists, propranolol and pindolol, on cold acclimatization. Propranolol is a non-specific β-adrenoceptor antagonist with no intrinsic sympathomimetic activity (ISA) or partial agonist activity, in other words it also blocks β-adrenoceptors in tissues other than the heart and does not cause any stimulation of these receptors (Aellig, 1982). Pindolol is also a non-specific β-adrenoceptor antagonist, but with ISA, i.e. it blocks β-receptors but also acts as a partial agonist (Aellig, 1982). This partial agonist activity of pindolol is said to be about as high as the normal resting sympathetic activity, and consequently it has been shown to reduce only enhanced sympathetic tone, whereas β-adrenoceptor antagonists without ISA reduce even normal sympathetic activity (Aellig, 1982).

The aim of this study was to find out whether cold-induced increase in the oxidative capacity of brown adipose tissue is attenuated by the administration of β-adrenoceptor antagonists capable of blocking extracardial β-receptors. In addition catecholamine excretion during cold acclimatization was studied.

Methods

Animals and urine samples

Adult male rats ($n = 39$) were used in the experiments. They were allowed free access to pelleted food and water and had a constant ambient temperature of +22 ± 1°C and a light cycle of 12 h. Before the start of the experiments each animal was weighed and its colon temperature was measured with an electric thermometer (Ellab) at a room temperature of +22°C. After these procedures, thirty-two rats were put in individual cages designed for the collection of a 24 h urine sample, during which time they were not allowed any food or water. On the following day the urine volume was measured and samples taken in test tubes. The urine was acidified by putting 0.5 ml 6 N HCl into the collection bottles beforehand. The urine samples were stored at −70°C until analysed.

β-Adrenoceptor blocking effects of propranolol and pindolol

To investigate the β-receptor blocking effects of the selected doses of propranolol (2.0 mg kg^{-1} i.p.) and pindolol (0.2 mg kg^{-1} i.p.), we used a group of 7 male rats weighing 367–420 g. The animals were anaesthetized with phentanyl-

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fluanisone (Hypnorm, Duphar), 1 ml i.m., and diazepam (Diapam, Orion), 0.5 ml i.m., and their heart rates monitored with a cardioscope (Olli 201) equipped with needle electrodes. After 20 min each animal was given propranolol (Propral, Medipolar) or pindolol (Visken, Sandoz) or 1 ml 0.9% NaCl i.p. Each animal received an i.p. injection of one of these three solutions, the time between the experiments being at least 48 h. The heart rate monitoring lasted for 40 min after the i.p. injection.

Cold-acclimatization and drug treatment

Twenty-four rats were divided into three groups, eight in each, and were transferred to a cold room with an ambient temperature of $+4 \pm 1$°C, where they were placed in individual cages. The cold acclimatization lasted for 42 days, during which time the rats received pelleted food and water *ad libitum*, except on the days of urine collection (once a week), when they were deprived of food and water.

The first group of rats was injected with 2.0 mg kg^{-1} propranolol (Propral, Medipolar) i.p. every day, the second with pindolol (Visken, Sandoz) 0.2 mg kg^{-1} i.p. and the third with 0.9% NaCl i.p. All injections took place at the same time of day, beginning at 13 h 30 min. The animals were weighed once a week and their colon temperature measured. A 24 h urine sample was also taken once a week.

The fourth group of rats ($n = 8$) was kept at room temperature ($+23 \pm 1$°C) for 42 days and they were treated with 0.9% NaCl. Urine was collected and the animals were weighed and their colon temperature measured as described above.

Brown adipose tissue

After the period of 42 days the rats were killed by a blow on the neck and the interscapular adipose tissue excised under a stereomicroscope. All white adipose tissue and other fragments of connective tissue were removed and the brown adipose tissue that remained was weighed, frozen in liquid nitrogen and stored at −70°C until analysed.

Isolation of mitochondria

Mitochondria were isolated from the BAT by the method of Cannon & Lindberg (1979). The BAT was homogenized at pH 7.2 in ice-cold 0.25 M sucrose containing 5 mM TES (N-trismethyl-2-aminoethanesulphonic acid) and the homogenate filtered through two layers of gauze and centrifuged at 8500 *g* for 10 min. The supernatant and hard-packed fat layer were discarded. The pellet, which consisted of cell debris, nuclei and mitochondria was resuspended in sucrose and the suspension centrifuged at 700 *g* for 10 min. The nuclear pellet was discarded and the supernatant was centrifuged at 8500 *g* for 10 min. The mitochondrial pellet was washed with sucrose.

Determination of oxidative enzymes

Succinate dehydrogenase (SDH) activity in the isolated mitochondria was determined by phenazinemethosulphate and 2,6-dichloroindophenol as the electron acceptor system, according to King (1967). In order to obtain the substrate-activated (maximal) enzyme activity, the suspension was preincubated at +37°C for 7 min in a mixture containing 50 mmol potassium-succinate in a total volume of 0.3 ml (Kimura *et al.*, 1967). The maximal enzyme activity was then determined from the incubated sample as described above.

Cytochrome oxidase (CyO) activity was determined spectrophotometrically by measuring the rate of aerobic oxidation of ferrocytochrome catalyzed by cytochrome oxidase at +21°C (Yonetani & Ray, 1965). Cytochrome c was reduced by dithionite and its excess absorbed with Sephadex. Protein was assayed by the method of Lowry *et al.* (1951).

Analysis of urinary catecholamines

The urinary catecholamines noradrenaline (NA), adrenaline (Ad) and dopamine were analysed according to the method presented by Taylor *et al.* (1983), by high-performance liquid chromatography with an electrochemical detector. The catecholamines were extracted from 30 µl of urine into 30 mg Al_2O_3 at pH 8.60 in conical test tubes. Dihydroxybenzylamine (DHBA) and isoprenaline (Iso) were used as internal standards. After the sample had been washed four times with 2 ml H_2O, the catechols were released into 100 µl of 0.2 M $HClO_4$. Thirty µl was injected into the chromatograph, which consisted of a M-45 pump (Waters Associates Inc.), a Rheodyne 7125 injector with a 50 µl sample loop, and Ultrasphere ODS 5 µl (150 × 4 mm) reversed phase analytical column (Beckman) protected with a guard column (50 × 2 mm) filled with Vydac RP (30–40 µm), a LC-4B amperometric detector (Bioanlytical Systems Inc.) with a glassy carbon working electrode and an Ag/$AgCl_2$ reference electrode. The oxidation potential was 0.6 V. The chromatograms were recorded on a double channel recorder (Kipp & Zonen). The ratio of the peak height of each catecholamine to the average peak heights of the internal standards was used in the concentration calculations.

Statistical analysis

The statistical significances of the differences in weight gain within each group were assessed by Student's paired *t* test and the overall significance between the four groups by a one-way analysis of variance. Where an overall significance of $P < 0.05$ was reached, individual group comparisons were made by Bonferroni's test. Two-way analysis of variance was used to see if any overall difference existed between the various treatments on heart rate. Where an overall significance of $P < 0.05$ was reached, the comparisons between individual treatments were made by Tukey's test.

Results

Heart rate

During the twenty minutes after the onset of anaesthesia heart rate remained around the same level in all rats. Heart rate was not modified by injection of 0.9% NaCl solution. Propranolol 2 mg kg^{-1} significantly decreased heart rate. Pindolol 0.2 mg kg^{-1} did not cause any apparent effect on heart rate, although a slight decrease was seen 30 min after an i.p. injection of the drug (Table 1).

Weight of the rats

The initial weights of the rats varied from 290 to 420 g, the average weights being about the same in each group. The NaCl-treated rats kept at room temperature gained weight significantly over the period of 42 days ($P < 0.05$), whereas the NaCl-treated rats kept in the cold lost some weight during the first two weeks. Thereafter a slight rise was seen and the average weight in this group on day 42 was practically the same as before the cold adaptation period. The propranolol-treated rats also lost some weight during the first two weeks in the cold, but after that their weights began to increase. The total weight gain was significant compared with the initial value ($P < 0.05$). The pindolol-treated animals gained weight throughout the acclimatization period, the differences compared with the initial weights being significant ($P < 0.01$) (Table 2). When all four groups were compared by analysis of variance, the weight gains differed significantly from each other ($F = 12.76$, $P = 0.000$). The weight gain was significantly greater in pindolol-treated animals than in the cold-exposed NaCl-treated ones ($P < 0.05$), but compared with the propranolol-treated group the difference was not significant.

Table 1 Changes in heart rate (HR) in propranolol, pindolol or 0.9% NaCl-treated rats

	Propranolol ($2.0\,mg\,kg^{-1}$) ($n = 7$)	*Pindolol* ($0.2\,mg\,kg^{-1}$) ($n = 7$)	*0.9% NaCl* (1 ml i.p.) ($n = 7$)
Pretreatment			
HR	321 ± 21	321 ± 15	311 ± 6
	($F = 2.9$, $P = 0.06$)		
10 min			
HR	309 ± 23	320 ± 14	309 ± 8
	($F = 3.0$, $P = 0.055$)		
Change	-12 ± 6[a]	-1 ± 8	-2 ± 2
	($F = 13.5$, $P < 0.001$)		
20 min			
HR	297 ± 20	319 ± 17	317 ± 6
	($F = 11.2$, $P < 0.001$)		
Change	-23 ± 14[b, c]	-1 ± 10	$+6 \pm 10$
	($F = 36.7$, $P < 0.001$)		
30 min			
HR	283 ± 33	310 ± 33	315 ± 9
	($F = 6.5$, $P = 0.004$)		
Change	-37 ± 19[b, c]	-11 ± 19	$+4 \pm 9$
	($F = 29.1$, $P < 0.001$)		
40 min			
HR	296 ± 25	318 ± 20	313 ± 7
	($F = 3.3$, $P = 0.047$)		
Change	-26 ± 9[b, d]	-3 ± 9	$+5 \pm 9$
	($F = 51.2$, $P < 0.001$)		

[a] $P < 0.05$ compared to NaCl-treated rats.
[b] $P < 0.01$ compared to NaCl-treated rats.
[c] $P < 0.05$ compared to pindolol-treated rats.
[d] $P < 0.01$ compared to pindolol-treated rats. These comparisons were made by Tukey's test.

No significant difference was found between the propranolol-treated and cold-exposed NaCl-treated rats. The total weight gain in all the groups of cold-exposed animals was significantly lower than in the rats kept at room temperature (Table 2).

Core temperature

All the cold-exposed animals had normal colon temperatures before the beginning of the exposure, and no differences were seen between the cold-exposed groups either then or during the cold acclimatization. The animals kept at room temperature for 42 days had an almost constant colon temperature throughout the experimental period, but the initial values were higher than in the other groups. Higher colon temperatures were measured later during the adaptation period in the cold-exposed rats (Table 3).

Brown adipose tissue and oxidative enzymes

The weight of interscapular brown adipose tissue (IBAT) was lowest in the NaCl-treated rats kept at room temperature and analysis of variance showed statistically significant differences between the four groups ($F = 31.87$, $P = 0.000$). The weight of IBAT in all cold exposed groups was significantly higher than in the room temperature group ($P < 0.01$ for all cold-exposed groups), but no statistically significant differences existed between the three groups kept at +4°C. The relative weight of IBAT expressed as mg BAT g^{-1} body weight was again lowest in the NaCl-treated animals kept at room temperature and the overall difference between the four groups was statistically significant ($F = 44.79$, $P = 0.000$). All cold-exposed groups had a significantly higher relative weight of IBAT than

Table 2 Weights of rats before and after acclimatization at +4°C or +23°C

	+4°C Propranolol ($2\,mg\,kg^{-1}\,day^{-1}$) ($n = 8$)	*+4°C Pindolol* ($0.2\,mg\,kg^{-1}\,day^{-1}$) ($n = 8$)	*+4°C NaCl 0.9%* ($1\,ml\,day^{-1}$) ($n = 8$)	*+23°C NaCl 0.9%* ($1\,ml\,day^{-1}$) ($n = 8$)
Initial weight (g)	319 ± 17	328 ± 29	333 ± 29	316 ± 17
	($F = 1.08$, $P = 0.374$)			
Weight, day 14 (g)	314 ± 16	340 ± 31	323 ± 30	341 ± 18
	($F = 2.32$, $P = 0.096$)			
Weight, day 42 (g)	335 ± 18[c]	366 ± 35	343 ± 35[c]	401 ± 52
	($F = 5.68$, $P = 0.004$)			
Total weight gain (g)	16.4 ± 18.4[b]	38.8 ± 22.5[a, b]	0.3 ± 27.3[c]	84.6 ± 42.0
	($F = 12.76$, $P = 0.000$)			

[a] $P < 0.05$ compared to NaCl-treated rats kept at +4°C.
[b] $P < 0.01$ compared to NaCl-treated rats kept at +23°C.
[c] $P < 0.05$ compared to NaCl-treated rats kept at +23°C. These comparisons were made by Bonferroni's test.

Table 3 Colon temperature (T_c) in rats at +23°C and during cold acclimatization at +4°C

	Ta = +4°C Propranolol ($2.0\,mg\,kg^{-1}$ i.p.) ($n = 8$)	*Ta = +4°C Pindolol* ($0.2\,mg\,kg^{-1}$ i.p.) ($n = 8$)	*Ta = +4°C 0.9% NaCl* (1 ml i.p.) ($n = 8$)	*Ta = +23°C 0.9% NaCl* (1 ml i.p.) ($n = 8$)
Initial T_c (°C)	37.3 ± 0.4[a]	37.3 ± 0.3[a]	37.4 ± 0.5[a]	38.6 ± 0.2
	$F = 26.80$, $P = 0.000$)			
T_c (°C) day 1	37.2 ± 0.5	37.2 ± 0.5	37.4 ± 0.2	—
	($F = 0.50$, $P = 0.687$)			
T_c (°C) day 7	37.6 ± 0.4	37.6 ± 0.4	37.9 ± 0.3	37.5 ± 0.4
	($F = 1.36$, $P = 0.275$)			
T_c (°C) day 14	37.7 ± 0.5	$38.2 \pm 0.$	37.9 ± 0.4	37.5 ± 0.2
	($F = 4.01$, $P = 0.017$)			
T_c (°C) day 21	37.3 ± 0.3	37.6 ± 0.5	37.4 ± 0.3	37.1 ± 0.3
	($F = 2.87$, $P = 0.053$)			
T_c (°C) day 29	38.0 ± 0.6[b]	38.1 ± 0.8	38.4 ± 1.0[a]	37.3 ± 0.4
	($F = 3.12$, $P = 0.041$)			
T_c (°C) day 35	37.8 ± 0.6	38.0 ± 0.6[a]	37.5 ± 0.4	37.0 ± 0.5
	($F = 6.08$, $P = 0.003$)			
T_c (°C) day 42	37.4 ± 0.4	37.5 ± 0.5	37.6 ± 0.4	—
	($F = 0.16$, $P = 0.918$)			

[a] $P < 0.01$ compared to NaCl-treated animals kept at +23°C.
[b] $P < 0.05$ compared to NaCl-treated animals kept at +23°C, by Bonferroni's test.
Ta = ambient temperature.

Table 4 Weight, relative weight and protein content in brown adipose tissue in rats acclimatized to +4°C for 42 days treated with propranolol, pindolol or 0.9% NaCl, or kept at +23°C for 42 days and treated with 0.9% NaCl

	Ta = +4°C *Propranolol* (2 mg kg^{-1} day^{-1}) (*n* = 8)	*Ta* = +4°C *Pindolol* (0.2 mg kg^{-1} day^{-1}) (*n* = 8)	*Ta* = +4°C *0.9% NaCl* (1 ml day^{-1}) (*n* = 8)	*Ta* = +23°C *0.9% NaCl* (1 ml day^{-1}) (*n* = 8)
Interscapular adipose tissue weight (mg)	676 ± 145[a]	609 ± 80[a] ($F = 31.87$, $P = 0.000$)	665 ± 104[a]	248 ± 56
Interscapular adipose tissue weight/body weight (relative weight) (mg g^{-1})	2.02 ± 0.46[a]	1.67 ± 0.20[a,b] ($F = 44.79$, $P = 0.000$)	1.98 ± 0.11[a]	0.64 ± 0.17
Protein content (mg)	70.58 ± 17.90[a]	61.26 ± 10.77[a] ($F = 51.34$, $P = 0.000$)	63.24 ± 9.00[a]	7.38 ± 3.18

[a] $P < 0.01$ compared to NaCl-treated rats kept at +23°C;
[b] $P < 0.05$ compared to NaCl-treated rats kept at +4°C, by Bonferroni's test. Ta = ambient temperature.

Table 5 Specific activities of oxidative enzymes in mitochondria of brown adipose tissue in rats acclimatized to +4°C for 42 days and treated with propranolol, pindolol or 0.9% NaCl, or kept at +23°C for 42 days and treated with 0.9% NaCl

	Ta = +4°C *Propranolol* (2 mg kg^{-1} day^{-1})	*Ta* = +4°C *Pindolol* (0.2 mg kg^{-1} day^{-1})	*Ta* = +4°C *0.9% NaCl* (1 ml day^{-1})	*Ta* = +23°C *0.9% NaCl* (1 ml day^{-1})
Cytochrome oxidase activity (μmol cytochrome c oxidized min^{-1} mg^{-1} protein)	2.01 ± 1.09	2.16 ± 0.85 ($F = 1.67$, $P = 0.194$)	1.93 ± 0.85	2.81 ± 0.65
Succinate dehydrogenase activity (μmol min^{-1} mg^{-1} protein)				
Actual	0.78 ± 0.15	0.77 ± 0.19 ($F = 0.86$, $P = 0.526$)	0.88 ± 0.32	0.70 ± 0.20
Maximal	0.88 ± 0.16	0.96 ± 0.25 ($F = 0.90$, $P = 0.544$)	0.92 ± 0.37	0.76 ± 0.20

Ta = ambient temperature.

the room temperature group ($P < 0.01$). Pindolol-treated rats had significantly lower relative weight of IBAT than the NaCl-treated rats kept at +4°C ($P < 0.05$).

The protein content of the interscapular brown adipose tissue varied significantly between the four groups ($F = 51.34$, $P = 0.000$), being smallest in the room temperature group. However, no statistically significant differences existed between the cold-exposed groups (Table 4).

There were no statistically significant differences seen in the activities of succinate dehydrogenase or cytochrome oxidase between the four experimental groups (Table 5).

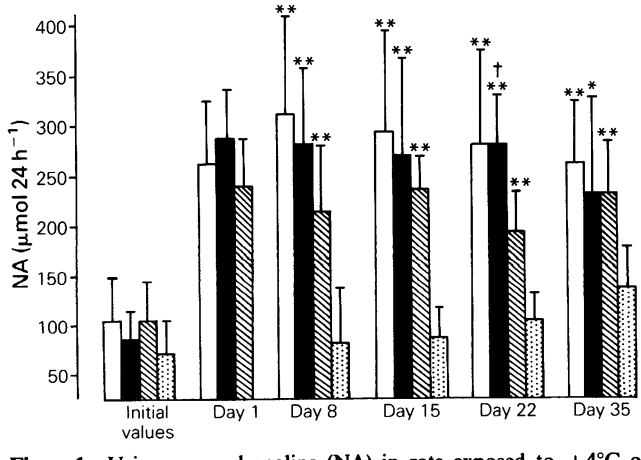


Figure 1 Urinary noradrenaline (NA) in rats exposed to +4°C or +23°C for 42 days and treated with propranolol, pindolol or 0.9% NaCl. Open columns, propranolol at +4°C; solid columns, pindolol at +4°C; hatched columns, 0.9% NaCl at +4°C; stippled columns, 0.9% NaCl at +23°C. Columns represent means for 8 rats and vertical lines show s.e.mean. *$P < 0.05$; **$P < 0.01$ compared to NaCl-treated rats kept at +23°C, Bonferroni's test. †$P < 0.05$ compared to NaCl-treated rats kept at +4°C, Bonferroni's test.

Excretion of catecholamines

An initial rise in noradrenaline (NA) excretion was seen in all three cold-exposed groups after the first week at +4°C, this being about 3.2 fold in the pindolol group, 2.5 fold in the propranolol group and 2.2 fold in the NaCl group. NA excretion was constant in the NaCl-treated rats kept at room temperature, except that the last two values were a little higher. Significant differences existed between room temperature-exposed rats and cold-exposed rats throughout most of the experimental period, but the initial values measured at room temperature were the same in all four groups. NA excretion during cold acclimatization was highest in the pindolol-treated animals and lowest in the NaCl-treated ones, but the only statistically significant difference between pindolol and NaCl treatment was seen on day 22 (Figure 1).

Adrenaline excretion followed the same pattern in all three cold-exposed groups, an initial rise during the first two weeks in the cold followed by a decline to about the same level. The initial adrenaline values were significantly smaller in the room temperature group and no marked rise was seen later with this group (Figure 2).

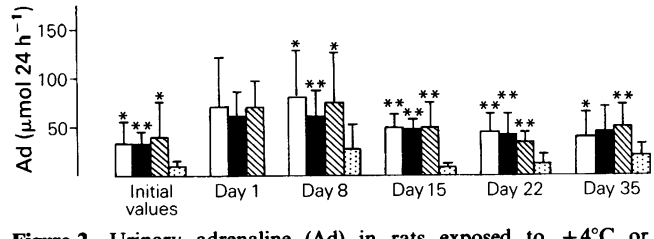


Figure 2 Urinary adrenaline (Ad) in rats exposed to +4°C or +23°C for 42 days and treated with propranolol, pindolol or 0.9% NaCl. Open columns, propranolol at +4°C; solid columns, pindolol at +4°C; hatched columns, 0.9% NaCl at +4°C; stippled columns, 0.9% NaCl at +23°C. Columns represent means for 8 rats and vertical lines show s.e.mean. *$P < 0.05$; **$P < 0.01$, compared to NaCl-treated rats kept at +23°C, Bonferroni's test.

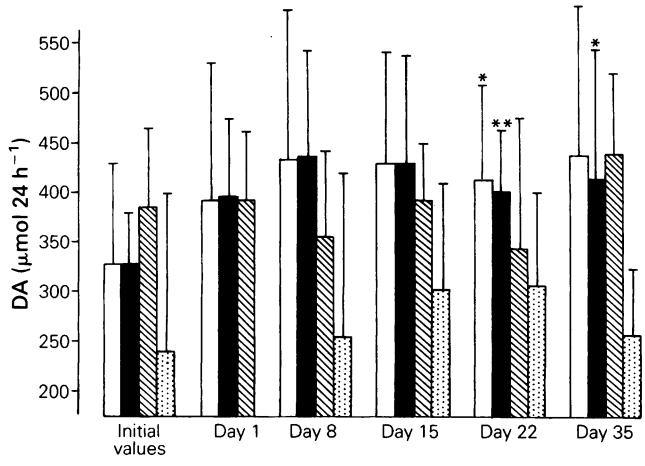


Figure 3 Urinary dopamine (DA) in rats exposed to +4°C or +23°C for 42 days and treated with propranolol, pindolol or 0.9% NaCl. Open columns, propranolol at +4°C; solid columns, pindolol at +4°C; hatched columns, 0.9% NaCl at +4°C; stippled columns, 0.9% NaCl at +23°C. Columns represent means for 8 rats and vertical lines show s.e.mean. $*P < 0.05$; $**P < 0.01$, compared to NaCl-treated rats kept at +23°C, Bonferroni's test.

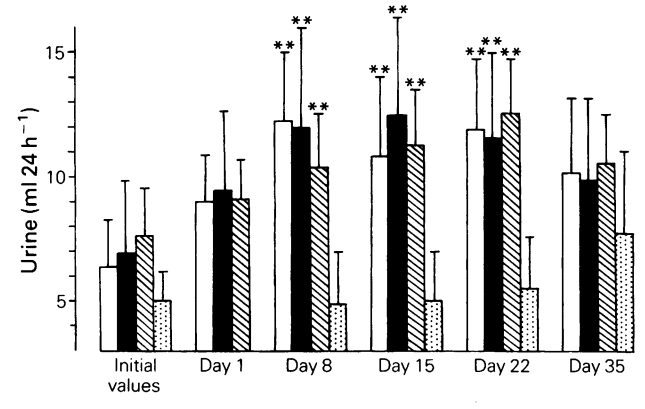


Figure 4 Urinary volumes in rats exposed to +4°C or +23°C for 42 days and treated with propranolol, pindolol or 0.9% NaCl. Open columns, propranolol at +4°C; solid columns, pindolol at +4°C; hatched columns, 0.9% NaCl at +4°C; stippled columns, 0.9% NaCl at +23°C. Columns represent means for 8 rats and vertical lines show s.e.mean. $**P < 0.01$ compared to NaCl-treated rats kept at +23°C, Bonferroni's test.

Dopamine-excretion remained around the same level throughout cold exposure, but a slight rise was seen in the two groups treated with β-adrenoceptor antagonists after two weeks at +4°C compared with the initial values measured at room temperature. Dopamine excretion was a little, but significantly, higher in the cold than at room temperature during the last two weeks (Figure 3).

The 24 hour urinary volumes were approximately the same in the three cold-exposed groups throughout the experiment. A rise was seen during the first three weeks and the final values were still a little higher than the initial ones. In the room temperature control group the urinary volumes remained at a constant level until a slight rise was seen on day 35 (Figure 4).

Discussion

The dose of propranolol used in this study caused a decrease in heart rate. Pindolol did not cause any apparent change in heart rate, probably because of the intrinsic sympathomimetic activity of the drug. The haemodynamic effects of the different β-adrenoceptor agonists have been studied previously, e.g. Clark (1982) found that in dogs, the doses chosen for pindolol and propranolol produced an equivalent reduction of isoprenaline-induced tachycardia, the dose of propranolol being ten times that of pindolol. As the dose of propranolol used in our experiments blocked β-adrenoceptors, we decided to use a ten times lower dose of pindolol in the acclimatization experiments despite its non-significant effect on the heart rate of the rat.

Regular administration of the non-specific β-adrenoceptor antagonist, propranolol, was not seen to have any inhibitory effect on the hypertrophy and oxidative capacity of brown adipose tissue induced by cold exposure. Since an acute inhibitory effect on non-shivering thermogenesis has been verified for propranolol in many previous experiments (Clark, 1979), this result was not to be expected. However, it is possible that the blockade of β-receptors in the BAT was no longer effective after a long period of time. The daily dose of propranolol ($2.0\,mg\,kg^{-1}$) was quite small, and possibly did not provide an effective β-adrenoceptor blockade in brown adipose tissue, although doses of this size have been shown to inhibit non-shivering thermogenesis in mammals, e.g. in reindeer (Soppela *et al.*, 1986) and rats (Griggio, 1982).

The pindolol-treated rats, on the other hand, had smaller amounts of interscapular brown adipose tissue (IBAT) than the other cold-acclimatized animals, which is indicative of an effect in this tissue. Due to its intrinsic sympathomimetic activity, pindolol has been shown to act as a partial β-adrenoceptor agonist in other tissues as well as the heart, e.g. peripheral blood vessels (Thulesius *et al.*, 1982), and its effects on blood lipids are probably also related to this partial agonist activity (Leren *et al.*, 1982). The slight reducing effect of pindolol on IBAT hypertrophy observed here is indicative of a potent β-adrenoceptor blocking effect in this tissue. A pindolol-induced reduction of the thermogenic capacity of brown adipose tissue cannot be ruled out, although no changes in BAT protein contents or activities of CyO and SDH were seen. There are controversial results in the literature on changes in specific activities of oxidative enzymes in response to cold-acclimatization (Thomson *et al.*, 1969; Skala *et al.*, 1970; Bukowiecki *et al.*, 1982). Also chronic alcohol intake has been found to increase the specific activities of CyO and SDH, in addition to causing BAT-hypertrophy, without increasing BAT protein content per unit of tissue (Huttunen & Kortelainen, 1988).

Although statistical analysis revealed only a few significant differences between the three cold-exposed groups in urinary catecholamine excretion, treatment with the β-adrenoceptor antagonists produced slightly higher values for NA. This is in accordance with previous observations in which elevated plasma NA levels were obtained during propranolol treatment in man (Trap-Jensen *et al.*, 1982). On the other hand, pindolol did not cause any reduction in the cold-induced rise in NA, although this drug has been shown to prevent the rise in NA during mental stress (Trap-Jensen *et al.*, 1982).

The excretion of NA and Ad followed the same pattern during cold acclimatization as found earlier (LeBlanc, 1975) and no obvious drug effects were seen. Dopamine excretion was relatively unchanged in the cold-exposed groups during the period of 42 days, but had a tendency to rise towards the end of the cold-acclimatization period and during the last two weeks significant differences existed between these groups and the control group at +23°C. It is unlikely that the consistently lower outputs of these three catecholamines in the +23°C control group could be due to a reduced stability of urinary catecholamines at this temperature over the 24 h period. Urinary catecholamines are known to be stable under acidic conditions (Moleman, 1985), but storage of catecholamines at −30°C or less has been shown to be essential for longterm preservation of urine samples (Weiber *et al.*, 1983).

The decreased weight gain in NaCl-treated rats during cold-acclimatization could be explained by increased metabolism for heat production. However, the opposite phenomenon was seen in the propranolol and pindolol-treated rats; they gained

weight in the cold, the increase being more marked in the pindolol-treated animals. This may be related to the anti-lipolytic properties of β-adrenoceptor antagonists, derived from their inhibition of catecholamine-induced lipolysis (Harms et al., 1982). On the other hand, the possibility that these β-blockers increased food consumption, which was not measured in our experiments, cannot be ruled out. However, users of β-blockers have not been found to gain weight and have therefore been assumed to reduce their food consumption (Björntorp, 1982). The relative abundance of white adipose tissue, which has insulating properties in the cold (LeBlanc, 1975), could also be a compensatory feature for the inhibitory effect of β-blockers on BAT.

The higher initial colon temperature in the room temperature group might have been caused by differences in the handling of the animals, which were not yet accustomed to temperature measurements. No signs of infection or other diseases were observed either before or during the experimental period.

In conclusion, the present results show that pindolol, a potent non-selective β-adrenoceptor antagonist with intrinsic sympathomimetic activity, prevents the reduction in body weight gain in rats during cold-acclimatization and slightly reduces the amount of interscapular brown adipose tissue, without having any effects on the other BAT parameters studied here. Another non-selective β-adrenoceptor antagonist without ISA, propranolol, also caused some weight gain compared with the saline-treated rats in the cold, but did not affect the oxidative capacity of BAT. The differences in these drug-effects might also be due to other pharmacological properties of these drugs, e.g. differences in plasma clearance rates, rather than to the presence or absence of ISA. These results suggest a possible connection between weight gain, cold-acclimatization and the administration of β-adrenoceptor antagonists. In addition, pindolol induced a reduction in brown adipose tissue hypertrophy and the effect of different doses of this drug on this parameter may be worthy of further study.

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Antigen challenge induces pulmonary airway eosinophil accumulation and airway hyperreactivity in sensitized guinea-pigs: the effect of anti-asthma drugs

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1 Guinea-pigs were sensitized with 3 injections of ovalbumin (OA) (1 or 10 μg per animal) using $Al(OH)_3$ and pertussis vaccine as adjuvants at two week intervals.

2 Sensitized guinea-pigs were challenged with an aerosol of OA (0.1%) over a one hour period and both airway reactivity and cellular content of bronchoalveolar lavage (BAL) fluid were assessed at intervals for up to 7 days.

3 Guinea-pigs sensitized with 1 μg of ovalbumin responded to an aerosol of OA with increased pulmonary airway eosinophilia, which was evident 1 day after challenge and was present for up to 7 days. Airway hyperreactivity was not detectable in these animals.

4 Guinea-pigs sensitized with 10 μg of ovalbumin responded to an aerosol of OA with increased pulmonary airway neutrophilia and eosinophilia and with increased airway reactivity which was maximal between 8 and 24 h after exposure to OA.

5 Depletion of circulating platelets or neutrophils, by use of selective antisera, did not alter either the magnitude of eosinophilia or the intensity of airway reactivity in sensitized guinea-pigs (10 μg) exposed to an aerosol of OA.

6 Pretreatment of sensitized guinea-pigs (10 μg) for 6 days with AH 21-132, aminophylline, dexamethasone or ketotifen inhibited pulmonary airway eosinophilia, but did not diminish airway hyperreactivity. Neither eosinophil accumulation nor development of airway hyperreactivity was influenced by treatment with mepyramine or salbutamol over a 6 day period before OA inhalation.

7 Although eosinophilia may occur in association with increased airway reactivity in this animal model, there is no evidence of a causal relationship.

Introduction

The presence of eosinophils in airways of asthma patients was recognised over sixty years ago in histological preparations of lung tissue (Huber & Koessler, 1922). Increased eosinophil numbers in peripheral blood, sputum or lung tissue are now widely acknowledged to be characteristic of asthma (Dunnill, 1960; Epstein, 1972; Horn *et al.*, 1975; Frigas & Gleich, 1986). In addition, toxic eosinophil products, such as major basic protein (MBP) (Frigas *et al.*, 1981) and eosinophil cationic protein (ECP) (Dahl *et al.*, 1978) have been demonstrated in the sputum and blood of asthma patients. Further evidence of eosinophil involvement in inflammatory reactions within the asthmatic lung has been provided, by demonstration of an increased bronchial eosinophilia in patients exhibiting a late asthmatic response (De Monchy *et al.*, 1985) and of a positive correlation between airway reactivity to methacholine and blood eosinophilia in asthmatics who respond to allergen with a biphasic airway obstruction (Durham & Kay, 1985). Because of this association, it is widely considered that airway eosinophilia leads to epithelial desquamation as a consequence of MBP and ECP release, which in turn would lead to airway hyperreactivity (Flavahan *et al.*, 1988).

The present investigation has sought to study the relationship between airway eosinophilia and hyperreactivity in sensitized guinea-pigs after exposure to antigen. The guinea-pig has been chosen as a test animal because of histological similarities that exist between lungs of antigen exposed guinea-pigs and asthmatic lungs in man (reviewed by Kallos & Kallos, 1984) and because this species can exhibit early and late-onset airway obstruction (Hutson *et al.*, 1988a), bronchial eosinophilia (Aoki *et al.*, 1988; Dunn *et al.*, 1988) and increased airway reactivity following exposure of sensitized animals to antigen (Aoki *et al.*, 1988; Daffonchio *et al.*, 1989). The effect of a number of established anti-asthma drugs, and a novel substance AH 21-132 (Bewley & Chapman, 1988; Sanjar *et al.*, 1990), upon antigen-induced airway eosinophilia and hyperreactivity has been studied. In addition, the role of circulating neutrophils and platelets in the development of these events has been assessed by use of lytic antisera for selective depletion of these cells.

Methods

Animals

Male Dunkin-Hartley guinea-pigs (400–500 g) were used for study of lung function. Male New Zealand White rabbits (2.5–3.5 kg) were used for raising antibodies.

Active sensitization of guinea-pigs to ovalbumin

Guinea-pigs received cyclophosphamide (100 mg kg^{-1} i.p.) 1 day before sensitization. Animals were sensitized by i.p. injection of 1 ml of an emulsion of $Al(OH)_3$ (2 mg), pertussis vaccine (0.25 ml) and 1 or 10 μg of ovalbumin (OA), with supplementary injections of such emulsion 3 and 6 weeks later. After 7 weeks, OA (10 μl per site) was injected intradermally and skin thickness was measured 1 h later. A doubling of skin thickness indicated sensitization. Animals which did not respond to intradermal OA were not used.

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Antigen exposure

Sensitized guinea-pigs were placed in a 16 litre plexiglass chamber which was partitioned into 4 equal sections. Each section housed a guinea-pig, which was restrained by the neck. The snout of each animal was placed in a nose-cone attached to the outlet of a DeVilbiss nebuliser. Animals were exposed for one hour to an aerosol of OA (10 ml of 0.1% solution) which was generated in the nebuliser using compressed air ($7 l min^{-1}$). This procedure does not lead to an anaphylactic response. At various times after exposure, airway reactivity to intravenous spasmogens was measured in anaesthetized animals and lungs were subsequently lavaged. Guinea-pigs sensitized with 1 μg of OA were tested at 24, 48, 72 h and 7 days after exposure to OA, whereas guinea-pigs sensitized with 10 μg were tested additionally at 4, 8 and 12 h after exposure to OA. Control groups included sensitized guinea-pigs not exposed to allergen and naïve guinea-pigs exposed to OA and tested 24, 48 or 72 h after exposure.

Measurement of lung function and airway reactivity to histamine and $PGF_{2\alpha}$

Guinea-pigs were anaesthetized with sodium phenobarbitone ($100 mg kg^{-1}$ i.p.) and sodium pentobarbitone ($30 mg kg^{-1}$ i.p.) and paralysed with gallamine ($10 mg kg^{-1}$ i.m.). Animals were ventilated ($8 ml kg^{-1}$, 1 Hz) via a tracheal cannula with a mixture of air and oxygen (1:1 v/v). Air flow was monitored by a pneumotachograph (type 0000, Fleisch, Switzerland) in line with the respiratory pump). Pressure changes within the pneumotachograph were measured with a differential pressure transducer (MP45-871, Validyne, U.S.A.). Pressure changes within the thorax were monitored directly via an intrathoracic cannula, and the differential pressure between the trachea and thorax was measured with a differential pressure transducer (PM45-24, Validyne, U.S.A.). From these measurements of flow and differential pressure, both airway resistance (R_L) and compliance (C_{dyn}) were calculated breath by breath with a respiratory analyser (Model 6, Buxco, U.S.A.). Preparations were allowed to stabilize for 10 to 15 min, after which the lungs were hyperinflated for 3–5 respiratory cycles by increasing the respiratory pump output to $16 ml kg^{-1}$. After this manoeuvre, values for basal R_L and C_{dyn} were recorded. To assess airway reactivity, spasmogens (histamine, 1.0, 1.8 $\mu g kg^{-1}$ and $PGF_{2\alpha}$ 56 $\mu g kg^{-1}$) were administered intravenously at 10 min intervals via a cannulated jugular vein.

Bronchoalveolar lavage and cell counting

After measurement of lung function parameters, guinea-pigs were killed with sodium pentobarbitone ($100 mg kg^{-1}$ i.v.) and lungs were lavaged with 6 × 10 ml aliquots of Ca^{2+} and Mg^{2+} free Hank's balanced salt solution (HBSS), containing bovine serum albumin (BSA, 0.3%), EDTA (10 mM) and HEPES (10 mM). Lavage fluid was centrifuged (200 g for 10 min) and the cell pellet resuspended in 1 ml of supplemented HBSS. Twenty μl of the cell suspension was added to 180 μl of Turk's solution (1:20 dilution) and total cells were counted in an haemocytometer. Differential cell counts were made from smears stained by Leishman's stain. Cells were identified and counted under oil immersion (× 1000), approximately 500 cells per smear were counted and the total population of each cell type was calculated.

Drug administration

Drugs were administered for seven days (6 days before antigen exposure) via an osmotic mini-pump (Alzet), implanted subcutaneously in the nuchal region under ether anaesthesia. Animals receiving saline or a solvent mixture (polyethylene glycol and ethanol, 30/70 v/v) acted as controls. AH 21-132, dexamethasone, ketotifen and mepyramine were administered at $1 mg kg^{-1} day^{-1}$. Aminophylline was administered at $10 mg kg^{-1} day^{-1}$ and salbutamol at 1.0 or $0.1 mg kg^{-1} day^{-1}$.

Passive cutaneous anaphylaxis

Blood (5 ml) was removed by cardiac puncture from a group of sensitized (10 μg OA) guinea-pigs ($n = 30$) under ether anaesthesia. Serum was collected 24 h after clot retraction, pooled and centrifuged (400 g for 10 min). Aliquots of serum were stored at −20°C. Naïve guinea-pigs were shaved on both flanks and one hour later, marked skin sites were injected with diluted (1:100) serum from hyperimmune (10 μg OA) or normal animals. Three days later, other sites were injected with diluted (1:100) hyperimmune serum. After a further 3 days, Evans blue dye (2.5% w/v in normal saline) and ^{125}I-human serum albumin (1 μCi per animal), in a final volume of 0.5 ml, were injected intravenously. Within 10 min, sensitized and non-sensitized skin sites were injected with OA (10 μg per site). Thirty min later, guinea-pigs were killed by cervical dislocation and 1 ml of blood was withdrawn by cardiac puncture. The injection sites were removed with a wad-punch (15 mm diameter) and the radioactive content of blood and skin sites was determined in an automatic gamma counter (LKB). Plasma protein extravasation was expressed as μl whole blood equivalent (Williams & Morley, 1973). This procedure allowed measurement of the ability of serum from sensitized guinea-pigs to sensitize skin of naïve guinea-pigs after 3 or 6 days.

Preparation of antibodies to guinea-pig platelets and neutrophils

Preparation of lytic antibodies to guinea-pig platelets or neutrophils has been described in detail elsewhere (Sanjar *et al.*, 1990; Hutson *et al.*, 1989). Briefly, purified platelets (from blood, ca 5×10^8) or neutrophils (from peritoneal exudates, ca 5×10^7) from the guinea-pig were emulsified with Freund's complete adjuvant (1:1) and injected subcutaneously into the neck and flank of rabbits. This procedure was repeated on two further occasions at three-weekly intervals after which rabbits were anaesthetized (sodium pentothal, $30–50 mg kg^{-1}$ i.v.) and bled by cardiac puncture. Serum was collected 24 h later, heat inactivated (56°C for 30 min) and absorbed with guinea-pig erythrocytes. After centrifugation (2000 g for 10 min.), sera were divided into aliquots and stored at −20°C.

Materials

The following materials have been used: aluminium hydroxide (Sandoz), bovine gamma globulin (Miles), bovine serum albumin (Fluka), cyclophosphamide (Sigma), EDTA (Merck), ether (Siegfried), Evans blue dye (Merck), Freunds complete adjuvant (Difco), gallamine (Davis & Geck), Hanks balanced salt solution (Gibco), HEPES (Gibco), histamine (Fluka), ^{125}I-human serum albumin (Amersham), Leishmans stain (Merck), ovalbumin (Fluka), percoll (Pharmacia), pertussis vaccine (Swiss serum & vaccine Institute), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, Sigma), sodium pentobarbitone (Merck), sodium phenobarbitone (Merck), sodium pentothal (Merck), Turks solution (Merck), AH 21-132, (*cis*-6-(*p*-acetoamidophenyl)-1,2,3,4,10b-hexahydro-8,9-demethoxy-2-methyl (benzo-[c][1,6]-naphthyridine, Sandoz), ketotifen, mepyramine (Sandoz), aminophylline (Siegfried), dexamethasone (Sigma), ethanol, polyethylene glycol, tartaric acid and ascorbic acid (Merck) and Tris buffer (Fluka).

Statistical comparison

Data are presented as means ± s.e.mean and Student's t test for groups of unequal size was used for comparison between treatments.

Results

Comparison of naïve and sensitized guinea-pigs

Only sensitized guinea-pigs in which there was a substantial increased skin thickness (>100%) following intradermal injection of OA (10 μg per site) were used in this study. Serum from these animals could sensitize the skin of naïve guinea-pigs for up to 6 days. Intradermal injection of OA (10 μg per site) into non-sensitized sites induced an increased plasma protein extravasation (IPPE) of 14 ± 1 μl whole blood equivalent, whereas injection at sites sensitized 3 or 6 days previously, induced IPPE of 148 ± 7 and 66 ± 12 μl whole blood equivalent, respectively ($n = 8$). These observations indicate that responses mediated by IgE-type antibodies contribute substantially to reactions in actively sensitized animals.

Basal airway function (R_L and C_{dyn}), airway reactivity to histamine (1.0–1.8 $\mu g\,kg^{-1}$) or $PGF_{2\alpha}$ (56 $\mu g\,kg^{-1}$) and cellular content of bronchoalveolar lavage (BAL) fluid were determined in naïve guinea-pigs and guinea-pigs sensitized with either 1 μg or 10 μg of OA. Sensitized animals did not differ from naïve animals in any of the lung function parameters measured (Table 1). However, sensitized guinea-pigs exhibited a small, but significant, increase in the total eosinophil numbers recovered in BAL (Table 1), and in the incidence of eosinophils, when compared as a percentage of total cells (naïve, $5.0 \pm 0.7\%$; 1 μg OA, $10.6 \pm 2.0\%$; 10 μg OA, $16.5 \pm 3.0\%$).

Exposure of naïve guinea-pigs to ovalbumin

Naïve guinea-pigs exposed to an aerosol of OA (0.1%) exhibited minimal changes in basal lung function or airway reactivity to histamine (1.8 $\mu g\,kg^{-1}$) or $PGF_{2\alpha}$ (56 $\mu g\,kg^{-1}$) (Table 2). There was a signficant increase in total cell numbers recovered in BAL at 24 ($46 \pm 7 \times 10^6$, $P < 0.001$, $n = 5$) and 48 ($31 \pm 3 \times 10^6$, $P < 0.024$, $n = 5$) hours after exposure to OA when compared with naïve guinea-pigs not exposed to OA ($21 \pm 3 \times 10^6$, $n = 5$) (Table 2).

Exposure of sensitized guinea-pigs to ovalbumin

Animals sensitized with 1 μg ovalbumin Exposure of this group of guinea-pigs to an aerosol of OA (0.1%) for 1 h did not induce overt respiratory distress. Basal lung function (R_L and C_{dyn}) and airway reactivity to histamine (1.8 $\mu g\,kg^{-1}$) or $PGF_{2\alpha}$ (56 $\mu g\,kg^{-1}$) remained unchanged at all time points (24, 48, 72 h and 1 week after challenge), when compared with sensitized guinea-pigs not exposed to OA (0.1%) (Table 3). However, there was an increase in all cell types in BAL fluid,

Table 1 Comparison of airway responses and bronchial cell populations in naïve and sensitized guinea-pigs

Sensitization	*Airway responses*					*Cell populations* ($\times 10^6$)			
(μg ovalbumin)	R_L	C_{Dyn}	*Histamine* (*1.0*)	*Histamine* (*1.8*)	$PGF_{2\alpha}$	*Macro*	*Eosin*	*Neut*	*Lymph*
None	130 ± 4	1.0 ± 0.1	27 ± 4	140 ± 20	120 ± 26	17 ± 2	1 ± 0.2	0.5 ± 0.1	2 ± 0.5
1	133 ± 4	1.3 ± 0.1	27 ± 8	131 ± 40	114 ± 36	20 ± 2	3 ± 1*	0.5 ± 0.1	4 ± 1
10	145 ± 4	1.0 ± 0.1	32 ± 4	166 ± 33	138 ± 33	16 ± 1	4 ± 1	0.4 ± 0.1	2 ± 0.4

R_L = airway resistance ($cmH_2O\,l^{-1}\,s^{-1}$), C_{dyn} = dynamic compliance ($ml\,cmH_2O^{-1}$). Histamine and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) indicate increases in R_L above basal values when histamine (1 or 1.8 $\mu g\,kg^{-1}$) or $PGF_{2\alpha}$ (56 $\mu g\,kg^{-1}$) was injected intravenously. Macro = macrophages; Eosin = eosinophils; Neut = neutrophils; Lymph = lymphocytes. Results are expressed as mean ± s.e.mean (obtained from at least 10 guinea-pigs). Student's *t* test was used to assess statistical significance of differences and * indicates a statistical significance, $P < 0.025$.

Table 2 Airway responses and bronchial cell populations after exposure of naïve guinea-pigs to an aerosol of ovalbumin (0.1%)

Time	*Airway responses*					*Cell populations* ($\times 10^6$)			
	R_L	C_{dyn}	*Histamine* (*1.0*)	*Histamine* (*1.8*)	$PGF_{2\alpha}$	*Macro*	*Eosin*	*Neut*	*Lymph*
0	130 ± 4	1.0 ± 0.1	27 ± 4	140 ± 20	120 ± 26	17 ± 2	1 ± 0.2	0.5 ± 0.1	2 ± 0.5
24	119 ± 6	1.7 ± 0.3	39 ± 6	86 ± 12	132 ± 58	28 ± 6	3 ± 2	2 ± 1	5 ± 1*
48	124 ± 8	1.6 ± 0.3	19 ± 3	112 ± 26	206 ± 112	24 ± 2	3 ± 1	1 ± 0.2*	4 ± 1*
72	ND	ND	ND	ND	ND	16 ± 2	1 ± 0.3	1 ± 0.3*	3 ± 1
1 week	ND	ND	ND	ND	ND	15 ± 2	2 ± 0.2	0.5 ± 0.2	1 ± 0.2

R_L = airway resistance ($cmH_2O\,l^{-1}\,s^{-1}$), C_{dyn} = dynamic compliance ($ml\,cmH_2O^{-1}$). Histamine and $PGF_{2\alpha}$ indicate increases in R_L above basal values when histamine (1.0 or 1.8 $\mu g\,kg^{-1}$) or $PGF_{2\alpha}$ (56 $\mu g\,kg^{-1}$) was injected intravenously. Macro = macrophages; Eosin = eosinophils; Neut = neutrophils; Lymph = lymphocytes; ND = not done. Each time point comprises 5 guinea-pigs and results are expressed as mean ± s.e.mean. Student's *t* test was used to assess statistical significance of differences and * indicates a statistical significance, $P < 0.025$.

Table 3 Airway responses and bronchial cell populations after exposure of sensitized guinea-pigs (1 μg) to an aerosol of ovalbumin (0.1%)

Time	*Airway responses*					*Cell populations* ($\times 10^6$)			
	R_L	C_{dyn}	*Histamine* (*1.0*)	*Histamine* (*1.8*)	$PGF_{2\alpha}$	*Macro*	*Eosin*	*Neut*	*Lymph*
0	133 ± 4	1.3 ± 0.1	27 ± 8	131 ± 40	114 ± 36	20 ± 2	3 ± 1	0.5 ± 0.1	4 ± 1
24	123 ± 4	1.3 ± 0.1	28 ± 7	146 ± 37	89 ± 37	31 ± 4*	20 ± 3*	6 ± 1*	4 ± 1
48	136 ± 7	1.0 ± 0.1	48 ± 13	187 ± 44	524 ± 196	24 ± 3	20 ± 3*	6 ± 1*	5 ± 1
72	126 ± 12	1.2 ± 0.2	28 ± 6	167 ± 39	107 ± 21	23 ± 2	17 ± 3*	3 ± 1*	4 ± 0.5
1 week	116 ± 4	0.9 ± 0.2	42 ± 21	206 ± 184	ND	16 ± 3	11 ± 3*	0.2 ± 0.1	2 ± 1

R_L = airway resistance ($cmH_2O\,l^{-1}\,s^{-1}$), C_{dyn} = dynamic compliance ($ml\,cmH_2O^{-1}$). Histamine and $PGF_{2\alpha}$ indicate increases in R_L above basal values when histamine (1.0 or 1.8 $\mu g\,kg^{-1}$) or $PGF_{2\alpha}$ (56 $\mu g\,kg^{-1}$) was injected intravenously. Macro = macrophages; Eosin = eosinophils; Neut = neutrophils; Lymph = lymphocytes; ND = not done. Each time point comprises 10 guinea-pigs except the $PGF_{2\alpha}$ column, where 5 animals received the spasmogen. Results are expressed as mean ± s.e.mean. Student's *t* test was used to assess statistical significance of differences and * indicates a statistical significance, $P < 0.025$.

Table 4 Airway responses and bronchial cell populations after exposure of sensitized guinea-pigs (10 μg) to an aerosol of ovalbumin (0.1%)

	Airway responses					*Cell populations* ($\times 10^6$)			
			Histamine						
Time	R_L	C_{dyn}	(1.0)	(1.8)	$PGF_{2\alpha}$	*Macro*	*Eosin*	*Neut*	*Lymph*
0	145 ± 4	1.0 ± 0.1	32 ± 4	166 ± 33	138 ± 33	16 ± 1	3 ± 1	0.4 ± 0.1	2 ± 0.4
4	183 ± 12*	0.8 ± 0.2	77 ± 15*	267 ± 49	392 ± 102*	19 ± 3*	5 ± 1	16 ± 3*	3 ± 0.4
8	170 ± 5*	0.8 ± 0.1	68 ± 16	463 ± 70*	339 ± 85*	35 ± 3*	16 ± 3*	17 ± 3*	5 ± 1*
12	165 ± 8*	0.7 ± 0.1	92 ± 25*	405 ± 72*	412 ± 109*	35 ± 3*	29 ± 4*	23 ± 5*	4 ± 1*
24	140 ± 5	1.1 ± 0.1	76 ± 14*	451 ± 116*	322 ± 111	34 ± 3*	33 ± 4*	14 ± 3*	7 ± 1*
48	145 ± 8	1.2 ± 0.2	60 ± 12	337 ± 58*	233 ± 61	33 ± 5*	30 ± 6*	10 ± 3*	5 ± 1*
72	134 ± 3	1.3 ± 0.1	52 ± 9	248 ± 45	221 ± 68	28 ± 3*	21 ± 2*	4 ± 1*	3 ± 1
1 week	136 ± 7	1.6 ± 0.2	34 ± 7	202 ± 24	260 ± 100	22 ± 2	14 ± 1*	0.4 ± 0.1	2 ± 0.3

R_L = airway resistance ($cmH_2O\,l^{-1}\,s^{-1}$), C_{dyn} = dynamic compliance ($ml\,cmH_2O^{-1}$). Histamine and $PGF_{2\alpha}$ indicate increases in R_L above basal values when histamine (1.0 or 1.8 $\mu g\,kg^{-1}$) or $PGF_{2\alpha}$ (56 $\mu g\,kg^{-1}$) were injected intravenously. Macro = macrophages; Eosin = eosinophils; Neut = neutrophils; Lymph = lymphocytes. Each time point comprises 10 guinea-pigs and results are expressed as mean ± s.e.mean. Student's *t* test was used to assess statistical significance of differences and * indicates a statistical significance, $P < 0.025$.

except lymphocytes (Table 3). The largest increase was observed in the eosinophil population, which comprised 33 ± 5% of total cells 24 h after exposure to OA, as compared with 11 ± 2% in sensitized animals not exposed to OA. The proportion of eosinophils in BAL fluid remained significantly elevated for up to one week after exposure. Epithelial cells were also observed in BAL fluid at all time points following antigen exposure; the incidence of detached epithelial cells was increased but these changes could not be quantified as these cells were usually found clumped together.

Animals sensitized with 10 μg ovalbumin Exposure of these animals to an aerosol of OA (0.1%) did not provoke obvious respiratory distress. However, in anaesthetized guinea-pigs, basal R_L was significantly higher between 4 and 12 h after exposure to OA, although C_{dyn} was unaffected (Table 4). Intravenous injection of histamine (1.8 $\mu g\,kg^{-1}$) or $PGF_{2\alpha}$ (56 $\mu g\,kg^{-1}$) revealed increased reactivity to both spasmogens (Table 4). Airway reactivity to histamine was significantly greater at 8, 12, 24 and 48 h. Airway reactivity to $PGF_{2\alpha}$ was significantly greater at 4, 8 and 12 h; reactivity to $PGF_{2\alpha}$ was increased at all time points after exposure to OA, but the difference did not achieve significance at 24, 48, 72 h and 1 week because of considerable inter-animal variation. Guinea-pigs exposed to OA exhibited a prompt accumulation of cells into the pulmonary airway lumen, such that 4 h after exposure to antigen, total cell numbers in BAL fluid had increased to 42.3 ± 3.1 × 10^6 and reached a maximum of 93 ± 8.3 × 10^6 by 12 h; for comparison, 22.7 ± 1.9 × 10^6 cells were recovered from sensitized guinea-pigs not exposed to OA. Neutrophilia was most pronounced between 4 and 12 h, with a steady decline thereafter (Table 4). Eosinophil accumulation into the airway lumen had a slower onset, but was more protracted than neutrophil accumulation, with a significant increase persisting for at least one week (Table 4). Expression of eosinophil counts as percentage of the total cell population, revealed the predominance of this cell type, since eosinophils comprised approximately 38% of the cells recovered in BAL fluid one week after exposure to OA. A temporal relationship between airway hyperreactivity to histamine and pulmonary airway eosinophilia was apparent (Figure 1). However, when airway reactivity to histamine was compared with airway eosinophilia for each animal between 4 h and 7 days following exposure to OA, no correlation was evident (Figure 2). There was also no correlation between airway reactivity and accumulation of any other cell type in BAL fluid.

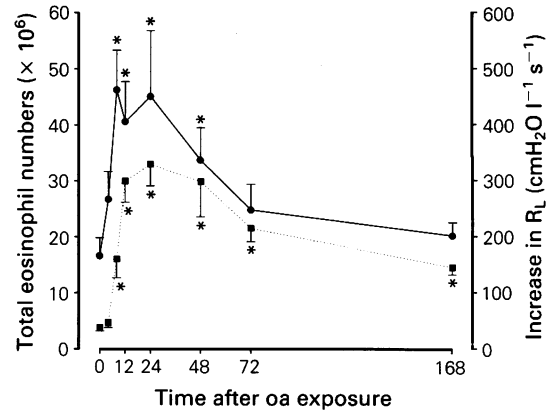


Figure 1 Exposure of sensitized guinea-pigs (10 μg ovalbumin) to an aerosol of ovalbumin (0.1%) induces pulmonary airway eosinophil accumulation (■) and increased airway reactivity to histamine (1.8 $\mu g\,kg^{-1}$) (●). Bars indicate s.e.mean and *denotes $P < 0.025$ (Student's *t* test). R_L = airway resistance.

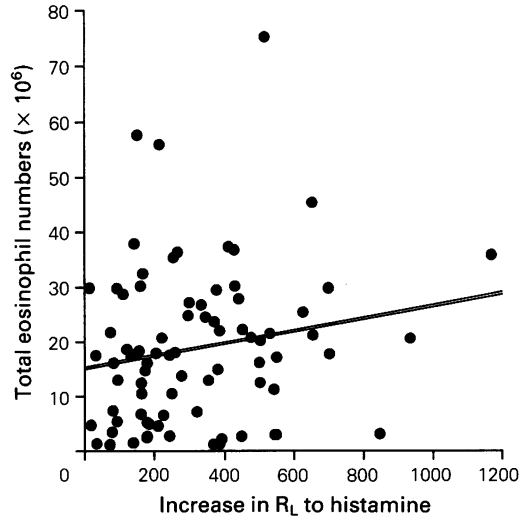


Figure 2 Absence of correlation ($r = 0.18$) between antigen-induced pulmonary airway eosinophil accumulation and airway hyperreactivity (measured as increase in airway resistance R_L) to histamine (1.8 $\mu g\,kg^{-1}$) in individual sensitized guinea-pigs (10 μg ovalbumin) at various time points (4 h to 7 days) following inhalation of antigen (ovalbumin 0.1%).

Drug effects on airway hyperreactivity

None of the drugs tested in this study diminished airway hyperreactivity as indicated by the reaction to $PGF_{2\alpha}$. Ketotifen and mepyramine fully inhibited responses to histamine,

Table 5 Effect of anti-asthma drugs upon antigen-induced airway hyperreactivity and eosinophilia in sensitized guinea-pigs (10 μg ovalbumin) 24 h after challenge

Treatment	n	Airway reactivity to Histamine (1.0)	Histamine (1.8)	$PGF_{2\alpha}$	Cells ($\times 10^6$) Neutrophil	Eosinophil
No challenge	10	32 ± 4	166 ± 33	138 ± 33	0.4 ± 0.1	3.8 ± 0.6
Solvent	20	128 ± 30	430 ± 57	502 ± 71	17.8 ± 3.0	32.4 ± 3.2
AH 21-132	10	87 ± 12	453 ± 50	446 ± 123	9.7 ± 1.7*	18.0 ± 1.7*
Aminophylline	10	117 ± 20	471 ± 65	470 ± 108	10.4 ± 2.4	16.6 ± 2.2*
Dexamethasone	10	113 ± 32	496 ± 122	433 ± 136	7.5 ± 1.7*	14.0 ± 3.0*
Ketotifen	10	0	0	328 ± 71	8.3 ± 1.6*	14.5 ± 2.5*
Mepyramine	5	0	0	397 ± 132	6.6 ± 1.7*	24.0 ± 2.0
Salbutamol	4	109 ± 30	457 ± 130	446 ± 180	33.8 ± 8.8	36.8 ± 11.9

Drugs were given at 1 mg kg^{-1} day^{-1} except aminophylline (10 mg kg^{-1} day^{-1}) and salbutamol (0.1 mg kg^{-1} day^{-1}). Histamine (1.0 or 1.8 μg kg^{-1}) and $PGF_{2\alpha}$ (56 μg kg^{-1}) were injected intravenously and an increased airway resistance (cmH_2O l^{-1} s^{-1}) above basal value was measured. Results are expressed as mean ± s.e.mean. Student's t test was used to assess statistical significance of differences and *indicates a statistical significance, $P < 0.025$.

which accords with their potency as histamine (H_1) antagonists (Table 5).

Drug effects on cellular content of BAL fluid

AH 21-132, aminophylline, dexamethasone and ketotifen significantly ($P < 0.025$) diminished eosinophilia in BAL 24 h after exposure to OA by 44%, 48%, 56% and 55% respectively (Table 5). Although mepyramine effected a small inhibition (25%), this did not achieve statistical significance. All guinea-pigs treated with salbutamol (1 mg kg^{-1} day^{-1}) died shortly after exposure to antigen. When a lower dose of salbutamol (0.1 mg kg^{-1} day^{-1}) was used, only one out of five animals died after exposure to OA. No inhibition of eosinophils or other cell types was observed in the BAL fluid of guinea-pigs that had been treated with salbutamol (Table 5). Interestingly, with addition of mepyramine, all drugs which inhibited eosinophil accumulation, also inhibited neutrophil accumulation (Table 5). Total cell numbers in BAL 24 h after exposure to OA ($106.7 \pm 9 \times 10^6$) were not significantly reduced by any of the drugs tested except for dexamethasone ($66.7 \pm 7 \times 10^6$, $P < 0.005$); conversely, animals treated with salbutamol showed a significant increase of total cell numbers ($154.8 \pm 8.14 \times 10^6$, $P < 0.025$) recovered in BAL fluid.

Effect of depletion of platelets or neutrophils

Intravenous injection of anti-platelet (APS) or anti-neutrophil (ANS) antiserum caused selective depletion of circulating platelets and neutrophils respectively without affecting resident cells in BAL fluid. Maximal depletion of each cell type was observed at 24 h and, whereas platelets returned to the circulation after a further 24 h, neutrophil depletion persisted for 48 h. Experiments with naïve animals revealed that platelet numbers decreased from 508,000 ± 27,859 to 61,500 ± 12,286 μl^{-1} 24 h after injection of APS ($n = 10$) and neutrophils decreased from 2,858 ± 273 to 166 ± 83 μl^{-1} 24 h after injection of ANS ($n = 10$). Guinea-pigs treated with APS or ANS were exposed to an aerosol of OA (0.1%) at 24 h, the time point of maximal depletion. Basal cell numbers in BAL fluid were not affected by these treatments and neither pulmonary airway eosinophilia nor airway hyperreactivity were diminished in thrombocytopaenic or neutropaenic guinea-pigs compared with untreated animals (Figure 3).

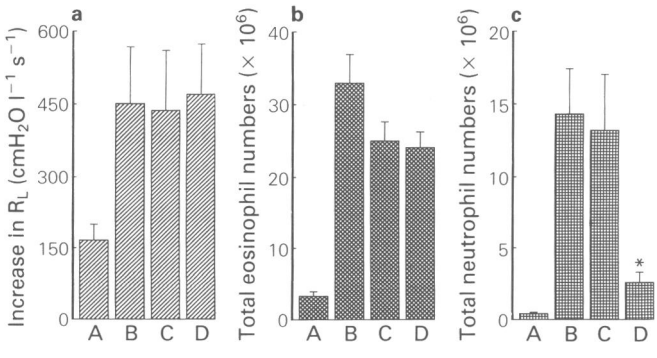


Figure 3 A comparison of airway response to histamine (1.8 μg kg^{-1} i.v.) (a), eosinophil numbers in bronchoalveolar lavage (BAL) fluid (b) and neutrophil numbers in BAL fluid (c) in (A) sensitized guinea-pigs (10 μg ovalbumin) not challenged; (B) sensitized guinea-pigs 24 h after challenge; (c) thrombocytopaenic sensitized guinea-pigs 24 h after challenge and (D) neutropaenic sensitized guinea-pigs 24 h after challenge. Bars indicate s.e.mean and * denotes $P < 0.025$ (Student's t test).

Discussion

Bronchial asthma is commonly characterised by increased airway reactivity to spasmogens (Cockcroft, 1983) and eosinophilia of sputum (Epstein, 1972), peripheral blood (Horn *et al.*, 1975) or bronchial tissue (Huber & Koessler, 1922; Dunnill, 1960; Filley *et al.*, 1982). Clinical studies strongly suggest a close link between airway eosinophilia and increased airway reactivity. During reactions to inhaled allergen, an eosinophil chemotactic factor has been detected in serum (Metzger *et al.*, 1986; Hakansson *et al.*, 1989), which accords with bronchial eosinophil accumulation found in patients exhibiting a late asthmatic response (De Monchy *et al.*, 1985; Metzger *et al.*, 1987) who are known to develop airway hyperreactivity (Durham & Kay, 1985; Diaz *et al.*, 1989). It has been proposed that eosinophils cause epithelial damage by releasing major basic protein (Filley *et al.*, 1982; Frigas & Gleich, 1986) which may lead to increased airway reactivity, either by exposing sensory nerve endings (Barnes, 1986) or by removing the protective effects of an epithelial derived relaxant factor (Flavahan *et al.*, 1988).

Other clinical studies indicate that eosinophilia and increased airway reactivity may be unrelated. Thus, increased airway reactivity is observed shortly after resolution of early airway obstruction following exposure to antigen (Thorpe *et al.*, 1987), when eosinophils are not evident in lavage fluid (De Monchy *et al.*, 1985). Increased airway reactivity occurs in normal subjects after viral infection of the upper respiratory tract (Empey *et al.*, 1976); a condition characterised by bronchial epithelial damage and shedding (Hers & Mulder, 1961; Walsh *et al.*, 1961), which is caused by a direct action of the virus (Negroni & Tyrrel, 1959), without an apparent involvement of eosinophils. Conversely, patients with chronic cough and elevated sputum eosinophils have been shown to exhibit no increased airway reactivity (Gibson *et al.*, 1989).

We have used sensitized guinea-pigs to study the relationship between increased airway reactivity and cellular inflammation of the airways, particularly eosinophil accumulation,

following antigen challenge. The guinea-pig is well suited for such studies since airway hyperreactivity and eosinophilia can readily be demonstrated in this species (Kallos & Kallos, 1984; Dunn *et al.*, 1988; Hutson *et al.*, 1988a; Aoki *et al.*, 1988; Daffonchio *et al.*, 1989). Exposure of sensitized guinea-pigs to aerosols containing a small dose of allergen induced an allergic reaction without anaphylactic bronchospasm. These guinea-pigs invariably developed a pronounced pulmonary airway eosinophilia which persisted for at least one week. Preliminary histological studies indicated that there was a good correlation between bronchial tissue eosinophilia and eosinophils collected in BAL fluid, in agreement with earlier studies (Snella *et al.*, 1987; Dunn *et al.*, 1988). Accordingly, we have chosen to assess eosinophil accumulation in BAL fluid rather than by histology, due to the difficulty in evaluating histological specimens of lung tissue from large numbers of guinea-pigs.

In animals sensitized with 10 μg of OA, airway eosinophilia and increased airway reactivity to histamine or $PGF_{2\alpha}$ followed a similar time course, yet there was no correlation between these two events, nor was accumulation of other cell types correlated with airway hyperreactivity. Dissociation between pulmonary airway eosinophil accumulation and increased airway reactivity was most clearly evident in guinea-pigs sensitized with 1 μg of ovalbumin, since these animals exhibited no airway hyperreactivity to spasmogens, despite a 7 fold increase in eosinophil numbers in BAL fluid. Such observations are in agreement with a recent study where endotoxin-induced airway inflammation, consisting of eosinophils and neutrophils, in the guinea-pig was not accompanied by airway hyperreactivity (Folkerts *et al.*, 1988). Furthermore, depletion of leukocytes in guinea-pigs by steroids did not suppress bronchial hyperreactivity induced by ozone (Murlas & Roum, 1985). It is interesting to note that stimuli such as ozone (Fabbri *et al.*, 1984; Holroyde & Norris, 1988) or viral infections, which cause increased airway reactivity in experimental animals or man, are more commonly associated with epithelial damage rather than eosinophil accumulation. Eosinophils may represent only one way of causing such damage, particularly in severe asthma. This proposition is supported by recent biopsy studies from mild or asymptomatic asthma patients (Laitinen *et al.*, 1985; Beasly *et al.*, 1989) in whom epithelial damage is a common finding with little or no eosinophil involvement. Our results indicate that eosinophil accumulation *per se* does not lead to increased airway reactivity, since the group of animals sensitized with 1 μg of OA responded to antigen exposure with a severe bronchial eosinophilia without increased airway reactivity. Eosinophils have been classified as normodense or hypodense on the basis of sedimentation on discontinuous metrizamide or Percoll gradients (Winqvist *et al.*, 1982). Hypodense eosinophils exhibit greater oxygen consumption (Winqvist *et al.*, 1982), are more reactive to a variety of stimuli, thus generating greater quantities of leukotriene C_4 (LTC_4), and kill parasite larvae more actively (Rothenberg *et al.*, 1988). Although the state of activation of cells obtained from BAL was not determined, it is possible that the group of animals which became hyperreactive (sensitized with 10 μg of OA) had more activated or hypodense eosinophils. However, on the basis of studies in patients (Winqvist *et al.*, 1982; Prin *et al.*, 1984), it is not unreasonable to assume that all eosinophils found in BAL were activated to a degree, since they had to cross several body compartments in order to arrive in the airway lumen.

Distinction between pulmonary airway eosinophilia and airway hyperreactivity could be confirmed by use of anti-asthma drugs. Aminophylline, dexamethasone, ketotifen and AH 21-132 significantly inhibited eosinophil and neutrophil accumulation in BAL without affecting airway hyperreactivity. Our data on inhibition of eosinophil accumulation are in agreement with those in the literature, since prophylactic anti-asthma drugs can inhibit eosinophilia, induced by different stimuli, in guinea-pigs (Murlas & Roum, 1985; Sanjar *et al.*, 1989, Sanjar *et al.*, 1990), rats (Spicer *et al.*, 1985) or man (Ohman *et al.*, 1972; Horn *et al.*, 1975; Gobel, 1979; Diaz *et al.*, 1984). It was surprising that none of the drugs used in this study inhibited airway hyperreactivity. Steroids are known to inhibit increased airway reactivity after antigen challenge (Cockcroft & Murdock, 1987) or exposure to toluene diisocyanate (Fabbri *et al.*, 1985). However, efficacy of compounds such as aminophylline or ketotifen at inhibiting acute increases in airway hyperreactivity is unclear and histamine (H_1) receptor antagonists and β-adrenoceptor agonists (Cockcroft & Murdock, 1987; Lai *et al.*, 1989) are ineffective. Of particular interest was the effect of salbutamol since animals treated with the high dose (1 mg kg^{-1} day^{-1}) did not survive antigen exposure. Increased mortality has previously been demonstrated in guinea-pigs treated with β-adrenoceptor agonists before exposure to an aerosol of histamine (Conolly *et al.*, 1971) or intravenous antigen (Izard *et al.*, 1971), which may reflect the capacity of these compounds to induce airway hyperreactivity (Sanjar & Morley, 1988). Guinea-pigs treated with a lower dose of salbutamol showed no inhibition of eosinophil accumulation, in agreement with an earlier study in which salbutamol (albuterol) was used (Hutson *et al.*, 1988b). However, fenoterol has been shown to inhibit eosinophil accumulation in airways of passively sensitized guinea-pigs (Fugner, 1989). The differences in the activity of these β-adrenoceptor agonists are not clear and may relate to relative potency or properties other than β-adrenoceptor stimulation. Animals treated with the lower dose of salbutamol showed a significantly greater increase of total cell numbers in BAL fluid 24 h after antigen exposure. This observation could relate to the bronchodilator efficacy of salbutamol which would prevent local airway constriction, thereby allowing greater entry of antigen into the airways. This proposition finds support in a recent clinical study, where the use of a short acting bronchodilator, rimiterol hydrobromide, allowed greater exposure of asthma patients to antigen (Lai *et al.*, 1989), thereby inducing late-onset airway obstruction in subjects who previously gave only an early airway obstruction. On the basis of these observations, the sensitized guinea-pig model differs from allergic asthma principally by the lack of inhibition of airway hyperreactivity by steroids.

Removal of circulating platelets (Pinckard *et al.*, 1977; Butler & Smith, 1981; Lellouch-Tubiana *et al.*, 1988) or neutrophils (Wedmore & Williams, 1981; O'Byrne *et al.*, 1984) has been shown to diminish inflammatory reactions. In the present study, neither platelets nor neutrophils contribute to either antigen-induced airway eosinophilia or hyperreactivity. These observations are in agreement with studies of platelet-activating factor (PAF)-induced pulmonary airway eosinophilia, when neither platelet nor neutrophil depletion influenced the eosinophilic response (Sanjar *et al.*, 1990). Furthermore, antigen-induced late-onset airway obstruction and pulmonary eosinophil accumulation was also not influenced by neutrophil depletion (Hutson *et al.*, 1989).

The present observations indicate that the association between pulmonary airway eosinophilia and acute airway hyperreactivity reflects a common origin (the allergic reaction) rather than a causal relationship, thus airway hyperreactivity and eosinophil accumulation occur simultaneously but independently of each other.

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RS 30026: a potent and effective calcium channel agonist

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1 A series of dihydropyridine derivatives has been evaluated for calcium channel agonist activity using reversal of nisoldipine-induced inhibition of beating of aggregates of embryonic chick myocytes. This test appears to be specific for calcium channel agonists since isoprenaline and cardiac glycosides are inactive.

2 RS 30026 was the most potent of the series, was significantly more potent than CGP 28392 and of similar potency to Bay K 8644 (pEC_{50} = 7.45, 6.16 and 7.20, respectively). RS 30026 increased edge movement of individual aggregates, in the absence of nisoldipine, by 50% at 2 nM.

3 Compounds were also evaluated for their effects on guinea-pig papillary muscle and porcine coronary artery rings. RS 30026 displayed positive inotropism at concentrations between 10^{-9} and 10^{-6} M (pEC_{200} = 8.21), but was a much more powerful inotrope than Bay K 8644, increasing contractility to 1300% of control at 10^{-6} M (compared to 350% of control for Bay K 8644). RS 30026 caused vasoconstriction at concentrations between 10^{-10} and 10^{-7} M.

4 Calcium channel currents in single embryonic chick myocytes were recorded by whole-cell voltage clamp techniques. RS 30026 (100 nM–500 nM) produced large increases in peak current amplitude and shifted the voltage for threshold and maximal currents to more negative values. RS 30026 (500 nM) also produced large increases in the inward tail currents evoked upon repolarization. The effects of Bay K 8644 (50 and 500 nM) were much less marked.

5 Analysis of the activation characteristics of currents showed parallel shifts in the activation curve to more negative potentials in the presence of 50 nM Bay K 8644, with a much smaller shift in the presence of 500 nM Bay K 8644. RS 30026 (100 and 500 nM) caused concentration-dependent shifts in the activation of the calcium channel currents with an increase of the slope of the curve.

6 RS 30026 appears to be the most potent and effective calcium channel agonist described to date.

Introduction

Schramm *et al.* (1983a) described the dihydropyridine Bay K 8644 which acted in the opposite manner to calcium channel antagonists, such as nifedipine and verapamil, in that it was positively inotropic and caused vasoconstriction. This compound and the dihydropyridine-lactone CGP 28392 have been shown to act directly at the calcium channel to increase the inward calcium current by increasing the probability of channel opening and the mean open time (Brown *et al.*, 1984; Hess *et al.*; 1984; Kokubun & Reuter, 1984; Nowycky *et al.*, 1985; Bechem & Schramm, 1987). In all studies CGP 28392 appears to be less potent and effective than Bay K 8644 (Erne *et al.*, 1984; Renaud *et al.*, 1984; Rogg *et al.*, 1985; Patmore & Duncan, 1988). In heart and neurones Bay K 8644 has been shown to shift the activation curve for the transient inward current to the left and increase the magnitude of the peak current (Sanguinetti *et al.*, 1986; Docherty & Brown, 1986; Hamilton *et al.*, 1987). However, large increases in calcium influx as a consequence of the increased open time of the channel result in larger tail currents being observed at the end of voltage clamp pulses. Under physiological rather than voltage clamp conditions the contributions from different types of calcium current will depend on the spike or action potential characteristics (Anderson & Patmore, 1988) and on the type of tissue and its resting membrane potential. Thus in neurones, which have short action potentials, the calcium current will be less affected than in smooth muscle, where many cells display slow spike activity. The more positive resting potential in smooth muscle is also closer to the threshold activation potential for the calcium current, which is shifted to more negative potentials in the presence of Bay K 8644.

The actions of dihydropyridine calcium channel agonists mirror the activity of the antagonists in that they display an apparent selectivity for vascular smooth muscle over cardiac muscle (Clarke & Patmore, 1986; Kwon *et al.*, 1989) and selectively enhance resistance artery myogenic tone (Hwa & Bevan, 1986). As potential inotropes these compounds are likely to cause vasoconstriction at lower concentrations than they would be positively inotropic.

It has been shown that low concentrations of dihydropyridine calcium channel antagonists can display agonist effects (Brown *et al.*, 1986) and at high concentrations racemic Bay K 8644 behaves as an antagonist (Thomas *et al.*, 1984). This appears to be due, at least in part, to the fact that one of the stereo-isomers displays agonist activity. Thus the (+)-isomer of Bay K 8644 has been shown to behave as an antagonist whilst the (−)-isomer displayed agonist activity (Franckowiak *et al.*, 1985; Hamilton *et al.*, 1987; MacDonald *et al.*, 1987). Interestingly, the actions of the isomers are not so clear, since MacDonald *et al.* (1987) demonstrated that at high concentrations the (−) (agonist)-isomer of Bay K 8644 displayed antagonist activity. Similarly the (+)- and (−)-isomers of PN 202-791 have been described as agonist and antagonist respectively (Hof *et al.*, 1985; Kongsamut *et al.*, 1985; Williams *et al.*, 1985). In the present study we have evaluated the agonist activity of a series of dihydropyridine-lactone compounds related to CGP 28392. We have tested for agonist or facilitator activity by assessing the ability of compounds to reverse nisoldipine-induced inhibition of beating of aggregates of embryonic chick myocytes, as described by Patmore & Duncan (1988). The most potent compound of the series has been evaluated for its vasoconstrictor and positive inotropic

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activities and for its actions on the calcium channel currents in myocytes. A preliminary account of the effects of RS 30026 on cardiac calcium channel currents has been presented to the Physiological Society (Anderson & Patmore, 1990).

Methods

Cell cultures

Primary cultures of myocytes were prepared as described previously by Patmore & Duncan (1988). Briefly, myocytes were dispersed from the hearts of eleven day old Leghorn K chicken embryos by agitation in Hanks calcium- and magnesium-free balanced salt solution (Gibco) with 0.05% trypsin. Cells were resuspended in culture media containing 20% nutrient media M-199 (Gibco), 6% heat-inactivated foetal calf serum, 1% penicillin/streptomycin solution and 73% low potassium salt solution, the composition of which was (mM): NaCl 116, $NaHCO_3$ 26.2, $MgSO_4$ 0.8, NaH_2PO_4 0.9, $CaCl_2$ 1.8 and dextrose 5.5, adjusted to pH 7.3. For measurements of antagonist-facilitator interactions and edge movement the cells were seeded into 96 well trays and the bottom of each well was coated with a layer of silicon elastomer resin to promote the formation of aggregates of 100–200 μm diameter. For electrophysiological experiments the cells were plated onto 35 mm plastic petri dishes at low density to allow recording from isolated myocytes. Cultures were maintained for 2–4 days before use.

Assessment of beating

Competition experiments between nisoldipine and potential calcium channel facilitators were performed as described by Patmore & Duncan (1988). Aggregates of myocytes displayed automaticity and the edge movement associated with contractions could be detected in each cluster on observation under low power (40×) microscopy. Contractility was assessed by scoring the % of aggregates in each well which were beating. Drugs were applied at 11 times the final concentration in a volume of 20 μl of salt solution, to wells containing 200 μl of media. The salt solution was designed to resemble the ionic composition of the culture media and contained (mM): NaCl 110, KCl 1.07, $CaCl_2$ 1.7, $MgSO_4$ 0.73, HEPES 19.3, $NaHCO_3$ 0.83 and dextrose 5.5. Following drug application, cultures were incubated for 30 min at 37°C and then identified wells scored again for % of aggregates beating. Different treatments (drugs or concentrations) were applied to each of 40 wells. For each concentration of each drug replicate tests were made on 3 further trays. On each tray a series of wells were treated as 'sham' controls where salt solution containing no drug was added. This caused no inhibition of beating.

Competition between calcium channel facilitators and antagonists was investigated by treating aggregates with varying concentrations of facilitator and 0.3 μM nisoldipine, a concentration of antagonist which caused approximately 85% inhibition of beating. Calcium channel facilitators caused a concentration-dependent reversal of nisoldipine-induced inhibition of beating. Data were fitted to a sigmoidal function between 15 and 100% of control and negative logarithms of concentrations causing 50% reversal of antagonist-induced inhibition of aggregate beating (pEC_{50} values) calculated to express the potency of the calcium channel facilitator. Where appropriate pEC_{50} values were compared by a Student's t test.

Detection of edge movement

Contractility was estimated by measuring edge movement of aggregates of embryonic chick heart cells, as described by Patmore & Duncan (1988). Aggregates, initially grown on silicon elastomer, were transferred to normal tissue culture treated plates where they developed contacts with the dish such that they could be superfused at 2.5 ml min^{-1} (chamber volume = 200 μl) with a salt solution prewarmed to 37°C. The salt solution contained (mM): NaCl 110, KCl 1, $MgSO_4$ 0.8, HEPES 19.3, $NaHCO_3$ 0.83 and dextrose 5.5, pH adjusted to 7.4 with NaOH. An area of the aggregate which contained an edge which displayed significant movement was selected for analysis, by use of a video motion detection system described previously (Patmore & Whiting, 1985; Patmore et al., 1989a). Dynamic recordings (sampled every 20 ms) of edge movement of the aggregate were recorded.

Electrophysiological recordings

Whole-cell voltage clamp recordings were made on single myocytes. Pipettes were made from Garner glass (Corning No. 7052) and filled with a solution containing (mM): CsCl 100, $MgCl_2$ 1, EGTA 11, $CaCl_2$ 1, HEPES 10 and glucose 5, pH 7.2. Experiments were carried out at room temperature. Recordings were made according to the methods of Hamill et al. (1981) by an EPC-7 amplifier (List Electronics, F.R.G.). Pipette resistances were 2–3 MΩ. No compensation for series resistance (Rs) was made. Rs was assessed to be at a maximum at 6 MΩ, based on the estimation of Rs during whole cell voltage clamp of twice the pipette resistance in the bath (Marty & Neher, 1983). Cells were superfused at a rate of 5–7 ml min^{-1} with a solution containing (mM): NaCl 110, KCl 1, HEPES 20, $MgCl_2$ 0.8, glucose 5.5 and $NaHCO_3$ 10, pH 7.4 when gassed with O_2 containing 5% CO_2. $BaCl_2$ 10 mM was added to the pregassed solution to avoid precipitation. Tetrodotoxin (TTX, 0.5 μM; Sigma) was also added to the superfusate. Calcium channel currents were evoked every 15–20 s by rectangular depolarizations from a holding potential of −80 mV. Current records were stored on magnetic tape (Racal Store 4DS), filtered at 0.25 kHz and subsequently digitised through the 12-bit A-D converters of a CED 1401 interface linked to an IBM AT computer. For all records shown in this paper, linear leak and capacitative currents have been subtracted from the whole cell current record. Conductance data were fitted to a Boltzmann function by the Simplex method of Nelder & Mead (1964), as implemented in the subroutine AMEOBA (Press et al., 1986).

Guinea-pig papillary muscles

Female guinea-pigs (Dunkin Hartley, 200–300 g) were killed by cervical dislocation. Right ventricular papillary muscles were dissected from the hearts and placed in oxygenated ice-cold salt solution. The muscles were suspended in a chamber of volume 1.8 cm^3 and superfused at 5 ml min^{-1} with salt solution containing (mM): NaCl 145, KCl 6, $CaCl_2$ 2, $MgCl_2$ 1, Trizma buffer 10 and glucose 5.5, pH 7.4 at 37°C. The tendon end of the muscle was attached to a miniature force transducer (AME 875, Norway) and the preparation was stimulated at 1 Hz via platinum field electrodes (0.5 ms, 2× threshold voltage ~ typically 3 V). Muscles were stretched to generate maximum developed tension. Following a period of equilibration (1–1.5 h) the muscles were superfused with the drugs under test. Responses to increasing concentrations of a drug were displayed on a galvanometric chart recorder. Measurements of twitch magnitude were made after 30 min exposure to each drug concentration and were expressed as a percentage of the twitch magnitude at the end of the control (equilibration) period. From concentration-response curves, concentrations increasing contractility to 200% of control levels (EC_{200}) were calculated.

Porcine coronary artery rings

Porcine hearts were obtained from the local abbatoir and left anterior descending coronary artery was dissected free. Rings of 2–3 mm length were cut and suspended between two fine needles, one of which was attached to a miniature force transducer (AME 875) in a perspex chamber (volume = 2 cm^3) and

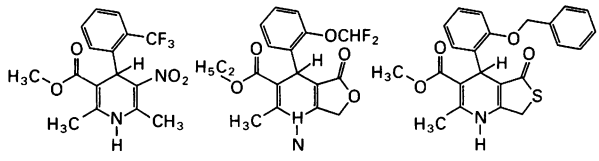


Figure 1 Molecular structures of Bay K 8644, CGP 28392 and RS 30026

superfused at 5 ml min^{-1} with salt solution (as described for papillary muscles) prewarmed to 37°C. The rings were placed under an initial resting tension of 1 g and were stimulated with 10 min pulses of high potassium (120 mM) salt solution at 30 min intervals. Increased potassium levels in these solutions were obtained by increasing the concentration of potassium at the expense of sodium. The preparations were allowed to equilibrate for 2–3 h, during which time successive high potassium responses of similar magnitude were obtained; this was taken as a maximal response. The rings were then superfused with 30 mM potassium salt solution to obtain a partial tonic contracture. These were typically around 30% of maximum; this was taken as a basal level for each tissue. Drugs were then added to the 30 mM potassium salt solution and increases in tonic contracture recorded. Measurements were made after 30 min drug exposure and increasing concentrations were applied to each tissue. Contractures were expressed as a percentage of the maximum contracture to 120 mM potassium observed during the equilibration period. Concentration-response curves were constructed and concentrations which increased the tone to 50% of the maximum level (EC_{50}) were calculated from an iterative fit of a sigmoid function to the data.

Drugs

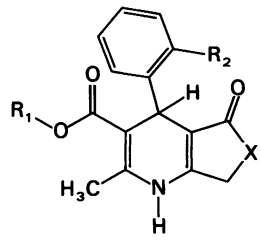
Bay K 8644 (see Figure 1), RS 30026 (methyl 2-methyl-4-(2-benzyloxyphenyl)-5-oxo-1,4,5,7-tetrahydrothieno [3,4-b] pyridine-3-carboxylate; Figure 1) and analogues were synthesised in the Institute of Organic Chemistry, Syntex Research, Palo Alto (Contribution No. 782). Details of the synthesis of RS 30026 will be published separately (R. Greenhouse). Both compounds were used as racemates. Nisoldipine was obtained from Bayer. All dihydropyridines were dissolved in ethanol and added to salt solutions to attain the concentrations required. The final concentration of ethanol was always less than 0.1%.

Results

Interactions between calcium channel antagonist and facilitators on cell beating

The use of embryonic chick heart cell aggregates in the investigation of interactions between calcium channel antagonists and facilitators has been previously described (Patmore & Duncan, 1988). We have examined the interactions between a novel series of dihydropyridines and the antagonist nisoldipine. From concentration-response relationships derived for each compound, a potency value has been calculated as the concentration causing reversal of inhibition of beating in 50% of the aggregates under test (pEC_{50}). Data for RS 30026 analogues and the standards Bay K 8644 and CGP 28392 are shown in Table 1. The RS 30026 series are based on the lactone-dihydropyridine structure of the calcium channel facilitator CGP 28392 (Figure 1). Reversal of nisoldipine-induced inhibition of beating by RS 30026 and Bay K 8644 is shown in Figure 2.

Table 1 Structure-activity relationships for RS 30026 series



RS No.	R_1	R_2	*X*	pEC_{50}
Bay K 8644*				7.20
CGP 28392* [RS 71684]				6.16
31003	C_2H_5	Cl	O	5.61
31227	C_2H_5	Br	O	5.84
31617	C_2H_5	CF3	O	6.03
71684	C_2H_5	$OCHF_2$	O	6.21
30421	C_2H_5	Ph	O	5.83
31156	C_2H_5	OCH_2Ph	O	6.67
30570	C_2H_5	$O(CH_2)_2Ph$	O	43% at 10^{-5} M
30321	C_2H_5	$O(CH_2)_3Ph$	O	5.95
31611	CH_3	CH_3	O	5.76
34674	CH_3	$OCH_2CH=CH_2$	O	5.18
34667	CH_3	OPh	O	6.21
30124	CH_3	OCH_2Ph	O	7.10
30026	CH_3	OCH_2Ph	S	7.45
34389	CH_3	Cl	S	6.15

Data shown are negative logarithms of concentrations of compounds causing a 50% reversal of nisoldipine-induced inhibition of beating. Values are calculated from a least squares non-linear regression fit to data from 4 experiments. Reproducibility of these potency values is shown by comparison of values for CGP 28392 *from Patmore & Duncan (1988) with that obtained for the same compound RS 71684 in a separate series of experiments. Only partial reversal (43%) of nisoldipine-induced inhibition of beating was observed with RS 30570 at the highest concentration tested (10^{-5} M).

Effects of RS 30026 on contractility of embryonic chick myocytes

The contractility of embryonic chick myocytes can be assessed by measuring edge movement of aggregates of myocytes, as described by Clusin (1981), and in this study edge movement and contractility have been assumed to be synonymous. Edge movement was recorded by a video tracking device which measures the movement of a discrete area of the aggregate,

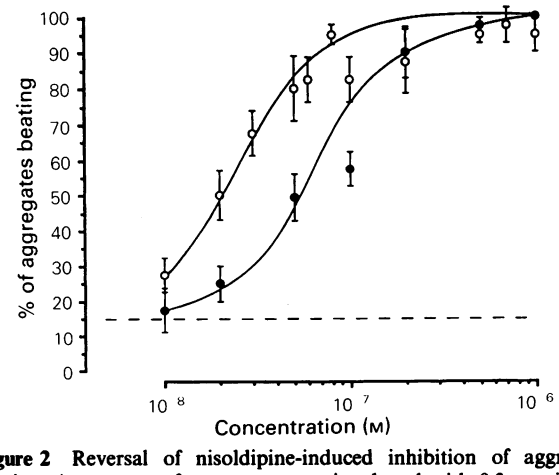


Figure 2 Reversal of nisoldipine-induced inhibition of aggregate beating. Aggregates of myocytes were incubated with 0.3 μM nisoldipine throughout, a concentration which causes inhibition of beating in 85% of aggregates. Addition of increasing concentrations of Bay K 8644 (●) or RS 30026 (○) reversed nisoldipine-induced inhibition of beating. pEC_{50} values were 7.20 for Bay K 8644 (n = 16) and 7.45 for RS 30026 (n = 12). Vertical lines show s.e.mean.

chosen on the basis of it displaying most movement. Typical control responses were around 2–5 μm. The method used has been described in detail elsewhere (Patmore *et al.*, 1989a). Positive inotropic responses in embryonic chick myocytes can be difficult to measure, since in normal extracellular calcium concentrations of 2 mM the myocytes are near the top of the calcium-twitch contraction relationship (Patmore *et al.*, 1989a). Thus in order to observe positive inotropic responses to calcium facilitators the external calcium was reduced to 1 mM, which is around 50% of maximal. It has previously been shown that embryonic chick myocyte aggregates respond rapidly to positive inotropic agents (Patmore & Whiting, 1985; Patmore *et al.*, 1989b). The effects of RS 30026 on edge movement are shown in Figure 3 together with the concentration-response relationship. The concentration of RS 30026 which increased contractility by 50% above control levels (EC_{50}) was 2 nM which compares with 3 nM obtained for Bay K 8644 in similar experiments (Patmore *et al.*, 1989b). Whilst the potencies for initial positive inotropism for the two drugs were similar, RS 30026 caused a 5–6 fold increase in edge movement at 30 nM, compared with the 2 fold increase by Bay K 8644 obtained by Patmore *et al.* (1989b).

Effects on cardiac contractility (papillary muscle)

Concentrations of calcium facilitators which caused an increase in contractility of papillary muscle to 200% of control (pEC_{200} values) are shown in Table 2. Whilst pEC_{200} values for Bay K 8644 and RS 30124 were similar, RS 30026, the thiolactone derivative of RS 30124 was more than 10 times more potent. CGP 28392 displayed weak activity in these experiments. This increase in potency is shown in concentration-response relations for these compounds in Figure 4. Interestingly, the substitution of a sulphur for oxygen in the lactone group of this molecule increases the potency and the efficacy of the compound as a positive inotrope. At 10^{-6} M Bay K 8644 increased contractility to around 327 ± 38% ($n = 9$) of control whilst RS 30124 increased twitch magnitude to a mean value of 868 ± 110% ($n = 3$). RS 30026 increased contractility to 1318 ± 498% ($n = 4$) of control values (all values are mean ± s.e. of mean).

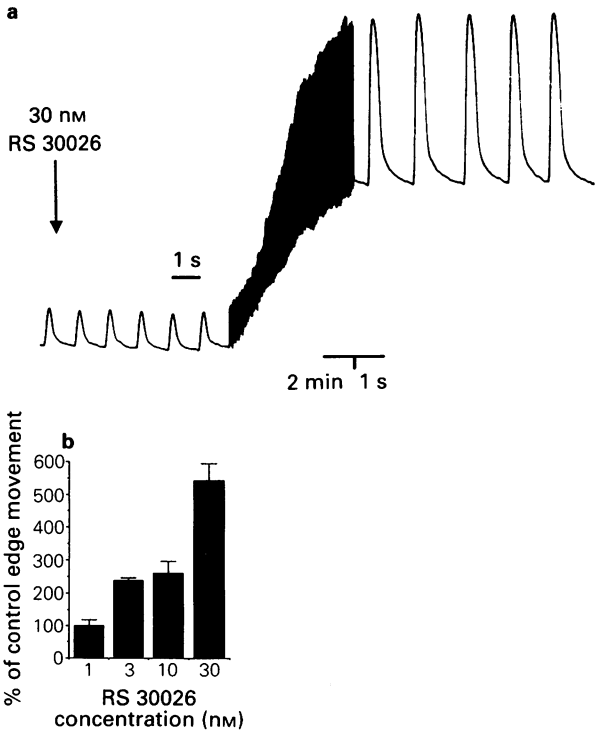


Figure 3 Effects of RS 30026 on edge movement of aggregates of myocytes. Measurements of edge movement were made from single aggregates of myocytes which display automaticity. (a) Addition of 30 nM RS 30026 to the superfusate caused a large increase in edge movement or contractility with a substantial increase in aggregate 'tone'. In the presence of RS 30026 the natural rate is slower. (b) Quantification of the inotropic effects of RS 30026. Data are means for $n = 4$ determinations at each concentration; vertical bars show s.e.mean. The superfusate contained 1 mM calcium throughout to allow observation of positive inotropism.

Table 2 Effects of calcium channel agonists on cardiac and vascular smooth muscle

	Cardiac ventricular muscle (Guinea-pig papillary muscle) pEC_{200}	*Coronary vascular smooth muscle* (Porcine coronary artery rings) pEC_{50}
Bay K 8644	6.79	8.26
CGP 28392	5.18	7.43
RS 30124	7.00	7.61
RS 30026	8.21	8.88

Data shown are negative logarithms of concentrations causing a 100% increase (doubling) of contractility of guinea-pig papillary muscle and a 50% of maximum augmentation of 30 mM potassium-induced contracture of porcine coronary artery rings. Values were obtained from a least squares non-linear regression fit of a sigmoid function to data obtained from 4–9 experiments.

Effects on coronary artery

Potencies for constriction of 30 mM potassium depolarized coronary artery rings (pEC_{50} values) by Bay K 8644, CGP 28392, RS 30124 and RS 30026 are shown in Table 2. In these experiments RS 30026 was more potent than Bay K 8644 with EC_{50} values of 1.3 and 5.5 nM respectively, differences in the maximum contraction were not observed as the design of the experiment only allowed contraction of the rings to a maximal 100% contracture. Concentration-response relationships for Bay K 8644 and RS 30026 are compared in Figure 5. CGP 28392 was also more active in relation to Bay K 8644 than was observed in experiments on papillary muscle.

Effects on calcium channel current

Since reversal of nisoldipine-induced antagonism of aggregate beating appears to be specific to agents which have a direct action on the calcium channel and is insensitive to isoprenaline or to cardiac glycosides such as ouabain (Patmore *et al.*,

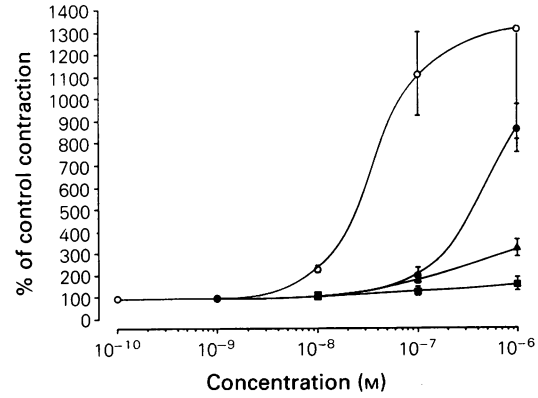


Figure 4 Effects of calcium channel agonists on contractility of guinea-pig papillary muscle. Positive inotropic responses to CGP 28392 (■, $n = 4$), Bay K 8644 (▲, $n = 9$), RS 30124 (●, $n = 3$) and RS 30026 (○, $n = 3$–6) were expressed as a percentage of the control contractility at the start of the experiment. Effects of each concentration were monitored over 30 min. Higher concentrations of Bay K 8644 caused no further increase in contractility whilst at 10^{-5} M CGP 28392 increased contractions to 220 ± 12% of control (mean ± s.e.mean, $n = 4$). Vertical lines show s.e.mean.

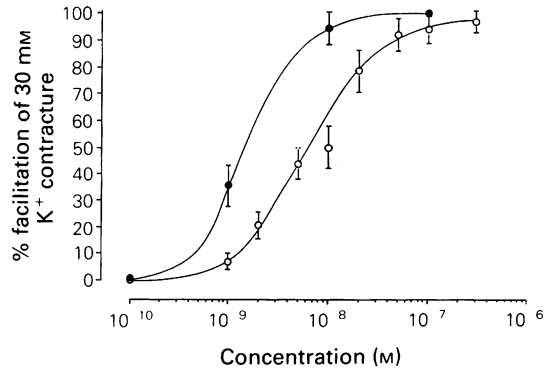


Figure 5 Effects of Bay K 8644 and RS 30026 on porcine coronary artery rings. Concentration-dependent facilitation of contractions of porcine left anterior descending coronary artery rings partially contracted with 30 mM K^+ by Bay K 8644 (○, $n = 4$) and RS 30026 (●, $n = 4$–8). Vertical lines show s.e.mean.

1989b), it seems that the positive inotropic and vasoconstrictor properties of RS 30026 are a feature of calcium channel activation and or facilitation. To demonstrate this activity we have made direct recordings of the voltage-activated calcium channel currents in embryonic chick myocytes. RS 30026 (100–500 nM) and Bay K 8644 (50–500 nM) produced large increases in calcium channel current amplitude, with a larger transient component to the current (Figures 6 and 7). RS 30026 (100 nM, $n = 3$; and 500 nM, $n = 4$) shifted the voltage at which current was maximal from +10 mV to −10 mV (Figure 6). The threshold at which current was observed was also shifted by 10 mV in a hyperpolarising direction by RS 30026 (100–500 nM). Depolarizations to −30 mV elicited little or no current under control conditions, but in the presence of RS 30026 a slowly activating sustained current was observed (Figure 6). The effects of Bay K 8644 were examined in a separate series of experiments. Bay K 8644 (50–500 nM) increased calcium channel currents as expected (Figure 7), but the magnitude and dose-dependency of the effects were less than those of RS 30026, at the concentrations tested. From the voltage-current relationship the conductance (g) was calculated for each potential (V_m) from $g = I/(V_m - V_{rev})$ where V_{rev} is the reversal potential and I the peak inward current. These values are plotted against voltage in Figure 8. These activation curves have been fitted with a non-linear least squares procedure to a Boltzmann distribution where $g = g_{max}/(1 + \exp((V_m - V_{1/2})/k))$. Voltages at which the current was half activated are compared in Table 3. RS 30026 caused a shift in the voltage at which the calcium current was half-maximally activated to more negative values with a 12.6 mV shift at 500 nM. This was similar to the shift of 11 mV observed with 50 nM Bay K 8644. Interestingly at 500 nM Bay K 8644 the shift in the activation curve was very much smaller (1.9 mV), presumably reflecting the antagonist properties of Bay K 8644 at this concentration. A marked difference in the slope of the calcium current activation curves was observed between Bay K 8644 and RS 30026. With Bay K 8644, the slopes were similar in the presence and absence of the drug. RS 30026 caused a concentration-dependent increase in slope (decrease in k) reflecting the activation of more current for a given depolarization. On repolarization from potentials of around 0 to +30 mV inward tail currents were usually observed. In the presence of 500 nM RS 30026 these became very large and slowly inactivating (Figure 6).

Discussion

Reversal of nisoldipine-induced inhibition of myocyte aggregate beating appears to be a useful and specific functional screen for identifying potential dihydropyridine calcium channel agonists. This assay is not sensitive to other positive inotropic agents such as isoprenaline or the cardiac glycoside strophanthin G (ouabain) (Patmore *et al.*, 1989b). Thus compounds which are positive in this test appear to have agonist

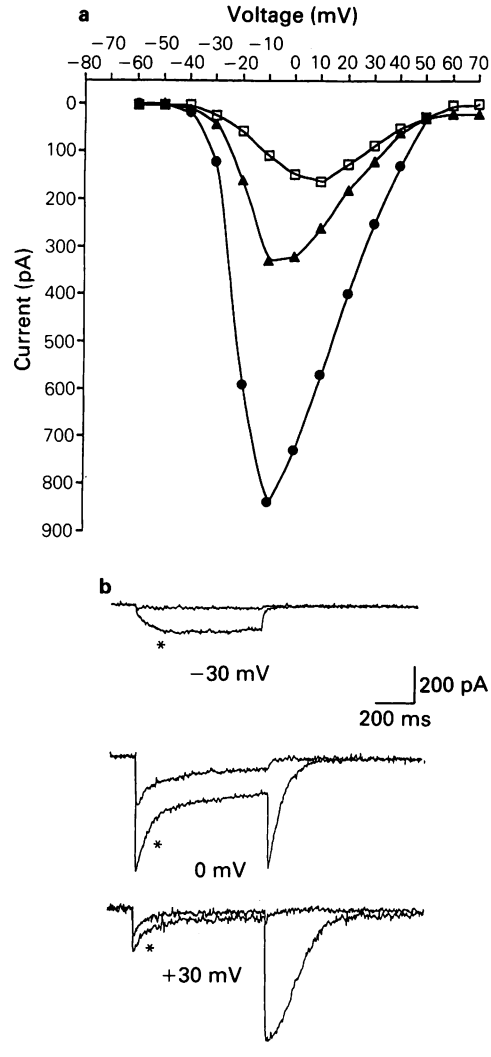


Figure 6 Effects of RS 30026 on whole-cell calcium channel currents in chick myocytes. Cells were held under voltage clamp at −80 mV and stepped to test potentials for 700 ms. (a) Current-voltage relationship for the peak inward current in the absence (□) and presence of 100 nM (▲) and 500 nM (●) RS 30026. Data are mean values from 4 experiments. (b) Representative current records evoked by depolarizations to test potentials as shown in the absence and presence (*) of 500 nM RS 30026. Note the very large tail currents on repolarization from 0 and +30 mV.

Table 3 Effects of Bay K 8644 and RS 30026 on activation of calcium currents in embryonic chick myocytes

	Bay K 8644			*RS 30026*		
	Control	50 nM	500 nM	*Control*	100 nM	500 nM
g_{max} (nS)	2.65	5.66	5.45	3.34	5.32	12.38
$V_{0.5}$ ($\Delta V_{0.5}$)	−15.2 (0)	−26.2 (11)	−17.1 (1.9)	−9.0 (0)	−18.1 (9.1)	−21.6 (12.6)
k (mV)	6.28	6.74	6.11	8.10	4.19	3.86

Conductance data are from fits of a Boltzmann function as described in the text to mean conductance-voltage relationships for 4 experiments for each treatment. Values shown in parentheses are shifts in the half-activation voltage ($V_{0.5}$) from control.

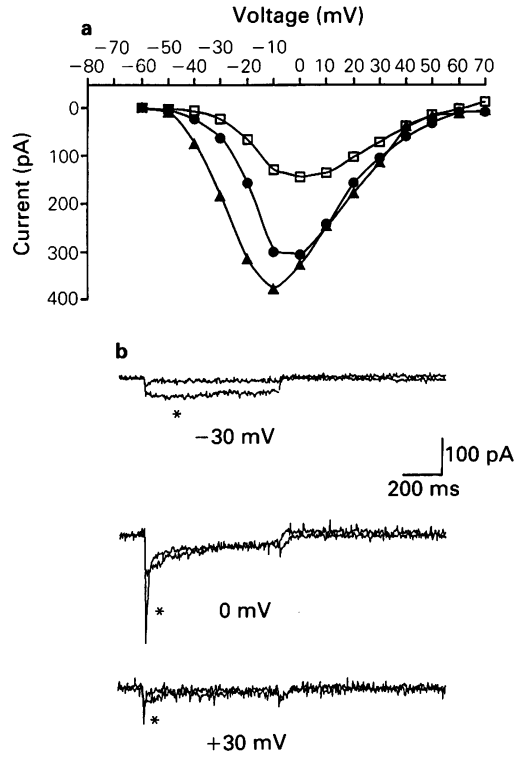


Figure 7 Effects of Bay K 8644 on whole-cell calcium channel currents in chick myocytes. Cells were held under voltage clamp at −80 mV and stepped to test potentials for 700 ms. (a) Current-voltage relationship for the peak inward current in the absence (□) and presence of 50 nM (▲) and 500 nM (●) Bay K 8644. Data are mean values from 4 experiments. (b) Representative current records evoked by depolarizations to test potentials as shown in the absence and presence (*) of 50 nM Bay K 8644.

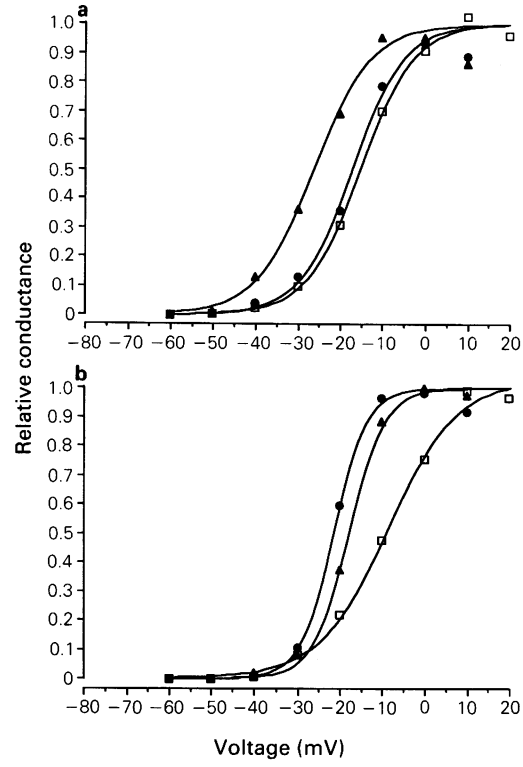


Figure 8 Effects of Bay K 8644 (a) and RS 30026 (b) on calcium channel current voltage-conductance relationships. Conductances (g) were calculated from the relation: $g = I_{peak}/(V_m - V_{rev})$ where the reversal potential (V_{rev}) for the calcium channel current was 60 mV from current-voltage data shown in Figures 6 and 7. Curves fitted to the data describe the Boltzmann function where $g = g_{max}/(1 + \exp((V_m - V_{1/2})/k))$. Values of g_{max}, $V_{1/2}$ and k are shown in Table 3. (a) Control (□) conductance-voltage relationship and in the presence of 50 nM (▲) and 500 nM (●) Bay K 8644. (b) Control (□) and in the presence of 100 nM (▲) and 500 nM (●) RS 30026.

characteristics. However, it might be considered that dihydropyridines which have reasonable affinity for the calcium channel but have low antagonist efficacy may displace nisoldipine and therefore appear positive in this test, but not necessarily be agonists at the calcium channel. In the series of dihydropyridine-lactone compounds described, we have evaluated several of the most potent for positive inotropic activity in guinea-pig papillary muscle (RS 30026 and 30124—Table 2; RS 31227, 30321, 34667 and 31156—data not shown). All these compounds were positively inotropic and thus appear to be agonists.

The dihydropyridine-type calcium channel activators described so far, such as the 3-nitro derivatives Bay K 8644 (Schramm *et al.*, 1983a) and 202-791 (Hof *et al.*, 1985), the lactone CGP 28392 (Erne *et al.*, 1984), the 2-amino derivative H 160/51 (Beyer *et al.*, 1985), and the amide YC-170 (Takenaka & Maeno, 1982) constitute a structurally heterogeneous group. Theoretical calculations (Mahmoudian & Richards, 1986) and comparisons between crystal structures of two of these agonists, Bay K 8644 and CGP 28392, with those of known antagonists, have led to proposals explaining the conformational differences between agonists and antagonists in terms of hydrogen bonding of the 1-NH group, ester group orientation and hydrophobic fit (Langs & Triggle, 1985). It is, however, not clear how structures such as YC-170 or H 160/51 fit into this picture. Furthermore, our own observation that optically inactive dihydropyridines with hydrogen atoms instead of methyl groups in the 2- and 6-positions also displayed agonist activity (R. Greenhouse, unpublished work) complicates this issue even further. In the present series, no clear-cut structure-activity relationship emerged apart from the observation that bulky, lipophilic substituents on the 4-phenyl group generally increased facilitator activity. Additionally, replacing the lactone oxygen with sulphur, as in RS-30026 and RS 34389, resulted in an enhancement of agonist activity.

A functional consequence of reversal of calcium antagonist-induced inhibition of aggregate beating is that the compounds should be positively inotropic in these myocytes. This activity has been demonstrated with the most potent compound from this series, RS 30026. In previous studies positive inotropism has only been observed when the calcium concentration was reduced to 1 mM (50% the normal concentration), since at 2 mM the contractility of aggregates is very close to the top of the external calcium response curve (Patmore *et al.*, 1989a,b). Under these conditions the standard calcium channel facilitator Bay K 8644 only increased contractions up to the level attained in 2 mM calcium (i.e. a 2 fold increase in contractility or edge movement). The effects of RS 30026 were more marked; whilst the EC_{50} (2 nM) was similar to that for Bay K 8644 (3 nM), RS 30026 increased edge movement to 5–6 times the control level in 1 mM. These data demonstrate that RS 30026 is a more effective calcium channel agonist than Bay K 8644.

In guinea-pig papillary muscle the effects of RS 30026 were even more marked. In this preparation Bay K 8644 caused considerable positive inotropism, increasing the magnitude of twitch contractions to 350% of control values at 1 μM, with an EC_{200} of 0.16 μM. At higher concentrations (10 μM) there was a reduction in the positive inotropic effect presumably indicating evidence of calcium channel antagonism. The RS 30026 series was based on the compound CGP 28392 which is a less potent and weaker agonist than Bay K 8644 (Rogg *et al.*, 1985; Truog *et al.*, 1985; Patmore & Duncan, 1988). CGP 28392 displayed less positive inotropic activity than Bay K 8644, only increasing contractility up to 220% of control at 10 μM, with an EC_{200} of 6.6 μM. RS 30124 is directly related to CGP 28392, both compounds are lactone-dihydropyridines with OCH_2Ph replacing the $OCHF_2$ group of CGP 28392.

The significance of this modification is demonstrated in Figure 4, RS 30124 increased contractility up to 868% of control levels whilst the EC_{200} of 0.1 μM was similar to that for Bay K 8644. Replacing the lactone oxygen atom with sulphur (RS 30026) makes a significant difference to the positive inotropic actions of the molecule. RS 30026 increased contractility up to 1318% of control levels at 1 μM with an EC_{200} of 6 nM; it was 26 times more potent than Bay K 8644 and over 3 times more effective in potentiating the magnitude of contractions.

Differences between the effectiveness of these compounds in potentiating contractions of 30 mM K^+ depolarized porcine coronary artery rings were not so obvious (Table 2). CGP 28392 was more potent in this assay (EC_{50} = 37 nM) and RS 30124 displayed a similar potency (EC_{50} = 24 nM). Bay K 8644 was more potent than RS 30124, with an EC_{50} of 5.5 nM, and RS 30026 was 4 times more potent than Bay K 8644, with an EC_{50} of 1.3 nM. It was not possible to observe differences in the magnitude of contraction in this preparation since rings were only able to constrict to a maximum 100% level. Whilst the compounds showed a similar rank order of potency as vasoconstrictors, the differences were not so marked as those observed in cardiac muscle. This may be attributed to the different state of the calcium channels in this preparation, since in 30 mM potassium the membrane potential will be considerably depolarized (around -20 to 30 mV as estimated from the Nernst equation) and this is considerably different from the -85 mV resting potential observed in papillary muscle preparations.

To demonstrate that RS 30026 does facilitate calcium currents in myocytes, direct measurement of the calcium channel currents which underlie contractility was made with the whole-cell voltage clamp technique. RS 30026 at 100 and 500 nM caused marked increases in the magnitude of the calcium channel current, and produced a shift in the threshold for activation of current and a negative (-20 mV) shift of the peak current potential. Similar but smaller increases in current magnitude were observed with Bay K 8644 and these were maximal at 50 nM, a higher concentration of 500 nM caused a smaller increase in the peak current. Bay K 8644, at 50 nM, also shifted the threshold for activation of currents to more negative potentials and caused a -10 mV shift in the potential at which the current was maximal. These observations were quantified in an analysis of the conductance-voltage relationships, which were fitted to a Boltzmann distribution describing current activation with depolarization. The activation characteristics of the calcium channel currents of embryonic chick myocytes are similar to those observed in adult guinea-pig myocytes. In embryonic chick myocytes half-maximal activation occurred at -15 to -9 mV with a slope (k) of 6–8 mV for an e fold increase in conductance. This compares with -14.9 mV (range = -25 to -5 mV), k = 6.2 for guinea-pig myocytes found by Kamp *et al.* (1989). Bay K 8644 (50 nM) caused a parallel shift of the activation curve by -11 mV. Similar parallel displacements have been obtained by Hamilton *et al.* (1987) (-12 mV for 1 μM (+)-Bay K 8644) in guinea-pig myocytes. The threshold for conductance activation was also shifted to more negative potentials in the presence of RS 30026, but the most noticeable effect was on the slope of the conductance-voltage relationship, with k decreasing from 8.1 mV to 4.2 mV in 100 nM and to 3.9 in 500 nM. Thus the shift of the activation curve in the presence of RS 30026 was not parallel and in this respect the action of RS 30026 is different from Bay K 8644 and the agonist isomer of the dihydropyridine (+)-202-791, which has been shown by Kamp *et al.* (1989) to shift the activation curve by -10.5 mV at 400 nM, whilst k values were similar in the presence (k = 5.41) and absence (k = 6.17) of the drug. It is possible that an increase in the slope may be a function of uncompensated series resistance of the recording pipette, giving rise to larger voltage steps than the command potentials when the magnitude of the current was increased with RS 30026. At the worst case where the conductance is maximal the current increases from 140 pA at +20 mV to 740 pA at 0 mV, this would lead to a 3.6 mV shift of the voltage based on an estimated series resistance of 6 MΩ (see Methods). At the half activation voltage the shift due to uncompensated series resistance would be only 0.4 compared with the 9.1 mV observed. Thus errors associated with series resistance artefacts would be small compared to the shifts in conductance observed in the presence of RS 30026. It may be considered that the series resistance could be higher than our estimate, but with similar increases in current in the presence of Bay K 8644 no changes in slope were observed.

These observations indicate that as the membrane potential depolarizes, the activation of the calcium channel conductance will be more rapid in the presence of RS 30026 than in the presence of Bay K 8644 or (+)-202-791, and that RS 30026 has different effects to other dihydropyridine agonists on the gating of the calcium channel. However, it does not seem likely that differences in activation characteristics can entirely account for the very large increases in contractility observed with RS 30026. Evidence to support this view comes from the very slowly decaying tail currents observed with RS 30026. These enhanced tails were also observed to a lesser extent in the presence of Bay K 8644, and are a reflection of the increased open time of the channel so that channels remain open after the membrane potential has been repolarized. The magnitude of the tail currents in the presence of RS 30026 indicates that this compound has marked effects on channel open time and it will be of obvious interest to investigate these actions at the single channel level. Thus these two phenomena of increased activation of the calcium current and marked prolongation of channel open time would result in a considerable increase in calcium influx in myocardial cells and can explain the extreme positive inotropism observed with RS 30026.

Throughout these studies we have used the racemic forms of Bay K 8644 and RS 30026. Previous work has shown that the individual enantiomers of Bay K 8644 (Franckowiak *et al.*, 1985), 202-791 (Hof *et al.*, 1985) and H 160/51 (Beyer *et al.*, 1985) have opposite effects on the calcium channel; one isomer being an antagonist and the other an agonist. If the same is true with RS 30026 then the agonist isomer may be even more potent and effective than we have demonstrated here for the racemate. In the present experiments high concentrations of Bay K 8644 showed antagonist properties, which may be a reflection of the actions of the (+)-antagonist isomer or, as suggested by MacDonald *et al.* (1987), may also reflect antagonism by high concentrations of the (−)-agonist isomer. No such effects were observed with the racemic RS 30026 and we have described considerable differences in the efficacy of Bay K 8644 and RS 30026. This may indicate that either the isomers of RS 30026 are both agonists or the antagonist isomer is less effective than the (+)-antagonist isomer of Bay K 8644 or, perhaps, there are significant differences in the intrinsic efficacy of the agonist isomers of the two agents, with Bay K 8644 behaving as a partial agonist and RS 30026 as a full agonist. The resolution of the isomers of RS 30026 is currently being undertaken and we will shortly be able to address these possibilities.

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Ciguatoxin enhances quantal transmitter release from frog motor nerve terminals

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1 Ciguatoxin (CTX), a marine toxin produced by the benthic dinoflagellate *Gambierdiscus toxicus*, is responsible for a complex endemic disease in man known as ciguatera fish poisoning. In the present study we have investigated the effects of purified CTX extracted for *Gymnothorax javanicus* moray-eel liver on frog isolated neuromuscular preparations with conventional electrophysiological techniques.

2 CTX (1–2.5 nM) applied to cutaneous pectoris nerve-muscle preparations induced, after a short delay, spontaneous fibrillations of the muscle fibres that could be suppressed with 1 μM tetrodotoxin (TTX) or by formamide to uncouple excitation-contraction.

3 In preparations treated with formamide, CTX (1–2.5 nM) caused either spontaneous or repetitive muscle action potentials (up to frequencies of 60–100 Hz) in response to a single nerve stimulus. Recordings performed at extrajunctional regions of the muscle membrane revealed that during the repetitive firing a prolongation of the repolarizing phase of the action potential occurred. At junctional sites the repetitive action potentials were triggered by repetitive endplate potentials (e.p.ps).

4 CTX (2.5 nM) caused a TTX-sensitive depolarization of the muscle membrane.

5 In junctions equilibrated in solutions containing high Mg^{2+} + low Ca^{2+}, addition of CTX (1.5 nM) first induced an average increase of 239 ± 36% in the mean quantal content of e.p.ps. Subsequently CTX reduced and finally blocked nerve-evoked transmitter release irreversibly.

6 CTX (1.5–2.5 nM) increased the frequency of miniature endplate potentials (m.e.p.ps) in junctions bathed either in normal Ringer, low Ca^{2+}-high Mg^{2+} medium or in a nominally Ca^{2+}-free solution containing EGTA. Extensive washing with toxin-free solutions did not reverse the effect. Furthermore, Cd^{2+} (0.1 mM), a potent calcium channel blocker, neither antagonized nor abolished the increase in transmitter release caused by CTX.

7 TTX (1 μM) completely prevented the effect of CTX (2.5 nM) on m.e.p.p. frequency. This effect was independent of the presence of extracellular Ca^{2+}. TTX, when added after CTX (2.5 nM) exposure, antagonized the increase in m.e.p.p. frequency. The antagonism was complete in Ca^{2+}-free medium. These results strongly suggest that increased permeability of the nerve terminal to Na^+ is responsible for the increase in m.e.p.p. frequency caused by CTX. It is likely that CTX may trigger calcium release from internal stores due to an increase of intraterminal Na^+ concentration.

8 It is concluded that CTX exerts, in the nanomolar concentration range, a selective action on sodium channels of the neuromuscular junction causing both pre- and postsynaptic effects.

Introduction

It has long been known that the eating of certain coastal fishes which inhabit sub-tropical and tropical seas causes, in man, a complex endemic disease known as ciguatera fish poisoning (Bagnis, 1968; Bagnis *et al.*, 1979; Withers, 1982; Anderson & Lobel, 1987).

Ciguatoxin (CTX) and maitotoxin (MTX), both produced by the benthic dinoflagellate *Gambierdiscus toxicus* (Yasumoto *et al.*, 1977; Bagnis, *et al.*, 1977) are the two main toxins transmitted to fish through the marine food chain (Taylor, 1979; Bagnis, 1981) which are responsible for ciguatera poisoning. Both CTX and MTX are among the most potent marine toxins known (Tachibana *et al.*, 1987; Yasumoto, 1980). While the effects of MTX are well characterized on excitable membranes (Freedman *et al.*, 1984; Miyamoto *et al.*, 1984; Kobayashi *et al.*, 1985), secretory cells (Takahashi *et al.*, 1983; Login, *et al.*, 1985; Schettini *et al.*, 1984) and at the neuromuscular junction (Kim *et al.*, 1985), the pharmacological actions of CTX (reviewed by Legrand & Bagnis, 1984) are less well known mainly due to difficulties in obtaining adequate quantities of chromatographically pure toxin.

CTX, first purified by Scheuer *et al.* (1967), is a complex lipid-soluble compound. Although H^1 nuclear magnetic resonance data suggest a molecular weight of 1111.7 ± 0.2 daltons, its molecular structure has not yet been completely established (Tachibana *et al.*, 1987). CTX has been shown to affect selectively sodium channels inducing membrane depolarization and spontaneous action potentials in neuroblastoma cells and myelinated axons (Bidart *et al.*, 1984; Benoit *et al.*, 1986).

In the present study we have investigated the effects of CTX at the frog neuromuscular junction in order to establish its effects on spontaneous and evoked quantal transmitter release from motor nerve endings. A preliminary account of part of this work has been previously communicated at the Joint Meeting of Association Française des Pharmacologistes and Nederlandse Vereniging voor Farmacologie (Molgó *et al.*, 1989).

Methods

All experiments were performed on isolated cutaneous pectoris nerve-muscle preparations removed from male frogs (*Rana esculenta*). The normal Ringer solution had the following composition in mM: NaCl 110.0, KCl 2.1, $CaCl_2$ 1.8 and N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES)/NaOH 5.0 (to give a pH of 7.25). In some experiments the Ca^{2+} concentration was reduced and Mg^{2+} added to the solution in concentrations specified in the Results. In

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other experiments Ca^{2+} was substituted by Mg^{2+} (2 mM) and 1 mM ethyleneglycol-bis-(β-amino-ethyl ether)N,N'-tetra-acetic acid (EGTA) was added to the medium. When changes were made of the ionic composition of the bathing solution, osmolarity was maintained by changing NaCl concentration. The experiments were performed at ambient room temperature which ranged from 20–22°C.

In some cases the mechanical activity of cutaneous pectoris muscles was uncoupled from the membrane surface depolarization by equilibrating the preparation in Ringer solution containing 2 M formamide (see del Castillo & Escalona de Motta, 1978). Excitation-contraction uncoupling occurred 12–15 min after the addition of formamide. After this treatment preparations were bathed in normal Ringer solution and the resting membrane potential of the muscle fibres was depolarized by 20–30 mV. With a few exceptions, preparations were not used until complete recovery of the resting membrane potential of the muscle fibres, which occurred within 2–4 h of continuous washing with normal Ringer solution.

Membrane potential was recorded with intracellular glass capillary microelectrodes filled with 3 M KCl of 8–12 MΩ resistance. Recordings were made either at endplate regions, visually identified by locating terminal nerve branches where miniature endplate potentials (m.e.p.ps) had rise times of 1 ms or less, or at extrajunctional areas of the muscle fibres. The nerve was stimulated with supramaximal current pulses of 0.05–0.1 ms duration through a suction electrode at frequencies of 0.5 Hz.

In order to follow the time course of changes in m.e.p.p. frequency two protocols were generally used: (i) continuous recording at an individual junction; (ii) multiple sampling of different junctions from the same muscle.

Data were collected by an IBM-AT microcomputer equipped with a TM 100 Labmaster analogue interface board (Scientific solutions, U.S.A.) and modified pClamp software (Axon Instruments, U.S.A.). An event detector (AI 2020, Axon Instruments) was used to detect random events. Synaptic potentials were also stored on video tape with the aid of a modified digital audio processor (Sony PCM 701 ES) and a video cassette recorder for later analysis if needed.

The mean quantal content (m) of e.p.ps was calculated either by the failure method (m = ln Nt/No) in which Nt is the total number of stimuli delivered to the motor nerve and No the number of failures of release or by the direct method (m = e.p.p. amplitude/m.e.p.p. amplitude). Usually 30-100 e.p.ps and 30–100 consecutive m.e.p.ps were used in the calculations.

Ciguatoxin was extracted for *Gymnothorax javanicus* moray-eel liver and purified by column chromatography by use of silicic acid, Florisil, DEAE-cellulose, Sephadex LH_{20} and C_{18} reversed phase silica successively (Legrand *et al.*, 1989). Pure CTX thus obtained had a mouse LD_{50} (intraperitoneally) of 0.4 μg kg^{-1} with a mean survival time of 10 ± 1.4 h (Legrand *et al.*, 1988). The toxin sample was dissolved in ethanol and divided into several fractions. The ethanol was then evaporated under nitrogen flux. The fractions were kept at −18°C and diluted immediately before the experiment in Ringer solution to give final toxin concentrations of 1–2.5 nM.

Tetrodotoxin, formamide and (+)-tubocurarine were obtained from Sigma (St. Louis, MO, U.S.A.) and all salts were of analytical grade.

Statistical analysis of data was performed with Student's *t* test (two tailed). Values are expressed as mean ± s.e.mean with the number of observations (*n*) in parentheses. Data were considered significant at $P < 0.05$.

Results

Effects of ciguatoxin on neuromuscular transmission

Addition of CTX (1–2.5 nM) to neuromuscular preparations bathed in normal Ringer solution induced within seconds visible spontaneous uncoordinated contractions of the muscle fibres. This spontaneous activity occurred in random isolated groups of muscle fibres but with much less intensity in Ca^{2+}-free medium and in the presence of 30–60 μM (+)-tubocurarine. The contractile activity was not observed when excitation-contraction was uncoupled with formamide (see Methods) and was either prevented or suppressed by 1 μM tetrodotoxin (TTX). These results suggest that the contractile activity induced by CTX is mediated both pre- and post-synaptically.

Intracellular recordings in preparations previously treated with formamide rapidly showed, upon addition of CTX (1–2.5 nM) to the normal Ringer solution, bursts of spontaneous muscle action potentials at rates varying from a few Hz to tens of Hz. In this condition, a single nerve stimulus elicited trains of repetitive action potentials of variable frequency and duration at extrajunctional recording sites (Figure 1A). During repetitive nerve stimulation at 0.5 Hz, action potentials usually appeared as short trains in a given fibre (Figure 1Aa). With continued stimulation the trains became longer (Figure 1Ab, Ac) and the firing reached frequencies of about 60 to 100 Hz. The time course of long trains usually exhibited two phases. During the first repetitive action potentials appeared at a faster rate than during the second (Figure 1Ab, Ac). Analysis of the time course of the action potentials within the second phase of the train revealed that their decay times increased with little change in the amplitudes or the times to peak (Figure 1Bb and Bc). As these changes evolved during the second phase of a train the firing rate decreased and the train finally ceased. The trains were evoked by nerve stimulation only during 1 to 2 min following application of the toxin because the resting membrane potential depolarized. As shown in Table 1, CTX (2.5 nM) caused depolarization of skeletal muscle fibres bathed either in a normal Ringer solution or in a Ca^{2+}-free medium. The depolarization was antagonized by 1 μM TTX. Thus when muscles were bathed in TTX (1 μM) subsequent addition of CTX (2.5 nM) did not elicit depolarization of the muscle fibres (Table 1).

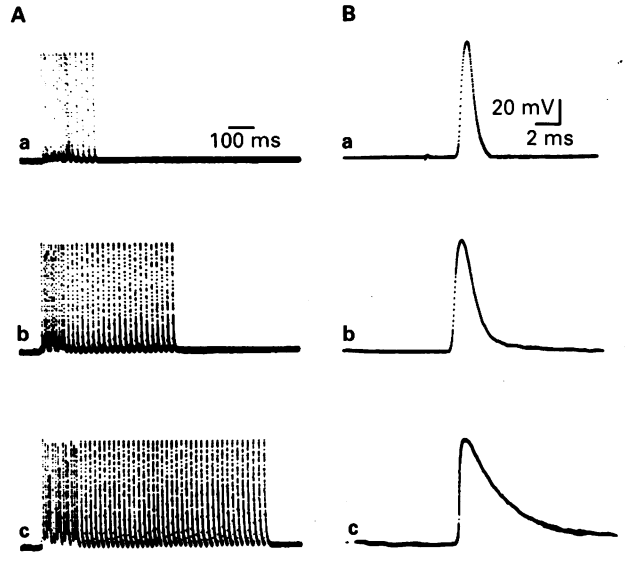


Figure 1 (A) Trains of repetitive muscle action potentials evoked by stimulation of the motor nerve at 0.5 Hz in the presence of ciguatoxin (CTX) (2.5 nM). (Aa–Ac) Action potential trains recorded at extrajunctional sites of a single fibre, 30–60 s after the addition of CTX to normal Ringer solution. Note the two phase firing pattern in (Ab) and (Ac). (B) Fast sweep recording of the action potentials during a single train such as those shown in (A). (Ba) First action potential of the train. (Bb) At about mid train and (Bc) just before the train stopped. Note the slowed repolarization in (Bb) and (Bc) as compared to (Ba). In (A) and (B) formamide was used to uncouple excitation from contraction. Vertical calibration applies to all recordings. Horizontal calibration in (Aa) applies to (Ab) and (Ac), and in (Ba) to (Bb) and (Bc). Resting membrane potential was −75 mV.

Table 1 Effect of ciguatoxin (CTX, 2.5 nM) on the resting membrane potential of frog cutaneous pectoris muscle fibres and the effect of tetrodotoxin (TTX, 1 μM)

Experimental condition	*Resting Membrane potential* (mV) *Controls*	*CTX*	*TTX + CTX*
Normal Ringer	−88.7±1.0 (49)	−57.4±2.2* (44)	−87.2±2.2 (15)
Ca^{2+}-free	−77.5±1.5 (27)	−56.6±2.0* (41)	−79.7±1.9 (16)

*$P < 0.001$ from respective controls. The numbers in parentheses represent number of fibres sampled in 3 different muscles. Note that TTX was applied 15 min before CTX perfusion. Values were obtained after 1 h of CTX exposure.

Recordings made at junctional sites of the fibres showed the classical synaptically-triggered action potential when the motor nerve (0.5 Hz) was stimulated in formamide-treated preparations. Application of CTX produced firing of action potentials in trains upon single nerve stimulation, as in the previous experiments. Soon after train activity was obtained, 2–5 μM (+)-tubocurarine was added to the perfusion medium. These experiments revealed that the trains of action potentials were triggered by repetitive e.p.ps. The timing of the application of (+)-tubocurarine was critically important because the effects of CTX were not long lived. The train firing in the presynaptic terminal revealed by recorded e.p.ps from a typical fibre are shown in Figure 2. The repetitive e.p.ps (Figure 2 b-h) usually exhibited some facilitation with respect to the first e.p.p. in each train and were separated by intervals ranging from 5 to 20 ms.

Effect of CTX on the quantal content of e.p.ps

In junctions equilibrated for 1 h in Ringer solution containing either 0.5 mM Ca^{2+} and 5 mM Mg^{2+} or 0.9 mM Ca^{2+} and 8 mM Mg^{2+}, CTX (1.5 nM) reduced the number of failures of release and increased the average amplitude of e.p.ps. The increase in quantal content of e.p.ps with respect to controls was 239±35.7% (range 135–350%, $n = 4$). In the junctions examined the increase in quantal content of e.p.ps reached a plateau in about 15–20 min and then declined during the subsequent 15–30 min. Concomitant with the decrease in quantal content of e.p.ps the frequency of spontaneous m.e.p.ps greatly increased, as shown in Figure 3. In most junctions examined

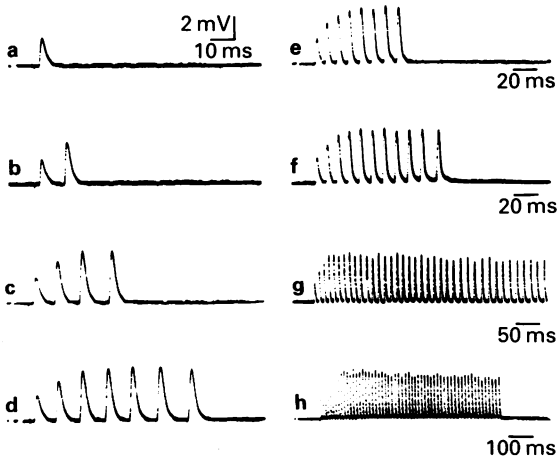


Figure 2 Sequential recording (a–h) obtained at a single junction 90 s after addition of ciguatoxin (CTX, 1.7 nM) to normal Ringer solution containing 3.5 μM (+)-tubocurarine. Note that in all records, a single nerve impulse applied to the motor nerve every 2 s elicited repetitive endplate potentials (e.p.ps) except in (a), which was recorded just before the repetitive firing occurred. Preparation treated with formamide.

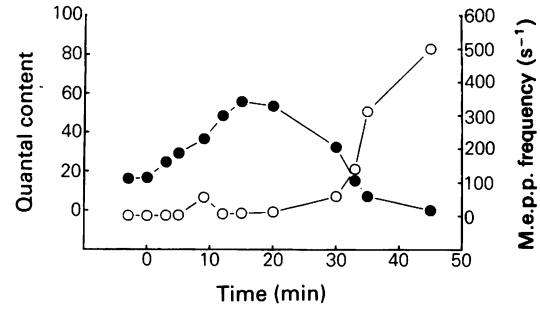


Figure 3 Effect of ciguatoxin (CTX, 2 nM) on the mean quantal content of endplate potentials (e.p.ps) (●) and on miniature endplate potential (m.e.p.p.) frequency (○). Data obtained on the same junction bathed in a Ringer solution containing low Ca^{2+} (1 mM) and 8 mM Mg^{2+}. The quantal content of e.p.ps was estimated by the direct method. Preparation treated with formamide.

after 45–60 min in CTX nerve stimulation did not evoke e.p.ps. Extensive washing of the preparation with CTX-free solution did not reverse either the blockade of evoked transmitter release or the increase in m.e.p.p. frequency.

Effect of CTX on spontaneous quantal transmitter release

CTX (1.5–2.5 nM) increased spontaneous m.e.p.p. frequency when added to normal Ringer solution (Table 2) in a time-dependent way. The increase in m.e.p.p. frequency usually developed after an initial delay of 20–40 min and was sustained at more than 100 times the basal level for up to 2 h and then declined towards zero within 3 h exposure in the continued presence of CTX. Extensive washing of the preparations (2 h) with a CTX-free solution did not reverse the effect of the toxin after it had been applied for 30 min.

Although CTX clearly increased spontaneous quantal release, the mechanism by which this occurred was uncertain. If CTX depolarized the nerve terminals, it might increase transmitter output by enhancing Na^+ and/or Ca^{2+} entry. This could be tested by blockade of either the influx of Na^+ with TTX, a highly specific blocker of voltage-dependent sodium channels (Narahashi, 1974), or the influx of Ca^{2+} by Cd^{2+}, a potent calcium channel blocker (Guan *et al.*, 1987).

In preparations equilibrated for 15 min with normal Ringer solution containing 1 μM TTX, addition of CTX (2.5 nM) caused no detectable change in m.e.p.p. frequency (Table 2). A similar lack of effect of 2.5 nM CTX in the presence of 1 μM TTX was observed after prolonged exposure (3 h). These results indicate that TTX prevents the presynaptic action of

Table 2 Miniature endplate potential frequency (s^{-1}) recorded under different experimental conditions just before and 45-60 min after addition of ciguatoxin (CTX)

Ionic medium	*Control*	*CTX* (1.4 nM)	*CTX* (2.5 nM)
Normal Ringer	1.1±0.1 (17/5)	157.5±34.0* (4/1)	208.6±4.2* (18/5)
Normal Ringer +TTX (1 μM)	—	—	0.8±0.07 (10/3)
Ca^{2+} (1 mM) Mg^{2+}(8 mM)	0.5±0.09 (7/2)	—	253.7±46.8* (3/1)
Ca^{2+}-free	0.3±0.07 (22/4)	—	233.2±49.5* (8/3)
Ca^{2+}-free +TTX(1 μM)	—	—	0.3±0.04 (19/3)

*$P < 0.001$ from respective controls. The figures in parentheses represent number of junctions sample and number of muscles. Note that TTX was applied before the addition of CTX.

CTX on spontaneous quantal release by blocking Na^+ entry through voltage-dependent channels.

It was of interest to study whether TTX antagonized the fully developed effect of CTX on m.e.p.p. frequency. As shown in a typical experiment (Figure 4A), although 1 μM TTX reduced the CTX-increased m.e.p.p. frequency by more than 90%, it did not suppress the effect of CTX completely.

In order to study the contribution of external Ca^{2+} to the increase in m.e.p.p. frequency caused by CTX, experiments were performed either on junctions bathed in normal Ringer solution to which Cd^{2+} (0.1 mM) was added or on junctions equilibrated for 60 min in a nominally Ca^+-free Ringer solution containing 1 mM EGTA and 2 mM Mg^{2+}. As shown by typical recordings of m.e.p.ps obtained in a junction bathed in normal Ringer solution containing 2.5 nM CTX (Figure 4Bb), addition of 0.1 mM Cd^{2+} did not antagonize the CTX-increase in m.e.p.p. frequency (Figure 4Bc). In addition, CTX (2.5 nM) also enhanced m.e.p.p. frequency in nominally Ca^{2+}-free medium (Table 2). Figure 5 shows representative experiments of the time course of CTX (2.5 nM) action on spontaneous m.e.p.p. frequency in a Ca^{2+}-free medium and the effect of TTX. As shown in Figure 5a, TTX (1 μM) completely antagonized the effect of CTX. However, removal of TTX by perfusion with a CTX-free and TTX-free solution induced the reappearance of an increased rate of m.e.p.ps, which indicates that the effect of TTX is more easily reversible than that of CTX. It is interesting to note that no reversal of CTX action was observed under those conditions, even after 2 h washing, and that subsequent addition of TTX could always reverse the CTX-induced increase on m.e.p.p. frequency (data not shown). As shown in Figure 5b, prior treatment with 1 μM TTX completely prevented the effect of CTX in a Ca^{2+}-free medium.

Analysis of the amplitude distribution of m.e.p.ps in the presence of CTX revealed that the distribution was monomodal as in controls. The coefficient of variation (mean $\pm$ s.d.) of the m.e.p.p. amplitude distribution in the presence of 1.5 nM CTX was 0.20 ± 0.01 ($n = 4$) and did not significantly differ from values obtained in control junctions (0.22 ± 0.018; $n = 4$). However, despite the similar distribution of m.e.p.p. amplitudes, in most junctions analysed there was a decrease in the mean m.e.p.p. amplitude with CTX as compared to controls (Figure 6). The reduction in amplitude can be accounted for by a reduction in the acetylcholine driving force through the activated channels of the endplate based on the assumption that the equilibrium potential for acetylcholine is 0 mV before and after CTX action.

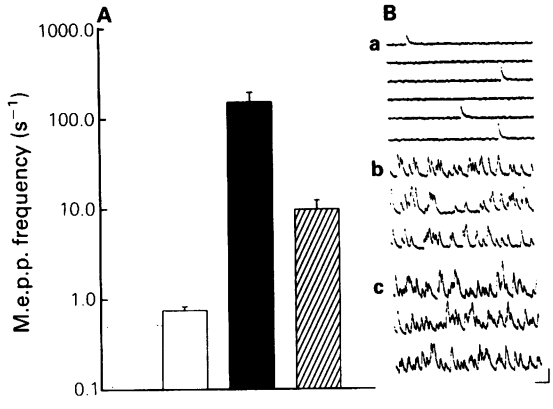


Figure 4 (A) Increase in miniature endplate potential (m.e.p.p.) frequency produced by 2.5 nM ciguatoxin (CTX) (solid column) over controls (open column) and the inability of tetrodotoxin (TTX, 1 μM) to reverse completely the effect of CTX (hatched column). Preparation bathed in a normal Ringer solution and previously treated with formamide. Each column represents values obtained by sampling 8–12 different junctions. CTX was applied for 30 min in normal Ringer solution before recording, while TTX was applied for 25 min in the continuous presence of CTX. Note the logarithmic scale of the ordinate. (B) Examples of m.e.p.ps recorded at a single junction just before (a), 60 min after the addition of 2.5 nM CTX (b), and after 15 min of 0.1 mM Cd^{2+} addition (c) to the preparation. Note that the addition of Cd^{2+} did not antagonize the effect of CTX. Vertical calibration in (c) is 1 mV and applies to all recordings. Horizontal calibration is 50 ms in (a) and 20 ms in (b) and (c).

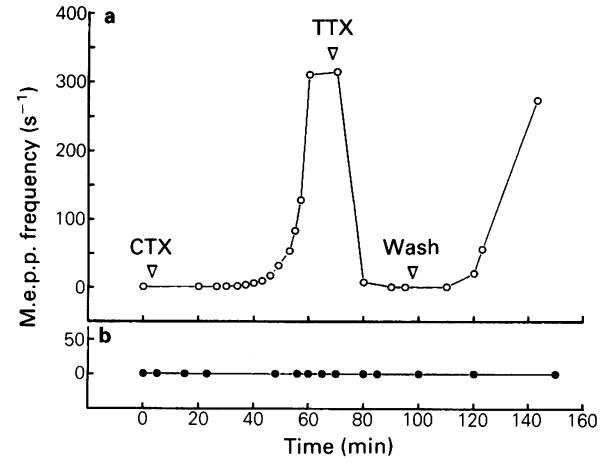


Figure 5 (a) Time course of the increase in miniature endplate potential (m.e.p.p.) frequency produced by 2.5 nM ciguatoxin (CTX, ○) in a Ca^{2+}-free Ringer solution and the ability of tetrodotoxin (TTX, 1 μM) to reverse completely and rapidly the effect of CTX. CTX was applied for 68 min and then the preparation was perfused with the same Ringer with TTX (1 μM) added. Note that washing with TTX-free and CTX-free solution markedly enhanced m.e.p.p. frequency. Arrow heads indicate changes in bathing solutions. All data were obtained from the same junction. (b) Failure of CTX (2.5 μM) to increase the rate of occurrence of m.e.p.ps (●) in another preparation exposed for 1 h to 1 μM TTX in a calcium-free Ringer solution. Data obtained by multiple sampling of different junctions in the same preparation.

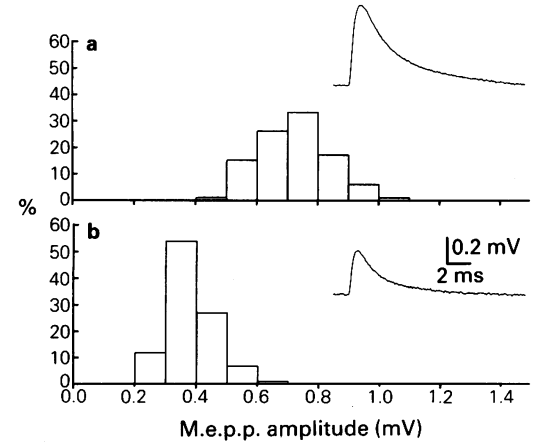


Figure 6 Histograms showing the distribution of miniature endplate potential (m.e.p.p.) amplitudes recorded before (a) and after (b) the action of 1.5 nM ciguatoxin (CTX). Values are expressed as a percentage of the total number of m.e.p.ps counted from a single junction. Preparation bathed in normal Ringer solution. Insert in (a) and (b) averaged digitized records of 67 m.e.p.ps, calibration in insert (b) applies also to insert (a). Resting membrane potential was −81.7 mV in controls and −45.5 mV during CTX action.

Discussion

The results presented here provide evidence that CTX, in the nanomolar range, affects the skeletal neuromuscular junction by a combination of pre- and post-junctional effects. Postsynaptically the toxin induces membrane depolarization and causes spontaneous contractile activity. Since both effects are prevented or suppressed by TTX, it is likely that an increase in Na^+ permeability is involved in these responses. Furthermore, a prolongation of the repolarizing phase of indirectly-

elicited action potentials was found to occur during repetitive firing. In this respect, the action of CTX is similar to that previously found with sea anemone, *Anemonia sulcata* toxin II (ATX-II) on frog skeletal muscle fibres (Khan *et al.*, 1986). However, in contrast to ATX-II we have been unable to detect action potentials with a long plateau in the presence of CTX. Further studies are required in order to understand direct effects of CTX on voltage-dependent ionic currents of the muscle membrane responsible for action potential generation.

One striking prejunctional effect of CTX is its ability to produce repetitive transmitter release, which triggers repetitive action potentials in the muscle fibre in response to a single nerve stimulus. These action potentials might be in part responsible for the spontaneous uncoordinated muscle contractions observed with CTX.

Several drugs and toxins, such as tetraethylammonium, guanidine, aminopyridines, dendrotoxins and ATX II, which affect presynaptic K^+ or Na^+ channels, induce repetitive e.p.ps at the neuromuscular junction (Koketsu, 1960; Matthews & Wickelgren, 1977; Molgó, 1982; Molgó & Mallart, 1985; Anderson & Harvey, 1988). However, to our knowledge the repetitive firing of the motor nerves produced by these agents did not attain the high frequency observed with CTX. At the frog node of Ranvier, it has been shown by Benoit *et al.* (1986) that CTX induces spontaneous action potentials at a frequency of about 100 Hz, with no modification of their time course. Such spontaneous activity has been attributed to a specific modification of a fraction of sodium channels which in the presence of CTX are activated at the normal resting potential and which fail to inactivate.

CTX in about the same range of concentrations used in the present study has been shown to stimulate the initial rate of TTX-sensitive $^{22}Na^+$ uptake by neuroblastoma cells and rat muscle cells in the presence of veratridine (Bidart *et al.*, 1984).

The present experiments show that CTX has a dual action on evoked transmitter release. The facilitatory effect on the mean quantal content of the e.p.p is not maintained and is followed, first, by a reduction, and finally by the irreversible blockade of nerve-evoked transmitter release. It is likely that the CTX-facilitatory effect on evoked transmitter release is not due to enhanced calcium entry during the presynaptic action potential, but to an increase in intraterminal calcium concentration due to sodium entry (see below). The reduction and finally the block of evoked transmitter release that follows the facilitatory action of CTX could be due both to a reduction in the driving force for Ca^{2+} entry and to the depolarization of the terminals caused by Na^+ entry. The blockade of evoked transmitter release can be explained neither by a depletion of transmitter stores in the nerve terminal nor by a change in the sensitivity to acetylcholine of the postsynaptic receptors, since at the time stimulation failed to elicit evoked release, m.e.p.ps could still be recorded and their frequency was markedly increased.

CTX increased the spontaneous quantal release of acetylcholine measured electrophysiologically as an increase in m.e.p.p. frequency. Nerve terminal calcium channel blockade with Cd^{2+} did not antagonize this effect. Furthermore, the acceleration of m.e.p.p. rate caused by CTX occurred independently of extracellular Ca^{2+} since it was observed even in a nominally Ca^{2+}-free solution containing EGTA. These results seem to rule out the possiblity that CTX enhances Ca^{2+} influx through voltage-sensitive Ca^{2+} channels of the nerve terminal, in contrast to the hydrosoluble MTX, whose stimulating effect on spontaneous transmitter release depends upon Ca^{2+} entry into motor endings (Kim *et al.*, 1985).

TTX, in the presence of extracellular Ca^{2+}, did not completely antagonize the increase in m.e.p.p. frequency caused by CTX. The reason for this may be that, even when TTX is able to block Na^+ influx through voltage-sensitive channels, the toxin does not prevent Ca^{2+} uptake through the reversed operation of the Na^+-Ca^{2+} exchange system that normally uses the Na^+ gradient to extrude Ca^{2+} (Blaustein & Nelson, 1982). However, the present study shows that application of CTX following TTX pretreatment failed to increase m.e.p.p. frequency and that TTX completely inhibited the CTX-induced increase in m.e.p.p. frequency in a Ca^{2+}-free medium. These results suggest that increased permeability of the nerve terminal to Na^+ is responsible for the enhancement of m.e.p.p. frequency. In synaptoneurosomes increases of intracellular Na^+ concentration can elicit phosphoinositide breakdown and stimulate phosphatidylinositol systems which are capable of mobilizing Ca^{2+} from internal stores (Gusovsky *et al.*, 1986). In addition, in various secretory systems increases in intracellular Na^+ may mobilize intracellular Ca^{2+} and support transmitter or hormone release (Lowe *et al.*, 1976; Rahamimoff *et al.*, 1980; Melinek *et al.*, 1982). Whether the enhancement of spontaneous quantal transmitter release by CTX is related to increases in phosphoinositide breakdown, intraterminal calcium mobilization or to other mechanism remains to be determined.

Effects on m.e.p.p. frequency similar to those obtained here with CTX have been observed previously with other marine toxins like ATX-II (Lemeignan *et al.*, 1981; Harris & Tesseraux, 1984; Molgó *et al.*, 1986). However, CTX is more potent than ATX-II on a molar basis. Among the toxins isolated from the dinoflagellate *Ptychodiscus brevis*, brevetoxin B has been shown to share a common receptor site with CTX on the neuronal voltage-dependent Na^+ channel protein (Lombet *et al.*, 1987) and T-17 toxin to increase the rate of occurrence of m.e.p.ps in frog and rat neuromuscular preparations (Atchison *et al.*, 1986). However, in contrast to CTX, Ca^{2+} in the external solution was found to be necessary to stimulate m.e.p.p. frequency in the presence of T-17 toxin.

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Release of atrial natriuretic peptide from rat myocardium *in vitro*: effect of minoxidil-induced hypertrophy

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1 Ventricular hypertrophy is characterized by stimulation of ventricular synthesis of atrial natriuretic peptide (ANP). To examine the role of ventricular ANP levels in the secretion of ANP into the circulation, atrial and ventricular levels of immunoreactive-ANP (IR-ANP) as well as ANP messenger RNA (mRNA), and the release of IR-ANP from isolated perfused hearts, both before and after atrialectomy, were measured simultaneously in control and minoxidil-treated Wistar-Kyoto (WKY) and spontaneously hypertensive (SHR) rats.

2 IR-ANP levels in the ventricles of untreated, 12 month-old SHR with severe ventricular hypertrophy were increased when compared to age-matched WKY rats. Minoxidil treatment for 8 weeks in both strains resulted in a decrease in mean arterial pressure and increases in ventricular weight to body weight ratios, plasma IR-ANP concentrations (in WKY from 133 ± 20 to $281 \pm 34\,pg\,ml^{-1}$, $P < 0.01$; in SHR from 184 ± 38 to $339 \pm 61\,pg\,ml^{-1}$, $P < 0.05$), and in ventricular IR-ANP contents (in WKY: 53%; in SHR: 41%). A highly significant correlation was found between ventricular IR-ANP content and ventricular weight to body weight ratio ($r = 0.59$, $P < 0.001$, $n = 26$).

3 When studied *in vitro*, in isolated perfused heart preparations, the hypertrophied ventricular tissue after atrialectomy secreted more ANP into the perfusate than ventricles of the control hearts; ventricles contributed 28%, 22%, 18% and 15% of the total ANP release to perfusate in the minoxidil-treated SHR, control SHR, minoxidil-treated WKY and control WKY, respectively. A significant correlation was found between the IR-ANP release from ventricles and ventricular weight to body weight ratio ($r = 0.56$, $P < 0.01$, $n = 24$).

4 These studies demonstrate that the ventricles contribute substantially to the circulating level of ANP, and that the amount released depends on the degree of ventricular hypertrophy.

Introduction

Atrial natriuretic peptide (ANP), a peptide hormone that regulates salt and water balance and blood pressure, is synthesized, stored, and secreted from mammalian myocytes (Cantin & Genest, 1985; Lang *et al.*, 1985; Brenner & Ballerman, 1986; Ruskoaho *et al.*, 1987; Needleman *et al.*, 1989). The major site of ANP biosynthesis is the atria of the heart, where it constitutes 1–3% of the total messenger RNA (mRNA) (Needleman *et al.*, 1989). Recent evidence shows that biosynthesis of ANP also occurs in extra-atrial tissues, particularly in cardiac ventricles (Bloch *et al.*, 1986; Gardner *et al.*, 1986a; Needleman *et al.*, 1989). In tissue culture, ventricular as well as atrial cardiocytes secrete ANP into the culture medium (Bloch *et al.*, 1986). Increased ventricular ANP synthesis has been demonstrated in patients with congestive heart failure (Edwards *et al.*, 1988; Tsuchimochi *et al.*, 1988; Saito *et al.*, 1989) and in animal models of pressure and volume overload. These models include spontaneous biventricular hypertrophy (Lee *et al.*, 1988), genetic hypertension (Arai *et al.*, 1988), congestive heart failure in cardiomyopathic hamsters (Edwards *et al.*, 1988; Ding *et al.*, 1988), hypoxia (Stockmann *et al.*, 1988), administration of high doses of pharmacological agents (Gardner *et al.*, 1986b; Day *et al.*, 1987; Ruskoaho & Leppäluoto, 1988), or sudden circulatory overload due to aortic constriction, myocardial infarction, or arteriovenous shunting (Lattion *et al.*, 1986; Day *et al.*, 1987; Michel *et al.*, 1988). Whether the increased synthesis and storage of ANP by ventricles results in significant secretion of ANP by the ventricles is unknown.

The aim of the present study was to investigate the relationship between ventricular levels of immunoreactive ANP (IR-ANP) and ventricular release of ANP, which was evaluated *in vitro* by measuring the amount of ANP secreted into the perfusate from the isolated perfused heart preparation both before and after atrialectomy. Minoxidil, known to cause ventricular hypertrophy and increase ventricular levels of ANP (Ruskoaho & Leppäluoto, 1988), was used to produce an increase in ventricular mass in genetically hypertensive and normotensive control rats. In addition, the relationship of ventricular weight and ventricular IR-ANP, as well as ANP mRNA, were studied.

Methods

Animals

Male, 12 month-old, spontaneously hypertensive rats (SHR) of the Okamoto-Aoki strain (F65) and age-matched Wistar-Kyoto (WKY) (F63) rats from the Department of Pharmacology colony at the University of Oulu, Finland, were used. Both strains were originally obtained from Mollegaards Avslaboratorium, Skensved, Denmark. The rats were housed in plastic cages in a room with controlled 40% humidity and a temperature of 22°C. A 6 h 00 min on–18 h 00 min off environmental light cycle was maintained. The experimental design was approved by the Animal Experimentation Committee of the University of Oulu.

Chronically instrumented rats

The surgical preparation and the experimental set-up, which have been described previously (Ruskoaho & Leppäluoto, 1988), were slightly modified. Under chloral hydrate ($300\,mg\,kg^{-1}$ i.p.) anaesthesia, a PE-60 catheter was placed in the abdominal aorta for measurement of blood pressure and heart rate and collection of blood samples, and a PE-50 catheter was inserted into the right atrium via the jugular vein for measurement of right atrial pressure. Both catheters were exteriorized behind the neck, filled with a heparinized (250 $iu\,ml^{-1}$) saline solution, and plugged with a stainless pin. After operation, rats were housed individually in the experimental cages and had continuous access to food and water.

The day after the operation, the arterial and right atrial catheters were attached to pressure transducers (model MP-15, Micron Instruments Inc., Los Angeles, CA) and a Grass polygraph (model 7D, Grass Instruments, Quincey, MA) for mean arterial pressure, heart rate and right atrial pressure recording. Haemodynamic variables were then recorded in the conscious, freely moving animals for 30 min. Then, 5 ml of blood was taken into pre-cooled EDTA tubes on ice for determination of plasma ANP, 2 ml into heparinized tubes with 100 μl of $Na_2S_2O_5$ for plasma noradrenaline and 2 ml into plain tubes for determination of serum sodium and osmolality. Samples were immediately centrifuged and the plasma and the serum stored at −20°C until assayed.

Isolated perfused heart preparation of the rat

To examine the proportion of ANP released from atria and ventricles of both untreated and treated WKY and SHR rats *in vitro*, the hearts were perfused according to the method of Langendorff as described previously (Ruskoaho *et al.*, 1986). Briefly, immediately after the last blood sample, rats were decapitated, the abdominal cavity opened, the diaphragm transected, lateral incisions made along both sides of the rib-cage and the heart cooled with perfusion fluid (4–10°C). The aorta was cannulated above the aortic valve and retrograde perfusion was begun with a modified Krebs-Henseleit bicarbonate buffer, pH 7.4, equilibrated with 95% O_2/5% CO_2 at 37°C. Final concentrations of the salts in the buffer (mmol l^{-1}) were: NaCl 113.8, $NaHCO_3$ 22.0, KCl 4.7, KH_2PO_4 1.2, $MgSO_4 \cdot 7\,H_2O$ 1.1, $CaCl_2 \cdot 2\,H_2O$ 2.5 and glucose 11.

During the equilibration period (60 min) the hearts were perfused by a peristaltic pump (Ismatec SA801, Ismatec Instruments, Zurich, Switzerland) at a flow rate of 8 ml min^{-1} and then switched to constant flow of 5 ml min^{-1}. Variations in perfusion pressure, arising from changes in coronary vascular resistance, were recorded on a Grass polygraph (model 7D, Grass Instruments, Quincey, MA) with a pressure transducer (model MP-15, Micron Instruments Inc., Los Angeles, CA) situated on a side-arm of the aortic cannula. Isometric force of contraction was recorded by a strain gauge transducer (Grass FT03) connected to the Grass polygraph. The output was damped to give the mean contractile force. The heart rate was counted from contractions by means of the Grass tachograph. The hearts were put under a resting tension of 2 g and stimulated (10 V, 0.5 ms) with a Harvard Apparatus Stimulator model 345 (Harvard Apparatus, Millis, MA) to increase heart rate 15–20% above the spontaneous beating rate. Each experiment began by perfusing hearts with the constant flow of 5 ml min^{-1} for 20 min. Then, all atrial tissue was carefully cut away along the ventricular surface and remaining tissue perfused again for 20 min. The coronary venous effluents were collected at 2 min intervals both before and after atrialectomy, and were placed immediately on dry-ice and stored at −20°C until assayed. When the proportion of the ventricular IR-ANP secretion was calculated, only the values of the last five samples of the 20 min period were used because during this period IR-ANP level in the perfusate remained constant.

Organ weights

After perfusion, the aorta and pulmonary artery were carefully cut close to the ventricular surface. The right and the left ventricles were not separated because, as we have shown (Ruskoaho *et al.*, 1989a), the right ventricle contributes only 19% of the total ventricular IR-ANP content in WKY rats and only 4% in SHR. Thus, only 2.8% and 0.9% of the IR-ANP released from ventricles in WKY and SHR, respectively, would be generated in the right ventricle. To avoid the possible contamination of the ventricular sample by atrial tissue, ventricles were cut into the superior (about 15–20% of total weight) and inferior part. Both ventricular samples, as well as the atrial tissue removed, were blotted dry, weighed and stored at −70°C until assayed. To rule out the possibility that changes in cardiac weights were a non-specific effect of minoxidil treatment, both kidneys were removed and weighed.

Assay of immunoreactive ANP (IR-ANP) in plasma and tissue

ANP was extracted from the atrial and ventricular samples as described previously (Ruskoaho & Leppäluoto, 1988). For the radioimmunoassay, the atrial and ventricular samples were diluted 3×10^5 and 1200 fold, respectively. Tissue ANP is expressed either as a concentration per mg wet weight or as content per organ.

The blood samples were centrifuged (2000 *g*, 10 min, at 4°C), and ANP was extracted as described previously (Ruskoaho *et al.*, 1989a). For the ANP radioimmunoassay the plasma and tissue extracts, and the unextracted perfusate samples were incubated in duplicates of 100 μl with 100 μl of the rabbit ANP antiserum in the final dilution of 1:25,000 (Ruskoaho *et al.*, 1989a). After incubation for 48 h at 8°C the immunocomplexes were precipitated with sheep antirabbit antiserum in the presence of 500 μl of 1.2 M ammonium sulphate, pH 7, followed by centrifugation for 40 min at 3000 *g*. The sensitivity of the assay was 0.8 pg per tube. The 50% displacement of the standard curve was at 20 pg per tube.

ANP messenger RNA determination

RNA was isolated from fresh ventricular tissues of WKY and SHR rats by the guanidine isothiocyanate-LiCl method (Cathala *et al.*, 1983). For the RNA blot analysis, 20 μg samples of the RNA from ventricles were transferred to the Schleicher & Schwell BAS 85 nitrocellulose membrane (Dassel, W. Germany) (Davis *et al.*, 1988). The full-length rat ANP cDNA probe Car-55 (Flynn *et al.*, 1985) (a generous gift from Dr Peter L. Davies, Queen's University, Kingston, Canada) was labelled with [^{32}P]-dCTP (deoxycytidine-5'-triphosphate; Amersham, Buckinghamshire, England) with a random primed labelling kit (Boehringer-Mannheim GMBH, Mannheim, F.R.G.). The membrane was hybridized overnight at 65°C in 6 × SSC (saline sodium citrate, 1 × SCC = 0.15 M NaCl, 0.015 M trisodium citrate, pH 7), 0.8% sodium dodecyl sulphate (SDS), 5 × Denhardt's solution, and 100 μg ml^{-1} sheared herring sperm DNA. After hybridization, the membrane was washed in 0.4 × SSC, 0.2% SDS 2–3 times for 20 min at room temperature, and once for 45 min at 65°C. The membrane was exposed at −70°C to XAR-5 X-ray film (Eastman Kodak, Rochester, NY) with a Cronex Lightning Plus intensifying screen (DuPont, Wilmington, Delaware) for three days. To quantify the amount of RNA loaded on agarose gels, a ^{32}P-labelled oligonucleotide probe complementary to rat 18 S ribosomal RNA was hybridized to all filters as described by Albretsen *et al.*, (1988). To estimate tissue ANP mRNA concentration, the ANP:18 S ratio was determined by dividing the absorbance corresponding to the ANP probe by the absorbance corresponding to 18 S probe hybridization.

Assay of plasma noradrenaline, and serum sodium and osmolality

Plasma noradrenaline with 5.0 pmol of dihydroxybenzylamine as an internal standard was extracted by the Plasma Catecholamine Analysis Kit (ESA Inc., U.S.A.). The liquid chromatographic-electrochemical detection (LE-EC) method as described by Turkka *et al.* (1986) was utilized for determination of noradrenaline. Validation and quantification were done as described in the Plasma Catecholamine Methodology book (ESA, Inc.). Serum sodium was determined with FLM3 Flame Photometer (Radiometer, Copenhagen, Denmark), and serum osmolality was measured with an Advanced wide-range osmometer 3W2 (Advanced Instruments, Inc., Needham Heights, MA, U.S.A.).

Antihypertensive treatment

Animals were treated with minoxidil (0.1 mg ml^{-1} in the drinking water) from the age of 10 months to 12 months. Each rat consumed 3–4 mg of minoxidil per day calculated from daily water consumption. The control animals received plain tap water. Before the treatment the rats were divided into two equal groups according to body weight, blood pressure and heart rate, which were measured by a tail-cuff method using a commercially available device (Blood pressure recorder no. 8002e, W + W Electronics, Basel, Switzerland) as described previously (Ruskoaho & Leppäluoto, 1988). Systolic blood pressure in the WKY rats was 139 ± 1 mmHg (n = 11) and in the SHR rats 212 ± 3 mmHg (n = 15, $P < 0.001$). Heart rate was 336 ± 10 beats min^{-1} in WKY rats and 339 ± 8 beats min^{-1} in SHR, and body weights were 425 ± 8 g and 431 ± 6 g, respectively.

Chemicals

The sources of chemicals were as follows: guanidine isothiocyanate (Fluka Chemie AG, Switzerland); LiCl and formaldehyde (JI Baker Chemicals BV, Holland); Agarose NA (Pharmacia, Sweden), heparin (Medica, Helsinki, Finland); synthetic rat ANP1-28 (Sigma Chemical Company, St. Louis, Missouri, U.S.A.); minoxidil (gift from Farmos Group Ltd, Turku, Finland). Minoxidil was first dissolved in deionized water and then added to the drinking water. Unless otherwise stated, other chemicals were from Sigma.

Analysis of data

The results are expressed as means ± s.e.mean. For the comparison of statistical significance between two groups Student's t test for unpaired or paired data was used. Linear regression correlation coefficients were calculated between ventricular weight to body weight ratios and ventricular IR-ANP content, and between ventricular weight to body weight ratios and ventricular IR-ANP release into the perfusate.

Results

Haemodynamics, plasma IR-ANP and noradrenaline in the control and treated WKY and SHR

Mean arterial pressure and heart rate, as measured directly in chronically cannulated conscious rats, were higher in untreated SHR than in WKY rats, whereas there was no difference in right atrial pressure between the strains (Table 1). Plasma IR-ANP concentration in the untreated SHR was 38% higher than in untreated WKY rats, but this difference between the strains was not statistically significant. The plasma IR-ANP values observed are comparable to those we obtained before (Ruskoaho & Leppäluoto, 1988). Plasma noradrenaline levels, serum osmolality and sodium did not differ between the strains.

Mean arterial pressure was significantly lower in the minoxidil-treated WKY and SHR when compared with the respective control WKY and SHR rats (Table 1). Heart rate did not differ significantly between the untreated and treated animals. Right atrial pressure of both the treated WKY and the treated SHR was higher than that of the corresponding untreated animals, the difference being statistically significant in SHR rats. Plasma IR-ANP concentration was 2.1 and 1.8 fold higher in the treated than in the control WKY and SHR, respectively. No significant differences in plasma noradrenaline or serum osmolality or sodium were noted between the control and treated WKY rats, but plasma noradrenaline was significantly higher in the treated SHR when compared to control animals (Table 1).

Body, atrial, ventricular and kidney weights in the control and treated WKY and SHR

Body weight as well as the atrial weight did not differ significantly between the untreated WKY and SHR rats (Table 2). The ventricular weight to body weight ratios and the kidney weight to body weight ratios were significantly higher in the untreated SHR, when compared with the WKY rats. The ventricular weight to body weight ratios were significantly higher in the treated WKY and SHR rats than in the untreated groups, whereas the kidney weight to body weight ratios were significantly lower in the treated rats, ruling out the possibility that changes in cardiac weight were a non-specific effect of minoxidil treatment. No statistically significant differences in body weights or in atrial weights were noted between the treated and control rats (Table 2).

Atrial and ventricular levels of IR-ANP and ANP mRNA in the control and treated WKY and SHR

As shown in Figure 1, total cardiac content of IR-ANP was slightly (31%) higher in untreated SHR than in WKY rats, but no differences were noted in cardiac IR-ANP concentrations between the strains. In the minoxidil treated WKY and SHR rats, cardiac content of IR-ANP was 1.5 and 1.4 fold higher,

Table 1 Haemodynamics, plasma levels of immunoreactive atrial natriuretic peptide (IR-ANP) and noradrenaline, serum osmolality and sodium in conscious control and minoxidil-treated Wistar-Kyoto (WKY) and spontaneously hypertensive (SHR) rats

	WKY		*SHR*	
	Control (n = 6)	*Minoxidil* (n = 5)	*Control* (n = 8)	*Minoxidil* (n = 7)
Mean arterial pressure (mmHg)	120 ± 2	109 ± 2**	186 ± 6***	143 ± 7***
Heart rate (beats min^{-1})	325 ± 15	318 ± 14	361 ± 8*	359 ± 5
Right atrial pressure (mmHg)	0.9 ± 0.3	1.4 ± 0.4	0.9 ± 0.3	2.4 ± 0.4**
Plasma IR-ANP (pg ml^{-1})	133 ± 20	281 ± 34**	184 ± 38	339 ± 61*
Noradrenaline (pg ml^{-1})	299 ± 33	246 ± 12	276 ± 8	432 ± 20**
Osmolality (mosm kg^{-1} H_2O)	281 ± 1	280 ± 2	281 ± 2	284 ± 1
Sodium (mmol l^{-1})	132 ± 1	133 ± 1	132 ± 1	133 ± 1

Results are expressed as means ± s.e.mean. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ control WKY vs control SHR, or treated vs control rats (Student's t test, unpaired).

Table 2 Body, cardiac and kidney weights of control and minoxidil-treated Wistar-Kyoto (WKY) and spontaneously hypertensive (SHR) rats

	WKY		SHR	
	Control ($n = 6$)	*Minoxidil* ($n = 5$)	*Control* ($n = 8$)	*Minoxidil* ($n = 7$)
Body weight (g)	444 ± 4	465 ± 7	461 ± 9	481 ± 10
Atria (mg)	165 ± 29	176 ± 18	183 ± 14	229 ± 17*
Ventricles (mg)	1273 ± 11	1539 ± 31**	1847 ± 41***	2094 ± 37***
Ventricular wt:body wt (mg g^{-1})	2.87 ± 0.02	3.31 ± 0.03***	4.02 ± 0.07***	4.36 ± 0.05**
Kidney (mg)	1376 ± 21	1278 ± 27	1636 ± 47**	1571 ± 23
Kidney wt:body wt (mg g^{-1})	3.10 ± 0.03	2.75 ± 0.02**	3.57 ± 0.15*	3.27 ± 0.06

Atria = total weight of atrial tissue, including weight of both auricles and non-auricular part of the atrium. Kidney wt = mean kidney weight. The results are expressed as means + s.e.mean. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ control WKY vs control SHR, or control vs treated rats (Student's t test, unpaired).

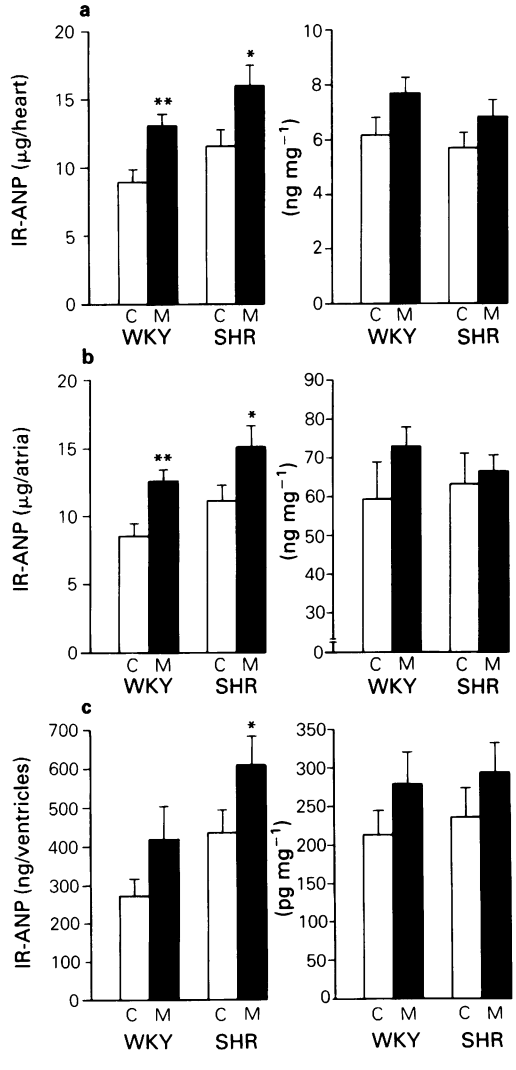


Figure 1 Total (a) cardiac, (b) atrial and (c) ventricular immunoreactive atrial natriuretic peptide (IR-ANP) in control (C) and minoxidil-treated (M) Wistar-Kyoto (WKY) and spontaneously hypertensive (SHR) rats. Control: open columns; minoxidil-treated: solid columns; pg or ng mg^{-1} = IR-ANP pg or ng mg^{-1} wet weight. Results are expressed as means and vertical bars show s.e.mean. *$P < 0.05$ and **$P < 0.01$ control vs minoxidil-treated (Student's t test, unpaired).

respectively, when compared to the untreated controls. Total atrial IR-ANP content (µg per atria) as well as atrial ANP concentration (ng mg^{-1} wet weight) did not significantly differ between the control WKY and SHR rats (Figure 1). In the minoxidil-treated WKY and SHR rats atrial IR-ANP content was significantly higher when compared to the untreated control rats, whereas no statistically significant differences were noted in atrial ANP concentrations.

Ventricular IR-ANP content was about 1.6 fold higher in untreated control SHR (433 ± 64 ng per ventricle) than in WKY rats (273 ± 45 ng per ventricle, $P < 0.05$), whereas no substantial differences were noted in ventricular ANP concentrations (Figure 1). In the minoxidil-treated WKY and SHR rats ventricular IR-ANP contents were 53% and 41% higher, respectively, than in the control groups, the difference being statistically significant in SHR rats. The differences in ventricular IR-ANP concentrations in the control and treated WKY and SHR (30% and 24%, respectively) were not significant (Figure 1). When ventricular IR-ANP contents (ng per ventricle) of all the rats used in this study were compared to their ventricular weight to body weight ratios, a statistically highly significant correlation was found ($r = 0.59$, $P < 0.001$; Figure 2).

RNA blot analysis with a full-length rat ANP cDNA probe identified a single 0.9-kb mRNA species in the ventricles. No apparent differences were noted in the ventricular ANP:18S ratios between the control WKY and SHR rats (0.126 ± 0.036 vs 0.123 ± 0.024 densitometric units, respectively). Minoxidil treated WKY and SHR rats had 1.4 and 2.1 fold higher ventricular ANP:18S ratios, respectively, when compared to the untreated controls (0.181 ± 0.044 densitometric units in the minoxidil-treated WKY and 0.254 ± 0.111 in SHR).

Release of IR-ANP from isolated perfused hearts in control and treated WKY and SHR

Basal perfusion pressure values were 24 ± 3, 25 ± 6, 30 ± 4 and 20 ± 1 mmHg in control WKY, minoxidil-treated WKY, control SHR and minoxidil-treated SHR, respectively. The basal release of IR-ANP before atrialectomy was significantly higher from the perfused hearts of the untreated SHR rats (4.25 ± 0.34 ng min^{-1}, $P < 0.01$, $n = 7$) compared to that from hearts from the untreated WKY rats (2.82 ± 0.19 ng min^{-1}, $n = 5$, Figure 3a). Atrialectomy decreased perfusate IR-ANP concentration significantly in both strains: in WKY rats from 564 ± 38 pg ml^{-1} to 82 ± 9 pg ml^{-1} ($P < 0.01$) and in SHR

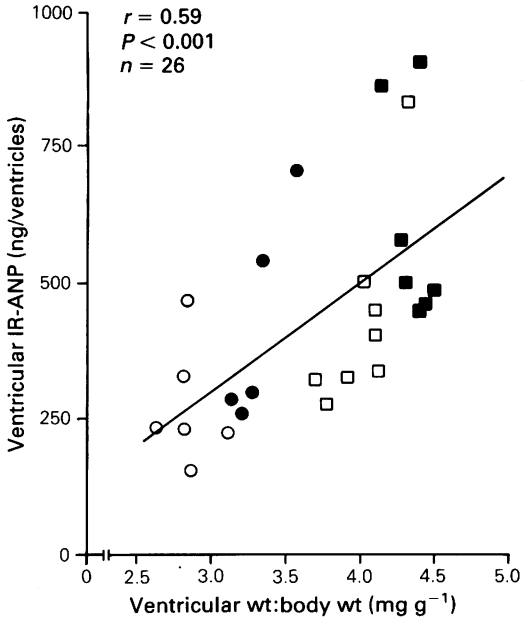


Figure 2 Relationship between ventricular immunoreactive atrial natriuretic peptide (IR-ANP) and ventricular weight to body weight ratios in control and minoxidil-treated Wistar-Kyoto (WKY) and spontaneously hypertensive (SHR) rats. Control WKY (○), minoxidil-treated WKY (●), control SHR (□) and minoxidil-treated SHR (■).

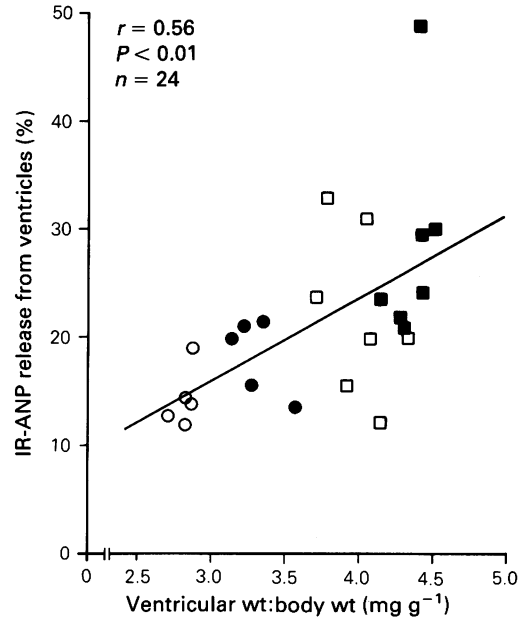


Figure 4 Relationship between the proportion of immunoreactive atrial natriuretic peptide (IR-ANP) released from the ventricles into the perfusion fluid and ventricular weight to body weight ratios in control and minoxidil-treated Wistar-Kyoto (WKY) and spontaneously hypertensive (SHR) rats. Control WKY (○), minoxidil-treated WKY (●), control SHR (□) and minoxidil-treated SHR (■).

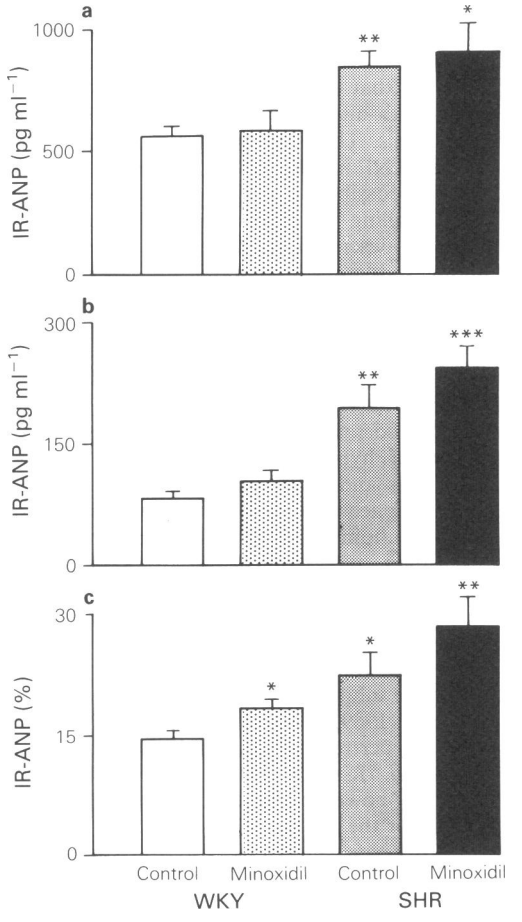


Figure 3 Immunoreactive atrial natriuretic peptide (IR-ANP) secreted into the perfusion fluid (a) before and (b) after atrialectomy, and (c) the proportion (%) of IR-ANP released from the ventricles in control and minoxidil-treated Wistar-Kyoto (WKY) and spontaneously hypertensive (SHR) rats. Results are expressed as means and vertical lines show s.e.mean. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ compared to control WKY (Student's t test, unpaired).

rats from $849 \pm 68\,pg\,ml^{-1}$ to $193 \pm 32\,pg\,ml^{-1}$ ($P < 0.001$). Thus, after atrialectomy control WKY rats secreted 15% ($0.41 \pm 0.04\,ng\,min^{-1}$, $P < 0.05$) and SHR hearts 22% ($0.97 \pm 0.16\,ng\,min^{-1}$) of the amount of IR-ANP secreted into perfusion fluid before atrialectomy (Figure 3).

The release of IR-ANP from the hearts of the minoxidil-treated WKY and SHR did not differ from their control groups. After atrialectomy, the release of IR-ANP from the perfused ventricles of the treated WKY and SHR rats was slightly higher in both strains when compared to their untreated controls. The hearts from the minoxidil-treated WKY and SHR secreted 18% and 28%, respectively, of the amount of IR-ANP secreted into perfusion fluid before atrialectomy (Figure 3). To exclude the possibility that the differences noted were due to contamination of the ventricles by atrial tissue, we measured the IR-ANP concentration in the superior part of the ventricular tissue separately, and found no differences between the control and treated animals (499 ± 162 vs $425 \pm 55\,pg\,mg^{-1}$ wet weight in SHR rats), whereas the IR-ANP concentration of the superior part was higher than that of the inferior part of the ventricles (236 ± 38 and $293 \pm 39\,pg\,mg^{-1}$ wet weight in control and minoxidil-treated SHR rats, respectively).

When compared to the hearts from the untreated control WKY rats, the hearts from both the treated and the untreated SHR rats secreted larger amounts of IR-ANP into the perfusate, both before and after atrialectomy, the difference being highly significant for the minoxidil-treated SHR rats after atrialectomy (Figure 3). When the IR-ANP release from ventricles (i.e. after atrialectomy) of all the rats used in this study was compared to their ventricular weight to body weight ratios, a satistically significant correlation was found ($r = 0.56$, $P < 0.01$; Figure 4).

Discussion

Ventricular hypertrophy is characterized by an increase in tissue mass because of exaggerated protein and RNA synthesis and can be induced by both pressure and volume overload. Ventricular tissue ANP levels and the synthesis of ANP in the

ventricles increase in several experimentally and pathologically induced models of ventricular hypertrophy (for references see Introduction). The present experiments *in vitro* with a rat isolated heart preparation indicate that the ventricular source contributes significantly to the circulating level of ANP and the amount released depends on the degree of ventricular hypertrophy. The results agree with the *in vivo* experiments (Ruskoaho *et al.*, 1989a), in which exercise caused release of ANP from the hypertrophied left ventricle.

To evaluate the relation between cardiac hypertrophy, ventricular ANP levels and ANP release, we treated WKY and SHR rats with minoxidil for 8 weeks. Ventricular ANP content of the untreated SHR was 1.6 fold higher than in the WKY rats. In contrast, the IR-ANP levels in the atria were not affected. The participation of the ventricle in ANP expression in SHR has also been described previously (Arai *et al.*, 1988). As expected, minoxidil treatment resulted in a decrease in mean arterial pressure and an increase in ventricular weight to body weight ratio, the most commonly used index of the degree of ventricular hypertrophy. The increased ventricular mass was associated with the augmentation of ventricular synthesis and storage of ANP, and there was a highly significant correlation between the ventricular weight to body weight ratios and ventricular ANP content. These results confirm the relationship between myocardial hypertrophy and ventricular ANP. However, these results differ from our earlier results (Ruskoaho & Leppäluoto, 1988). We found that the IR-ANP concentration of the ventricles of 12 month-old WKY rats was higher than that of the age-matched SHR rats. The reason for the discrepancy is not clear, but variations in the ventricular IR-ANP concentrations in the WKY strains and the biventricular hypertrophy occasionally observed in WKY rats (Lee *et al.*, 1988) are some possibilities. Consistent with this hypothesis is that the ventricular IR-ANP concentrations of the SHR rats measured in this study agree with our previous results (Ruskoaho & Leppäluoto, 1988). Treatment with minoxidil produced a 1.6–2.1 fold increase in ventricular ANP mRNA, and plasma IR-ANP levels were significantly elevated when compared to the untreated controls, as shown earlier by Stasch *et al.* (1987a). Hydralazine, another vasodilator, has been shown to have no effect on plasma IR-ANP levels (Oda *et al.*, 1988), and even to decrease ANP mRNA levels (Fukui *et al.*, 1989), but, on the other hand, it did not significantly change cardiac weight either.

The stimulus for ANP expression in the ventricle is not known. One hypothesis is that ventricular ANP expression may represent a regression of the ventricular myocyte to an earlier developmental form with reexpression of the foetal phenotype, as described for foetal forms of actin and myosin (Wei *et al.*, 1987; Zeller *et al.*, 1987). Alternatively, myocyte stretch, resulting from an increased after- or preload on the ventricle may represent the underlying stimulus for ANP expression in the ventricle. In the present studies, despite decreased blood pressure and afterload, minoxidil treatment aggravated ventricular storage and synthesis of ANP and hypertrophy, probably due to the increase in preload as reflected by an increase in right atrial pressure in SHR rats. Previous studies have demonstrated that sustained elevations in arterial pressure and volume overload are associated with enhanced left or right ventricular ANP production (Lattion *et al.*, 1986; Gardner *et al.*, 1986b; Ding *et al.*, 1987; Day *et al.*, 1987; Arai *et al.*, 1988; Stockmann *et al.*, 1988; Ruskoaho & Leppäluoto, 1988; Michel *et al.*, 1988). The observations that the endocardial half of the ventricle contained more ANP than the epicardial half (Edwards *et al.*, 1988; Ruskoaho *et al.*, 1989a) also supports the concept of stretch-induced reinduction, because wall tension in the endocardial half is greater than in the epicardial half (Hoffman, 1987). Thus, stretch may not only enhance the secretion of ANP (Lang *et al.*, 1985; Ruskoaho *et al.*, 1987), but may also serve as a signal for the induction of the ANP gene. Hypertrophy itself may also contribute to augmented expression of the ANP gene in the left ventricle. However, the increased left ventricular levels of ANP in Brattleboro rats, despite normal left ventricular weight to body weight ratio, suggest that increased ANP gene expression may occur in the ventricles independent of hypertrophy (Ruskoaho *et al.*, 1989b).

To examine the amount of ANP secreted from the heart, *in vitro*, we measured the perfusate IR-ANP concentration both before and after atrialectomy. This method allows the elimination of interfering factors that complicate *in vivo* studies by excluding other sources of ANP. To rule out the possibility that the IR-ANP released into the perfusate after atrialectomy would have been partly derived from contaminating atrial tissue, the ventricles were cut into superior and inferior parts and the IR-ANP concentration of the superior part was measured separately. The IR-ANP concentration of the superior part of the ventricles was higher than that of the inferior part and may, to some extent, increase the proportion of ventricular ANP release in this model. However, this finding does not explain the differences noted in IR-ANP release between the groups, since no apparent differences were noted between the control and treated animals, thus confirming the validity of our results.

When studied *in vitro* by this method, the hypertrophic ventricular tissue after atrialectomy secreted more ANP into the perfusate than ventricles of the control hearts. Further, there was a significant correlation between ventricular weight to body weight ratio and IR-ANP released from the ventricles into the perfusion fluid. Thus, these studies show that the ventricles contribute significantly to the circulating level of ANP, and the amount released, both *in vivo* (Ruskoaho *et al.*, 1989a) and *in vitro*, clearly depends on the degree of ventricular hypertrophy. However, in an isolated Langendorff perfused heart there is no load on the atria and because of this feature of our *in vitro* model it may overestimate the importance of ventricular ANP release *in vivo*. Recently, Yasue *et al.* (1989) obtained evidence that the secretion of ANP from the ventricles in patients with dilated cardiomyopathy augments circulating plasma levels. Thus, the cardiac ventricles synthesize, store and release ANP in man as well as in rats.

Although it is possible to restore left ventricular mass to normal by controlling hypertension with pharmacotherapy, several commonly described antihypertensive drugs, including diuretics and vasodilators, are unable to reverse ventricular hypertrophy (Tarazi, 1983; Ruskoaho, 1984; Messerli *et al.*, 1988). Other agents, including angiotensin converting enzyme inhibitors, calcium antagonists and most β-blockers, are capable of reversing hypertrophy (Tarazi, 1983; Ruskoaho, 1984; Messerli *et al.*, 1988). As the extent of synthesis and secretion of ANP correlates well with the degree of hypertrophy, regression of hypertrophy by treatment will lead to decreased synthesis and lower plasma levels. Experimentally, there is some evidence to support this concept. The calcium antagonists, nitrendipine and nisoldipine reduce the spontaneously developing hypertrophy and decrease the elevated plasma IR-ANP levels (Stasch *et al.*, 1986; 1987a, b). Others have demonstrated a decrease both in relative ventricular weight and in plasma IR-ANP after chronic treatment with a calcium antagonist (Kohno *et al.*, 1988) or angiotensin I converting-enzyme inhibitors (Kohno *et al.*, 1987; Oda *et al.*, 1988; Fukui *et al.*, 1989). Minoxidil treatment, in turn, aggravated cardiac hypertrophy and produced an additional increase in plasma IR-ANP (Stasch *et al.*, 1987a). None of these groups has, however, measured either the ventricular IR-ANP content or the release of IR-ANP from the ventricles. In this study we have shown that the changes in plasma IR-ANP observed by these groups could also reflect changes in ventricular IR-ANP content caused by changes in the degree of ventricular hypertrophy.

In conclusion, minoxidil can cause ventricular hypertrophy with an exaggerated ventricular synthesis and storage of ANP. When studied *in vitro*, a significant correlation was found between ventricular weight and ANP release from ventricles, indicating that the amount of the peptide released from the heart depends on the degree of ventricular hypertrophy. The

results show that the ventricle, in addition to cardiac hypertrophy and the dependence of tension on myofibre length, possesses a further compensatory mechanism, an increase in ANP secretion.

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Subclassification of β-adrenoceptors responsible for steroidogenesis in primary cultures of bovine adrenocortical zona fasciculata/reticularis cells

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1 Forty eight hour primary cultures of purified bovine adrenocortical zona fasciculata/reticularis cells secreted hydrocortisone in response to stimulation with β-adrenoceptor agonists. The observed order of potency was isoprenaline > noradrenaline > dobutamine > salbutamol > BRL37344.

2 Salbutamol acted as a partial agonist on these cells hence suggesting the presence of a β_1-adrenoceptor.

3 Schild analysis of the hydrocortisone response to isoprenaline showed that the selective β_1-antagonist practolol and the selective β_2-antagonist ICI118,551 gave pA_2 values of 6.85 and 7.17, respectively. These values were in close agreement with corresponding pA_2 values previously obtained for the β_1-adrenoceptor.

4 We conclude that β_1-adrenoceptors are responsible for mediating catecholamine-stimulated hydrocortisone secretion from primary cultures of bovine zona fasciculata/reticularis cells.

Introduction

Adrenocorticotrophic hormone (ACTH) and, in some species, angiotensin II are known regulators of steroidogenesis in the adrenal cortex (Gill *et al.*, 1977; Tait *et al.*, 1980). However, there is also evidence for innervation of the adrenal cortex in man (Mikhail & Amin, 1969), the rat (Kleitman & Holzwarth, 1985) and the mouse (Migally, 1979), and this has been suggested as another means of controlling adrenocortical steroidogenesis and possibly adrenal cell growth.

We have previously shown that primary cultures of bovine zona fasciculata/reticularis (ZFR) cells produce hydrocortisone in response to stimulation with catecholamines, and that this adrenergic stimulation of steroidogenesis is mediated by β-adrenoceptors (Walker *et al.*, 1988). The subclass of β-adrenoceptors which is involved in this response—either β_1 or β_2 as classified by Furchgott (1972), or the more recently characterised β_3 subclass (Arch *et al.*, 1984; Kaumann, 1989)—is unknown.

It is not yet clear in bovine or other species whether adrenergic control of hydrocortisone secretion at the level of the adrenal cortex occurs *in vivo* and, if so, whether this depends on adrenergic innervation, on the effects of circulating catecholamines, or even on catecholamines locally derived from the adrenal medulla. Ungar (1979) suggested that, in general, β_1-adrenoceptors tend to be innervated by adrenergic neurones, whereas β_2-adrenoceptors respond mainly to blood-borne catecholamines. On this basis, determination of the subclass of β-adrenoceptor should provide valuable evidence as to whether adrenergic control of the adrenal cortex is by direct innervation or via circulating catecholamines.

Traditional receptor classification is based on determination of antagonist pA_2 values (Schild, 1947; Kenakin, 1982), and comparison of a range of selective agonists (Lands *et al.*, 1967). Subclassification of β-adrenoceptors has been successfully demonstrated with the selective β_1-antagonist practolol (Dunlop & Shanks, 1968) and the selective β_2-antagonist ICI118,551 (Bilski *et al.*, 1983). These antagonists have been used in this present study. The effects of the selective β-agonists noradrenaline (β_1), salbutamol (β_2), dobutamine (β_1), isoprenaline (β_1/β_2), and BRL37344 (β_3) were also compared.

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Methods

Cell culture and stimulation

Bovine adrenal ZFR cells were prepared as described by Walker *et al.* (1988) using collgenase digestion, and purified by the column-filtration method developed by McDougall *et al.* (1979). This procedure gives a preparation essentially free from zona glomerulosa cell and medullary cell contamination (Williams *et al.*, 1989). The ZFR cells were dispersed into 12-well culture dishes (1.5 cm wells) at 250,000 cells per well in Ham's F10 medium containing 10% (v/v) CPSR5, penicillin ($50\,iu\,ml^{-1}$), streptomycin ($50\,\mu g\,ml^{-1}$) and amphotericin B ($2.5\,\mu g\,ml^{-1}$), and cultured at 37°C under 5% CO_2. After 24 h (day 2), the medium was replaced with 1 ml of identical fresh medium.

Experiments were carried out 48 h after initial plating (day 3). Medium was removed and cells were washed twice with 1 ml of Earl's balanced salt (EBS) solution containing 0.2% bovine serum albumin (BSA) and 0.1% added glucose (EBS/BSA/glucose). Agonists and antagonists were made up in EBS/BSA/glucose and added to the cells, giving a final volume of 1 ml per well. Antagonists were added 1 min before the addition of any agonists. Stimulation was carried out for 1 h under the same incubation conditions as those used to culture the cells and, at the end of this period, the medium overlying the cells was removed and stored at −20°C before assay for hydrocortisone.

Hydrocortisone was measured by radioimmunoassay as described by Gray *et al.* (1983). The inter-assay CV was 10% or less over the working range of the assay.

Statistical analysis

Experiments were carried out on cells from at least 3 separate cell preparations for determination of agonist potencies and for Schild analysis of each antagonist. Within each experiment, triplicate determinations were carried out for each combination of agonist and antagonist.

For the estimation of antagonist pA_2 values, dose-response curves were tested for parallelism by analysis of covariance

using the SPSS-X statistical package produced by Edinburgh University Computing Service. Schild regression lines were fitted with a least-squares fit by a Casio fx-180P programmable calculator and confidence limits calculated.

Materials

The source of all cell culture and radioimmunoassay materials is described in Walker *et al.* (1988). The controlled process serum replacement No. 5 (CPSR5) was obtained from Sigma Chemical Company, Poole, Dorset, U.K.

Noradrenaline was obtained from Winthrop, Guildford, Surrey, U.K.; salbutamol from Allen & Hanburys Ltd., London, U.K.; dobutamine from Eli Lilly & Co. Ltd., Basingstoke, U.K. and isoprenaline from Macarthy Medical, Romford, U.K. BRL37344 ((**R*R***)-(±)-4-[2-[2-hydroxy-2-(3-chlorophenyl)-ethylamino]propyl]phenoxyacetic acid. Na salt) was a generous gift from Beecham Pharmaceuticals, Epsom, Surrey, U.K. Practolol was obtained from ICI plc, Macclesfield, Cheshire, U.K. and ICI 118,551 (erythro-(±)-1-(7-methylindan-4-yloxy)-3-isopropylaminobutan-2-ol) was a generous gift from the same company.

Results

The effects of various selective β-agonists on hydrocortisone secretion

The effects of increasing concentrations of isoprenaline, noradrenaline, salbutamol, dobutamine and BRL37344 were tested on cells on day 3 of culture (Figure 1). The agonists had relative potencies as follows: isoprenaline > noradrenaline > dobutamine > salbutamol > BRL37344. Isoprenaline, noradrenaline and dobutamine all produced the same maximum response. Salbutamol and BRL37344 gave approximately 70% and 19% of the maximum response, respectively.

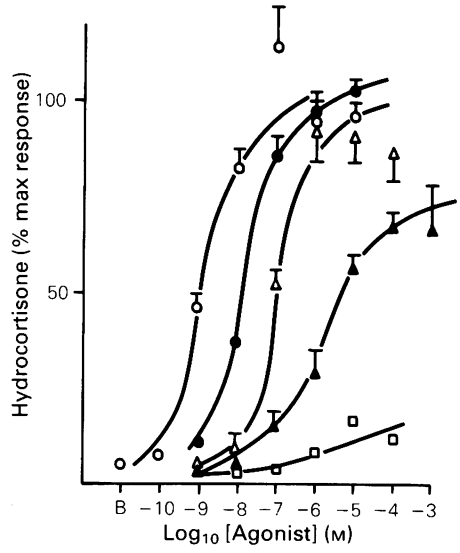


Figure 1 Dose-response curves for the secretion of hydrocortisone produced upon stimulation of purified bovine adrenocortical zona fasciculata/reticularis cells with isoprenaline (○), noradrenaline (●), dobutamine (△), salbutamol (▲) and BRL37344A (□) B = basal cortisol production.

The effects of the antagonists practolol and ICI118,551 on the isoprenaline dose-response curve

A series of dose-response curves for the effect of isoprenaline on hydrocortisone secretion were set up in the presence of increasing doses of either practolol or ICI118,551. Representative experiments for practolol and ICI118,551 are shown in Figures 2 and 3, respectively. The dose-response lines for each concentration of antagonist were judged to be parallel by analysis of covariance. Experiments were repeated on cells from 4 separate cell preparations for practolol and 3 separate cell preparations for ICI118,551.

The dose-ratio (DR) for each concentration of antagonist was obtained, Schild plots of $\log_{10}$ (DR − 1) versus antagonist

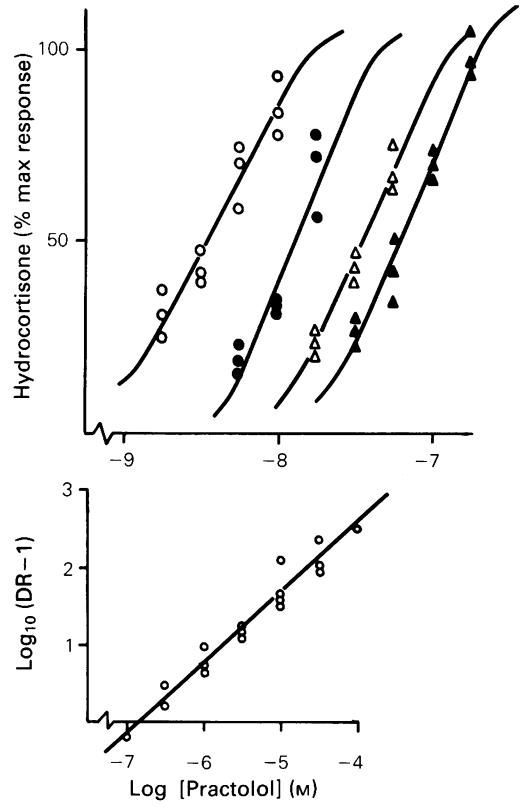


Figure 2 Representative experiment showing dose-response curves for the secretion of hydrocortisone produced on stimulation with isoprenaline, alone (○) and in the presence of increasing concentrations of practolol $10^{-6.5}$ M (●), 10^{-6} M (△), $10^{-5.5}$ M (▲). Inset: Schild regression—least squares fit of $\log_{10}$ (DR − 1) versus $\log_{10}$ [practolol] where DR = dose-ratio. Cumulative data from 4 separate cell preparations.

Table 1 Comparison of experimental and published data for practolol and ICI118,551

Antagonist	*Experimental* pA_2	*95% CL*	*Slope*	*95% CL*	*Published* pA_2
		6.67		0.84	
Practolol	6.85		0.90		6.80[1] (β_1)
		7.06		0.96	
		7.03		1.01	
ICI118,551	7.14		0.99		7.17[2] (β_1)
		7.28		0.97	9.26[2] (β_2)

95% CL = 95% confidence limits. Original references are [1]Kenakin & Black (1978), [2]Bilski *et al.* (1983).

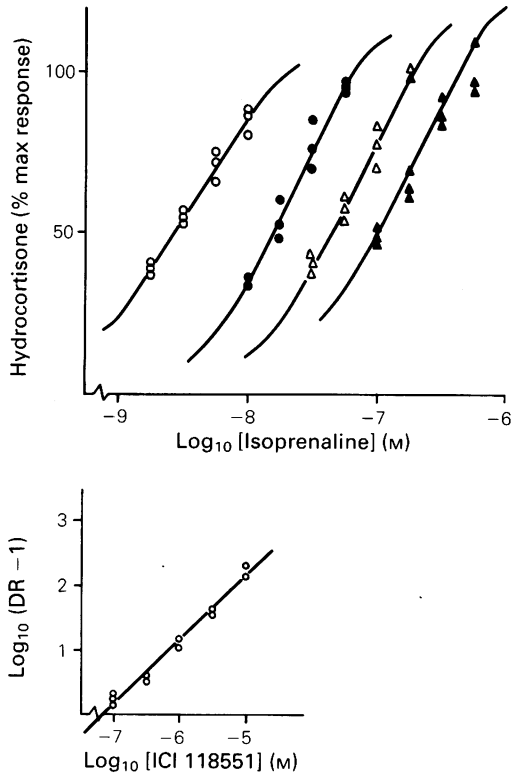


Figure 3 Representative experiment showing dose-response curves for the secretion of hydrocortisone produced on stimulation with isoprenaline, alonr (○) and in the presence of increasing concentrations of ICI118,551 $10^{-6.5}$M (●), 10^{-6}M (△) $10^{-5.5}$M (▲). Inset: Schild regression—least squares fit of $\log_{10}$(DR − 1) versus $\log_{10}$[ICI118,551] where DR = dose-ratio. Cumulative data from 3 separate cell preparations.

concentration plotted (lower sections in Figures 2 and 3) and used to calculate pA_2 values for each antagonist and to obtain 95% confidence limits. The slope of the regression line and 95% confidence limits were estimated similarly. Results for both antagonists are shown in Table 1.

Neither practolol nor ICI118,551 caused hydrocortisone secretion and therefore had no intrinsic agonist effects on the cells.

Discussion

The results establish the existence of β_1-adrenoceptors on bovine cultured adrenal ZFR cells, for two reasons, as discussed below.

Firstly, the effects of several selective β-adrenoceptor agonists on hydrocortisone secretion were consistent with this classification. Isoprenaline, noradrenaline and dobutamine were all full agonists, whereas salbutamol acted as a partial agonist, producing 70% of the maximum response. Salbutamol is known to act as a full agonist at β_2-adrenoceptors, but only as a partial agonist at β_1-adrenoceptors (Farmer *et al.*, 1970). This, in itself, suggests that the adrenergic stimulation of hydrocortisone secretion is mediated by β_1-adrenoceptors. Although BRL37344 produced stimulation of the cells at 10^{-5}M, it was the least potent of all the agonists studied and only produced 19% of the maximum response seen with isoprenaline. Previous studies have shown that, in systems thought to contain β_3-adrenoceptors, BRL37344 was a more potent agonist than isoprenaline (Arch *et al.*, 1984; Bond & Clarke, 1988). Hence, it is likely that BRL37344 is producing a non-specific stimulation, and that β_3-adrenoceptors are not present on bovine cultured adrenal ZFR cells.

Secondly, determination of the pA_2 values for the β_1-antagonist practolol and the β_2-antagonist ICI118,551 provided definitive evidence for the presence of β_1-receptors (Table 1). The pA_2 value for practolol of 6.85 (6.67–7.06) agrees well with published values (Table 1). The gradient of the Schild regression was significantly less than 1, slope = 0.90 (0.84–0.96), suggesting deviation from an ideal competitive antagonist. Practolol and ICI118,551 showed no partial agonist activity (results not shown), even though practolol is known to be a partial agonist in other systems (Kenakin & Black, 1978). It is possible that isoprenaline potentiated the partial agonist properties of practolol, leading to production of more hydrocortisone than expected and hence giving a Schild regression < 1.

The pA_2 value for the action of ICI118,551 at β_2-receptors has been found to be 9.26, and at β_1-receptors 7.17 (Bilski *et al.*, 1983). Hence, our experimental value of 7.14 (7.03–7.28) agrees well with the value for β_1-receptors. In this case, the gradient of the regression line of 0.99 (0.97–1.01) implies that ICI118,551 is acting as a pure competitive antagonist.

Although the occurrence of adrenergic control of steroidogenesis at the level of the adrenal cortex still remains to be established *in vivo*, an increasing number of observations suggest this possibility. Adrenergic innervation of the adrenal cortex has been demonstrated in man (Mikhail & Amin, 1969), the rat (Kleitman & Holzwarth, 1985) and the mouse (Migally, 1979). No similar studies on bovine adrenal cortex have been published.

Shima *et al.* (1984) have shown, using binding of [^{3}H]-dihydro-alprenolol, that membranes prepared from both the capsulated (zona glomerulosa, ZG) and decapsulated (ZFR) regions of the rat adrenal cortex contain β_1-adrenoceptors, and that only the latter are coupled to adenylate cyclase *in vitro*.

Previous work on primary cultures of adrenocortical cells has shown a steroidogenic response to catecholamines in rat ZG cells (DeLean *et al.*, 1984), in a bovine mixed adrenocortical preparation (Kawamura *et al.*, 1984), and in bovine purified ZFR cells (Walker *et al.*, 1988).

In conclusion, we have shown that β_1-adrenoceptors are responsible for mediating catecholamine-stimulated hydrocortisone secretion from primary cultures of bovine adrenal ZFR cells. Ungar (1979) suggested that β_1-adrenoceptors tend to be associated with adrenergic nerve endings so that the occurrence *in vivo* of catecholamine-stimulated steroidogenesis is probably mediated by direct adrenergic innervation of adrenocortical cells.

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Hypotensive effects of 8-hydroxy-2-(di-n-propylamino)tetralin and 5-methylurapidil following stereotaxic microinjection into the ventral medulla of the rat

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1 The effects of the 5-HT_{1a} receptor agonists 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT) and 5-methylurapidil (5-MU) on blood pressure and heart rate were investigated in the anaesthetized normotensive rat after local injection into the ventral medulla (medial part of the area of the B1/B3 cell group).

2 Both 8-OH-DPAT and 5-MU, decreased arterial blood pressure and heart rate. Local injection of the α_1-adrenoceptor antagonist prazosin did not affect cardiovascular parameters.

3 Subcutaneous injection of the 5-HT_{1a} receptor antagonists spiroxatrine or spiperone inhibited the cardiovascular response to 8-OH-DPAT and 5-MU.

4 Histological examination showed the injection sites which were associated with cardiovascular responses to the agonists to be contained within the medial part of the area of the B1/B3 cell group, corresponding to the region of the nucleus raphe magnus and pallidus. Injections of agonists into adjacent areas had no effect on blood pressure or heart rate.

5 The results support the hypothesis that activation of somatodendritic 5-HT_{1a} autoreceptors located on 5-hydroxytryptaminergic neurones in the ventral medulla of the rat results in a decrease in blood pressure and heart rate. The region of the B1/B3 cell group is suggested as a possible site for the hypotensive action of 5-HT_{1a} receptor agonists.

Introduction

It has been shown in several species that certain groups of 5-hydroxytryptaminergic neurones in the ventral medulla (Dahlström & Fuxe, 1964) project to the intermediolateral cell column of the thoracolumbar spinal cord (e.g. cat, Amendt *et al.*, 1979; rat, Loewy & McKellar, 1981), and it has been suggested that these neurones are involved in vasomotor control (for review see Coote, 1988). Of particular interest is the hypothesis that the 5-hydroxytryptaminergic neurones in the medulla exert an excitatory action on the sympathetic neurones in the spinal cord. For instance, Pilowsky *et al.* (1986) have demonstrated increased release of 5-hydroxytryptamine (5-HT) in the thoracic spinal cord of the rat associated with a pressor response following injection of an excitatory amino acid into the region of the B3 cell group. The authors suggested that this group of spinally projecting neurones had a sympatho-excitatory action.

Thus, inhibition of the medullary 5-hydroxytryptaminergic neurones would be expected to decrease sympathetic outflow and, in consequence, arterial blood pressure. One possible mechanism of inhibiting these neurones is by activation of their somatodendritic autoreceptors, which are described as belonging to the 5-HT_{1a} subtype (Verge *et al.*, 1985; Hjorth & Magnusson, 1988). McCall *et al.* (1987) have shown in the cat that peripheral administration of 5-HT_{1a} receptor agonists leads to a dose-related inhibition of sympathetic nerve discharge, hypotension and bradycardia. Decreases in blood pressure and heart rate have also been described in the rat following intracerebroventricular or intracisternal administration of 5-HT_{1a} receptor agonists (Mir & Fozard, 1988).

We present an investigation in which the potent and selective 5-HT_{1a} receptor agonist, 8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT; Middlemiss & Fozard, 1983), was injected directly into the medial part of the B1/B3 cell group of the ventral medulla of normotensive rats under concomitant monitoring of arterial blood pressure and heart rate. In addition to 8-OH-DPAT, 5-methylurapidil (5-MU), a structural congener of the antihypertensive drug urapidil, with high affinity for 5-HT_{1a} binding sites, was used as 5-HT_{1a} receptor agonist (Groß *et al.*, 1987).

Methods

Male normotensive Sprague-Dawley rats weighing 300–330 g were anaesthetized with pentobarbitone sodium (60 mg kg^{-1}, i.p.) and a polyethylene catheter was inserted into the left carotid artery. One to two days later the animals were anaesthetized again and placed in a David Kopf stereotaxic frame. Blood pressure was measured by a Miniature Pressure Transducer (Model CP-O2, Hugo Sachs Elektronik, BRD) and recorded on a Watanabe Polygraph (Graphtec Linearcorder mark VII WR 3101). Heart rate was triggered beat by beat from the blood pressure signal. A 25-gauge needle attached to a 0.5 µl Hamilton syringe was inserted through a craniotomy hole into the ventromedial medulla according to the Paxinos & Watson (1982) stereotaxic coordinates, using lambda as reference (modification: incisor bar at horizontal zero level): 2.2 caudal, 0.0 lateral, 10.1 ventral.

The insertion of the needle consistently caused a sharp, short-lasting drop in blood pressure followed by a period of stabilization in which, however, the value before insertion was not quite reached (see Figure 1). Drugs were injected only when this stabilization had taken place (usually 4–12 min). The substances or vehicle (solvent of 5-MU) were injected in a volume of 0.1 µl over a period of 30 s. Any one animal received only one dose of agonist.

Blood pressure and heart rate were recorded before and 15–30 min after the injections. Maximal effects of drugs were expressed as percentage decrease or increase in resting values of mean arterial blood pressure (MAP; mmHg) and heart rate (HR; beats min^{-1}). Resting values are defined as the values obtained at the end of the stabilization period immediately before the injection of drugs.

In some experiments spiroxatrine (SPX) or spiperone (SPP) were used to antagonize the effects of 8-OH-DPAT or 5-MU. SPX or SPP were injected at a dosage of 1 mg kg^{-1} s.c. or

0.25 mg kg^{-1} i.p., respectively, 60 min before stereotaxic injection of the agonists.

For histological verification of injection sites the brain stem was dissected and soaked overnight in 4% formaldehyde in 0.1 M phosphate buffer. Serial transverse sections of 5 μm thickness were cut and stained with cresyl violet.

Drugs used were: (±)-8-hydroxy-2-(di-n-propylamino)tetralin HBr (8-OH-DPAT; Research Biochemical, U.S.A.), 5-methylurapidil (5-MU; gift: Byk-Gulden, F.R.G.), prazosin HCl (gift: Pfizer, A), spiroxatrine and spiperone (SPX, SPP; Janssen, B), pentobarbitone sodium (Fluka, CH). 8-OH-DPAT and pentobarbitone sodium were dissolved in 0.9% saline, 5-MU and prazosin in 0.1 N HCl. All solutions were adjusted to pH 7.1–7.4 with 0.5 M phosphate buffer except spiroxatrine and spiperone. Spiroxatrine was dissolved in 0.1 N HCl, dimethylsulphoxide and distilled water, adjusted with phosphate buffer to a pH of 5.5. Spiperone was dissolved in 0.4 M lactic acid, adjusted with 1 M $NaHCO_3$ to a pH of 4.0.

Statistics

All values are given as means ± s.e.mean. Differences were analyzed for statistical significance by Student's *t* test for paired data.

Results

Microinjection of 8-OH-DPAT, 5-methylurapidil and prazosin into the B1/B3 region

In pentobarbitone-anaesthetized normotensive rats the resting mean arterial blood pressure was 105 ± 3 mmHg, and basal heart rate was 314 ± 10 beats min^{-1} (n = 32). Stereotaxic microinjection of the 5-HT$_{1a}$ receptor agonists 8-OH-DPAT and 5-MU into the ventral medulla caused significant decreases in blood pressure and heart rate. The results are summarized in Table 1. The maximal effects of the drugs generally occurred 4–16 min after injection and lasted for 20–25 min. Representative experiments illustrating the effects of 8-OH-DPAT (0.1 μg in 0.1 μl) and 5-MU (1 μg in 0.1 μl) are depicted in Figure 1.

Since 5-MU has been shown to act as an agonist at 5-HT$_{1a}$ receptors, but also as an antagonist at α_1-adrenoceptors (Groß *et al.*, 1987) the selective α_1-adrenoceptor antagonist prazosin was included in the present experiments for comparison. The stereotaxic injection of prazosin at a dosage of 0.133 μg in 0.1 μl had no significant effect on blood pressure (−0.07 ± 1.2%) or heart rate (−2.9 ± 1.9%, n = 3).

Antagonism of spiroxatrine or spiperone to the effects of 8-OH-DPAT and 5-methylurapidil

Pentobarbitone-anaesthetized normotensive rats were either pretreated with saline (1 ml kg^{-1}, s.c.) or with the 5-HT$_{1a}$ receptor antagonists SPX (1 mg kg^{-1}, s.c.) or SPP (0.25 mg kg^{-1} i.p.) 60 min before stereotaxic microinjection of 8-OH-DPAT or 5-MU.

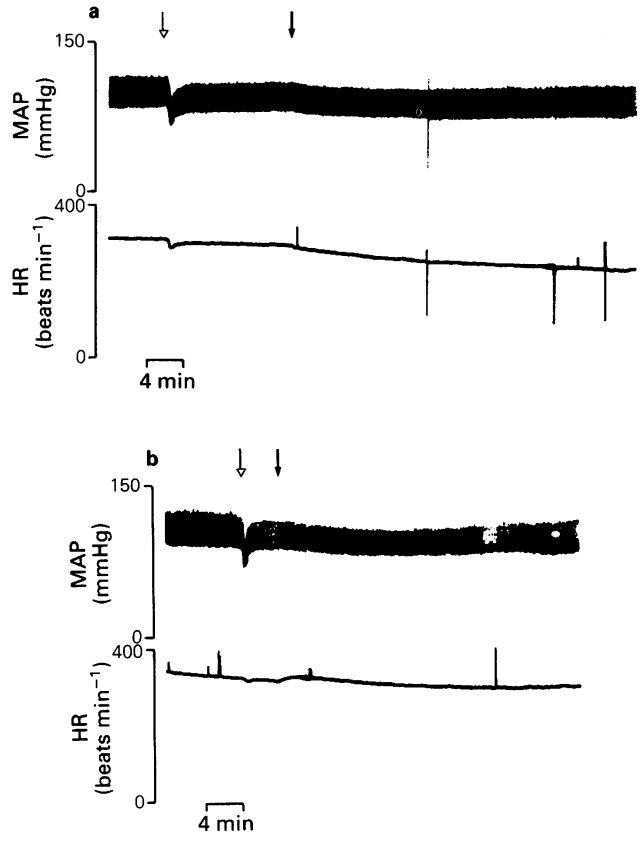


Figure 1 Experiments illustrating the effect of a stereotaxic injection of (a) 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT, 0.1 μg in 0.1 μl) and (b) 5-methylurapidil (5-MU, 1.0 μg in 0.1 μl) on cardiovascular parameters of pentobarbitone-anaesthetized normotensive rats. Mean arterial blood pressure (MAP) upper recording; heart rate (HR) lower recording; open arrow: insertion of needle; closed arrow: injection of drug.

There was no statistically significant difference in resting blood pressure (MAP) or basal heart rate (HR) between saline- and SPX-treated animals (saline: MAP 107 ± 4 mmHg, HR 320 ± 15 beats min^{-1}, n = 14; SPX: MAP 104 ± 6 mmHg, HR 334 ± 24 beats min^{-1}, n = 12). SPX markedly reduced the cardiovascular response following the intramedullary injection of 0.2 μg 8-OH-DPAT, and virtually abolished the response to 0.1 μg 8-OH-DPAT (Figure 2) or to 2 μg 5-MU (Figure 3).

In contrast to SPX, SPP induced a significant fall in mean arterial blood pressure and heart rate itself. Sixty min after the injection, mean arterial pressure was 81.0 ± 4 mmHg (n = 4; $P < 0.01$ vs saline), and heart rate 226 ± 5 beats min^{-1} (n = 4; $P < 0.05$ vs saline). The response following the intra-

Table 1 Hypotensive and cardio-inhibitory responses to 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT) or 5-methylurapidil (5-MU)

Drug	*Dose* (μg in 0.1 μl)	*% maximum change induced by drug* MAP	HR	n
8-OH-DPAT	0.1	−8.4 ± 1.6**	−6.5 ± 1.1**	6
8-OH-DPAT	0.2	−26.1 ± 5.9*	−24.2 ± 4.9**	4
5-MU	1.0	−8.1 ± 0.86***	−12.7 ± 2.7**	5
5-MU	2.0	−11.5 ± 1.04***	−10.9 ± 1.8**	7
Vehicle	—	+0.6 ± 2.8	−0.8 ± 2.2	10

Mean arterial blood pressure (MAP) and heart rate (HR) were measured following stereotaxic microinjection of drugs or vehicle into the area of the B1/B3 cell group of the ventral medulla of pentobarbitone-anaesthetized normotensive rats. Coordinates are given in Methods. MAP and HR before the injection of drugs (resting values; means ± s.e.mean) were 105 ± 3 mmHg and 314 ± 10 beats min^{-1} (n = 32), respectively. Results are expressed as percentages decrease or increase of resting values (means ± s.e.mean).
* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ vs resting value (see Methods).

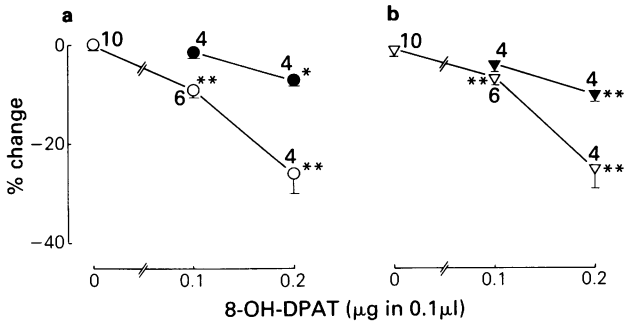


Figure 2 Cardiovascular effects of 8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT) in pentobarbitone-anaesthetized normotensive rats 60 min after the subcutaneous injection of saline or the 5-HT_{1a} receptor antagonist spiroxatrine (SPX; 1 mg kg^{-1}). 8-OH-DPAT (0.1 or 0.2 μg in 0.1 μl) was injected stereotaxically into the area of the B1/B3 cell group of the ventral medulla (coordinates see Methods). Changes in (a) mean arterial blood pressure (MAP) and (b) heart rate (HR) are expressed as percentage decrease of resting values (see legend to Table 1). Symbols represent means (with s.e.mean shown by vertical bar) of n observations.
(○): Saline-treated; (●): SPX-treated.
* $P < 0.05$; ** $P < 0.01$ vs resting values (see Methods).

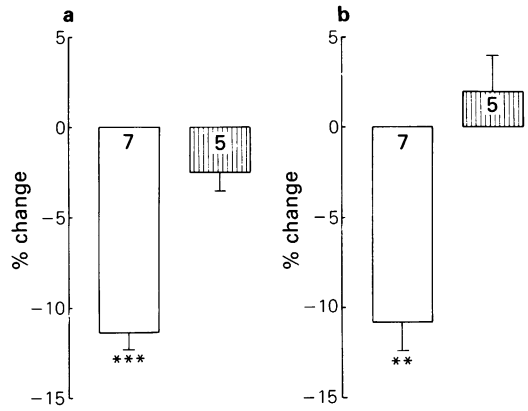


Figure 3 Cardiovascular effects of 5-methylurapidil (5-MU) in pentobarbitone-anaesthetized normotensive rats 60 min after the subcutaneous injection of saline (open columns) or the 5-HT_{1a} receptor antagonist spiroxatrine, hatched columns, (SPX; 1 mg kg^{-1}). 5-MU (2.0 μg in 0.1 μl) was injected stereotaxically into the area of the B1/B3 cell group of the ventral medulla (coordinates see Methods). Changes in (a) mean arterial blood pressure (MAP) and (b) heart rate (HR) are expressed as percentage decrease or increase of resting values (see legend to Table 1). Means with s.e.mean of n observations are shown.
** $P < 0.01$; *** $P < 0.001$ vs resting values (see Methods).

medullary injection of 0.2 μg 8-OH-DPAT was attenuated by SPP (MAP: −6.4%, HR: −12%, $n = 4$; $P < 0.05$ vs resting value immediately before the injection of 8-OH-DPAT). Higher doses of SPP, 0.5 and 1.0 mg kg^{-1}, i.p., caused such a marked decrease in blood pressure and heart rate that intramedullary injection of 8-OH-DPAT was not attempted.

Histological location of injection sites

Figure 4 shows the microinjection sites for 8-OH-DPAT 0.1 μg, 5-MU 2 μg, prazosin, and vehicle. All sites where the injection of 8-OH-DPAT or 5-MU resulted in a decrease of blood pressure and heart rate were restricted to the medial part of the area of the B1/B3 cell group, corresponding to the region of the nucleus raphe magnus and pallidus. The sites for prazosin and vehicle, which did not exert any effect, were located in the same area. Injections of 8-OH-DPAT and 5-MU into sites dorso/rostral (1.6/1.5 mm), lateral (0.8–1.2 mm) or ventro/lateral (1.0/0.6 mm) to the area of the B1/B3 cell group did not cause a change in blood pressure or heart rate.

Discussion

In the present experiments in the rat, 5-HT_{1a} receptor agonists were directly injected into an area of medullary 5-hydroxytryptaminergic cells that have been shown to project to the spinal cord and have been proposed to exert an excitatory action on sympathetic neurones in the intermediolateral cell column (for review see Coote, 1988).

8-OH-DPAT is a selective 5-HT_{1a} receptor agonist (Middlemiss & Fozard, 1983) inducing bradycardia and hypotension after peripheral (Fozard *et al.*, 1987), as well as intracerebroventricular or intracisternal administration (Martin & Lis, 1985; Mir & Fozard, 1988). The same effects were seen in the present experiments after local injection into the region of the B1/B3 cell group. Peripheral administration of drugs with 5-HT_{1a} receptor blocking properties such as SPX or SPP markedly reduced or abolished the fall in blood pressure and heart rate induced by stereotaxically injected 8-OH-DPAT. With respect to SPP, however, it must be pointed out that the drug depressed both systemic blood pressure and heart rate *per se* thus rendering difficult the interpretation of its antagonistic effects (see also Fozard *et al.*, 1987).

In addition, 5-MU, a structural congener of the antihypertensive drug urapidil, with high affinity for central 5-HT_{1a} binding sites (Groß *et al.*, 1987), was investigated. The compound also lowered blood pressure and heart rate after

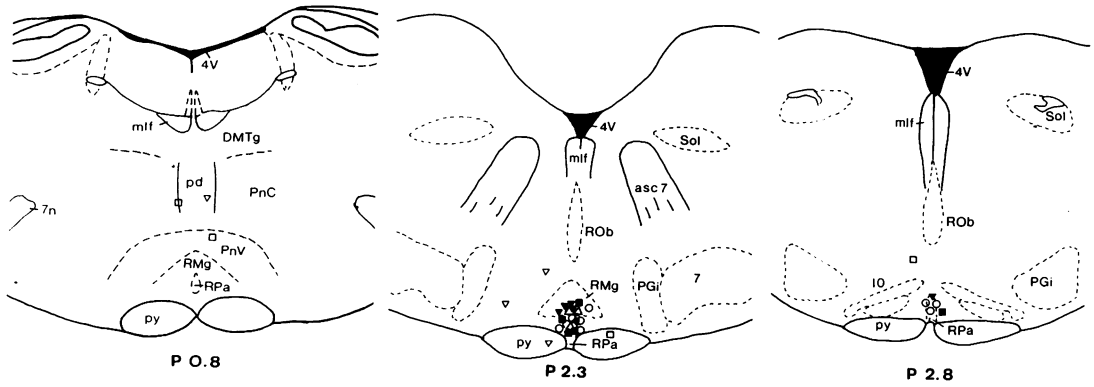


Figure 4 Coronal sections through the brain stem of the rat (adapted from the atlas of Paxinos & Watson, 1982) showing histologically verified sites of microinjections of 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT) 0.1 μg ((■) with effect, (□) without effect), 5-methylurapidil 1 μg ((▼) with effect, (▽) without effect), prazosin (△) or vehicle (○); all injection volumes 0.1 μl. The numerals at the bottom of each section represent the distance (in mm) posterior to the interaural line (for corresponding stereotaxic coordinates of injection sites see Methods). Abbreviations: 4V, fourth ventricle; asc7, ascending fibres of nervus facialis; 7, nucleus of nervus facialis; 7n, root of nervus facialis; DMTg, dorsomedial tegmental area; IO, inferior olive; pd, predorsal bundle; PGi, nucleus reticularis paragigantocellularis; PnC, caudal nucleus pontinus reticularis; PnV, ventral nucleus pontinus reticularis; py, pyramidal tract; RMg, nucleus raphe magnus; ROb, nucleus raphe obscurus; RPa, nucleus raphe pallidus; Sol, nucleus of the tractus solitarius; Sp5, spinal trigeminal nucleus.

stereotaxic microinjection into the ventral medulla, and its effects were antagonized by the 5-HT_{1a} receptor antagonist SPX. It is, therefore, conceivable that 5-MU, like 8-OH-DPAT, acts as 5-HT_{1a} receptor agonist. However, since 5-MU also possesses a high affinity for α_1-adrenoceptors and acts as antagonist at these receptors (Groß *et al.*, 1988), control experiments were performed with the selective α_1-adrenoceptor antagonist prazosin. The concentration of prazosin injected into the tissue was 3.17 mM (0.13 μg 0.1 μl^{-1}). Since the K_d of prazosin at central α_1-adrenoceptors is in the subnamolar range (Kellar *et al.*, 1984; Groß *et al.*, 1988) it is reasonable to assume that α_1-adrenoceptors in the injected area were blocked by the drug. The concentration of prazosin used and the concentration of 1 μg 0.1 μl^{-1} 5-MU can be considered approximately equipotent in blocking α_1-adrenoceptors (taking into account an approximately tenfold difference in K_ds; Groß *et al.*, 1988). The lack of effect of prazosin therefore suggests that α_1-adrenoceptors are not involved in the observed effects of 5-MU.

Our results agree with the findings of Gillis *et al.* (1987, 1989), who reported a central hypotensive action of urapidil and 8-OH-DPAT in the cat after application to the ventral surface of the medulla. These authors suggested the B1/B3 cell group as a possible site of action. In our study in the rat, histological examination showed all injection sites which were associated with a decrease in blood pressure and heart rate to be contained within a small pocket of tissue in the midline of the ventral medulla corresponding to the region of the nucleus raphe magnus and pallidus. Furthermore, injection of 8-OH-DPAT or 5-MU outside the B1/B3 region into adjacent sites (Figure 4) did not elicit any cardiovascular effects. The apparent importance of the midline component of the B1/B3 cell group in the regulation of blood pressure is also stressed in other studies. For instance, Franz *et al.* (1982) demonstrated a fall in blood pressure following electrical stimulation of the nucleus raphe magnus, and Aslanian *et al.* (1988) found in a study with *in vivo* voltammetry that after an increase in arterial blood pressure, an increase in extracellular 5-hydroxyindole acetic acid occurred in the area of the B3 cell group (nucleus raphe magnus).

On the other hand, Laubie *et al.* (1989) found in their study on the dog that microinjections of 8-OH-DPAT into the ventrolateral pressor area of the medulla caused long-lasting decreases in blood pressure and heart rate, whereas injections along the midline of the ventral medulla had no effect. On the basis of our data in the rat we cannot exclude the ventrolateral pressor area as a site participating in the cardiovascular effects of 5-HT_{1a} receptor agonists after systemic administration.

In conclusion, the present results support the hypothesis that activation of somatodendritic 5-HT_{1a} autoreceptors located on 5-hydroxytryptaminergic neurones in the medulla leads to a decrease in blood pressure and heart rate. The region of the B1/B3 cell group is suggested as a possible site for the hypotensive action of 5-HT_{1a} receptor agonists in the rat.

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Different effects of prostaglandins on adrenergic neurotransmission in atrial and ventricular preparations

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1 The effects of prostaglandin E_2 (PGE_2) and iloprost on the cardiac response to adrenergic nerve stimulation in guinea-pig atrial and ventricular preparations have been studied.

2 In guinea-pig isolated atria both PGE_2 (0.1–10 nM) and iloprost (0.1—3 μM) concentration-dependently reduced the cardiac response to adrenergic nerve stimulation.

3 The inhibition of cyclo-oxygenase by indomethacin and acetylsalicylic acid potentiated the response to nerve stimulation in the atrial preparations.

4 Arachidonic acid (1–10 μM) reduced the response to nerve stimulation in atria. This effect was prevented by indomethacin and acetylsalicylic acid.

5 In guinea-pig ventricles PGE_2 and iloprost were found to be effective at higher concentrations than in atrial preparations: arachidonic acid, indomethacin or acetylsalicylic acid did not modify the cardiac response to adrenergic nerve stimulation.

6 These results suggest a different modulator role for endogenous prostaglandins in atrial and ventricular tissue.

Introduction

It is widely accepted that prostaglandins of the E and I series are able to reduce the release of noradrenaline induced by sympathetic nerve stimulation in several tissues, including the rabbit and rat heart (Hedqvist *et al.*, 1970; Wennmalm, 1971; Fuder & Muscholl, 1974; Hedqvist, 1977; Khan & Malik, 1980; 1982).

Moreover, it has been demonstrated that stimulation of sympathetic nerves induces the output of a prostaglandin-like material from cardiac tissues (Junstad & Wennmalm, 1973; Khan & Malik, 1980), and that the inhibition of prostaglandin synthesis enhances the release of noradrenaline induced by adrenergic nerve stimulation (Samuelsson & Wennmalm, 1971; Chanh *et al.*, 1972; Fuder & Muscholl, 1974; Khan & Malik, 1982). These observations suggest that endogenous prostaglandins may have a physiological, modulator role on adrenergic neurotransmission in the heart.

In all previous studies the effects of prostaglandins have been tested on the whole isolated heart of the rabbit or rat. Hence, in the present study we decided to examine the effect of prostaglandins in two different sections of mammalian heart, namely atria and ventricular strips, in order to test whether the effects of prostaglandins may differ from section to section. Moreover, we have used two inhibitors of prostaglandin synthesis, indomethacin and acetylsalicylic acid, and arachidonic acid, to explore the possible modulator role played by endogenous prostaglandins in the atrial and ventricular tissues of guinea-pig heart.

Methods

Field stimulation of sympathetic nerve terminals during the functional refractory period was used in the experiments. Guinea-pig isolated preparations (atria or ventricular strips) were mounted in a glass chamber containing 15 ml of Tyrode solution bubbled with a gas mixture (95% O_2/5% CO_2) at a pH of 7.4 and at a temperature of 30°C. The preparations were driven at a constant rate (4 Hz for the atria, 2 Hz for the ventricular strips) by two pointed platinum electrodes. Field stimulation was applied through two platinum plates parallel to the preparation, during the absolute refractory period. A graded stimulation of sympathetic nerve terminals was induced by slightly different techniques in atrial and ventricular preparations. The method employed for the stimulation of ventricular preparations was almost identical to the one described by Blinks (1966): graded stimulus-inotropic response curves were obtained by applying one field pulse every 16 contractions (1/16) for 90 s, and then the frequency was doubled to one pulse every 8 contractions (1/8) and so on up to one pulse for every contraction (1/1), which produced the maximum increase in contractility. A second curve was determined after exposure to the selected drug, 40 min after the end of the first curve. For stimulation of atrial preparations a slight modification of the above-mentioned method was employed: trains of field pulses were applied at 3 min intervals, at a rate of one per contraction, and the number of field pulses per train was increased from 2 to 12, until the maximum positive inotropic response was reached (Ledda & Mantelli, 1984; Ledda *et al.*, 1984; 1985). The main reasons for this modification were the different responsiveness of atria and ventricles: in fact we previously observed that the total number of field pulses able to evoke a sympathetic response in atria was remarkably lower than that required for ventricular tissue, probably as a consequence of the greater innervation of atria. All the experiments in which the method of field stimulation was used were carried out in the presence of 1 μM atropine, in order to eliminate the parasympathetic component of the response (Blinks, 1966; Angus & Harvey, 1981). Dose-effect curves for noradrenaline were obtained by adding cumulative concentrations of the agonist. Statistical analysis was performed by Student's *t* test for comparisons between two groups of data. Analysis of variance and Tukey's test were used when a comparison between three or more groups was made. The following drugs were used: atropine sulphate (BDH), iloprost (kindly supplied by Schering), prostaglandin E_2 (PGE_2), acetylsalicylic acid, indomethacin, arachidonic acid (Sigma). All the drugs were dissolved in Tyrode solution except indomethacin and arachidonic acid, which were dissolved in ethanol and further diluted in Tyrode solution.

Results

Characteristics of the responses induced by field stimulation

In both atrial and ventricular preparations, trains of field pulses applied during the refractory period induced a graded

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positive inotropic effect. In preliminary experiments we verified the reliability of the two different methods of field stimulation, comparing the effects of a well-known prejunctional inhibitory agonist, i.e. clonidine, in atria and ventricles. The effectiveness of clonidine in reducing the positive inotropic effect of sympathetic stimulation in ventricular preparations was similar to that previously obtained in atrial preparations (Ledda & Mantelli, 1984). The IC_{50} value for clondine in ventricular tissue was 17.2 ± 1.8 nM (data not shown), a value very close to that previously detected in guinea-pig atrial preparations (Ledda & Mantelli, 1984).

Effect of PGE_2 on the sympathetic response to field stimulation

Exposure of guinea-pig atria to increasing concentrations of PGE_2 (0.1–10 nM) for 10 min before field stimulation produced a concentration-dependent and significant depression of the stimulus-response curve (Figure 1a). The concentration of PGE_2 able to induce a 50% inhibition (IC_{50}) of the response caused by an intermediate degree of sympathetic stimulation (i.e. the response induced by a train of 8 pulses) was 0.55 ± 0.11 nM. In guinea-pig ventricular preparations PGE_2 reduced the cardiac response to sympathetic nerve stimulation at higher concentrations than in guinea-pig atria (1–10 nM). The degree of inhibition of the cardiac response was lower in this tissue, and was significant at low and intermediate degrees of sympathetic stimulation but not at the maximal degree (see Figure 1b). The IC_{50} for PGE_2 (calculated for the response induced by intermediate stimulation, i.e. 1/2) was 4.06 ± 1.5 nM in ventricular tissue. In both tissues the inhibitory effect of PGE_2 was chiefly attributable to a prejunctional effect since, at the highest concentration tested (10 nM), PGE_2 did not modify cardiac contractility or the positive inotropic response to exogenous noradrenaline (see Table 1).

Effect of iloprost on the sympathetic response to field stimulation

A 10 min exposure of guinea-pig atria to iloprost, a synthetic stable analogue of prostacyclin, induced a concentration-dependent and significant rightward shift of the stimulus-response curve to sympathetic nerve stimulation (Figure 2a). Iloprost was active at concentrations ranging from 0.1 to 3 μM, and its IC_{50} (calculated as for PGE_2) was 0.74 ± 0.2 μM. In guinea-pig ventricular preparations iloprost was again active at ten fold higher concentrations; it was inactive at 0.1 μM (Figure 2b), and the IC_{50} = 1.5 ± 0.5 μM. It is interesting to note that the pattern of inhibition by iloprost of the sympathetic response in ventricular tissue was again different from

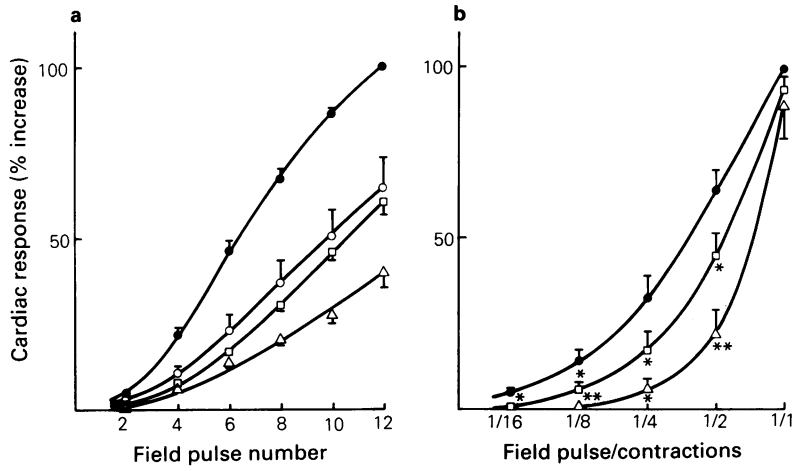


Figure 1 Effect of prostaglandin E_2 (PGE_2) on the stimulus-inotropic response curve obtained by increasing the number of field pulses in guinea-pig isolated atria stimulated at 4 Hz (a) and in ventricular strips stimulated at 2 Hz (b). (●) Control; (○) PGE_2 0.1 nM; (□) PGE 1 nM; (△) PGE_2 10 nM. Points represent means of 6 (a) and 5 (b) experiments; vertical lines show s.e.mean. In (a) all the points were statistically different from the control according to analysis of variance and Tukey's test ($P < 0.01$); symbols were omitted for clarity. In (b) $^*P < 0.05$; $^{**}P < 0.01$.

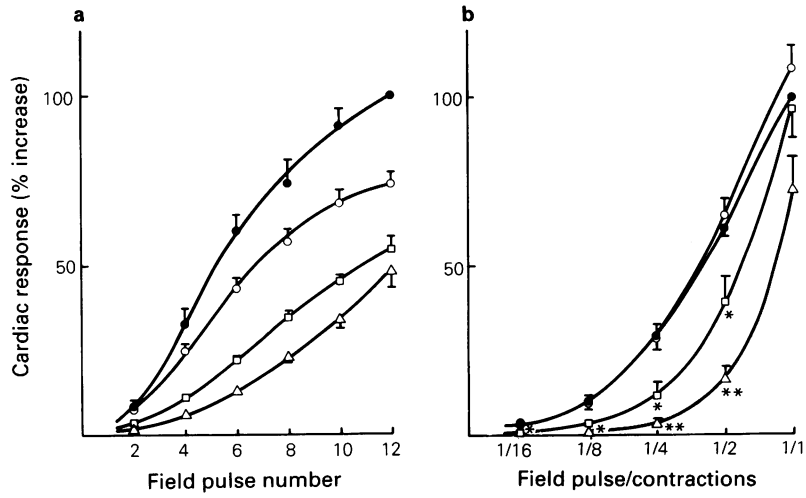


Figure 2 Effect of iloprost on the stimulus-inotropic response curve obtained by increasing the number of field pulses in guinea-pig isolated atria stimulated at 4 Hz (a) and in ventricular strips stimulated at 2 Hz (b). (●) Control; (○) iloprost 0.1 μM; (□) iloprost 1 μM; (△) iloprost 3 μM. Points represent means of 4 (a) and 6 (b) experiments; vertical lines show s.e.mean. In (a) all the points were statistically different from the control according to analysis of variance and Tukey's test ($P < 0.01$). In (b) $^*P < 0.05$; $^{**}P < 0.01$.

Table 1 Increase in contractile force induced by increasing concentrations of noradrenaline in guinea-pig atrial and ventricular preparations in the absence and presence of prostaglandin E_2 (PGE_2)

Noradrenaline concentration (μM)	*Control* (% *increase*)	PGE_2 (10 nM) (% *increase*)
Atrial preparations ($n = 6$)		
0.01	6.9 ± 1.2	6.1 ± 0.9
0.03	14.9 ± 2.2	14.4 ± 1.6
0.1	31.5 ± 3.8	32.2 ± 2.7
0.3	52.0 ± 5.1	51.0 ± 5.0
1	77.8 ± 5.4	76.8 ± 6.2
3	93.5 ± 2.6	92.5 ± 6.6
10	100.0 ± 0.0	100.4 ± 6.3
Ventricular preparations ($n = 4$)		
0.01	2.0 ± 0.7	2.0 ± 0.7
0.03	5.2 ± 1.4	4.3 ± 1.1
0.1	13.8 ± 2.6	11.9 ± 2.0
0.3	30.0 ± 5.2	26.3 ± 2.2
1	53.3 ± 5.0	52.7 ± 3.0
3	82.9 ± 6.6	87.9 ± 4.9
10	100.0 ± 0.0	109.4 ± 4.2

that observed in the atria, and was qualitatively similar to that observed with PGE_2 (compare Figures 1b and 2b). Iloprost (3 μM) was unable to affect the cardiac response to exogenous noradrenaline in ventricular preparations (data not shown); in atrial preparations, at the same concentration, it slightly but significantly reduced the positive inotropic effect of exogenous noradrenaline (Figure 3). However, its postjunctional inhibitory effect was very slight in comparison to the effect induced by the same concentration on the cardiac response to adrenergic nerve stimulation. Thus, the action of iloprost is mainly attributable to a prejunctional effect.

Effect of indomethacin and acetylsalicylic acid on the sympathetic response to field stimulation

A 40 min contact period of preparations with indomethacin and acetylsalicylic acid, at concentrations (3 μM and 500 μM respectively) able to block cyclo-oxygenase in isolated tissues (for a review see Flower, 1974), significantly potentiated the cardiac response to field stimulation in guinea-pig atrial tissue (Figure 4a). This effect was especially evident at a high intensity of nerve stimulation. On the other hand, neither indomethacin nor acetylsalicylic acid was able to modify the sympathetic response in guinea-pig ventricular preparations (Figure 4b).

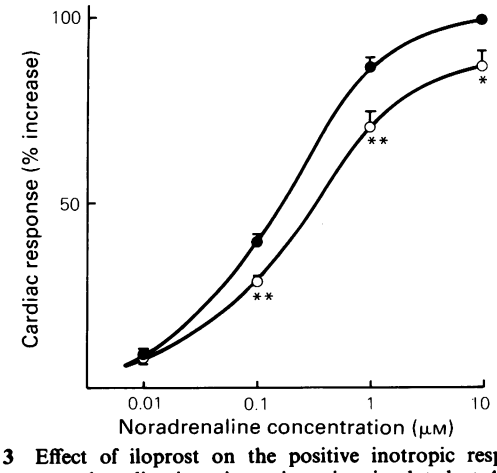


Figure 3 Effect of iloprost on the positive inotropic response to exogenous noradrenaline in guinea-pig atria stimulated at 4 Hz. The effect of the highest concentration of noradrenaline was taken as 100%. (●) Effect of noradrenaline alone and (○) in the presence of 3 μM iloprost. Points represent means of 14 experiments; vertical lines show s.e.mean. $*P < 0.05$; $**P < 0.01$.

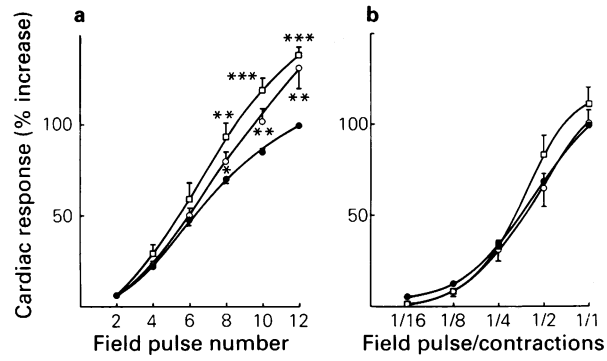


Figure 4 (a) Effect of indomethacin and acetylsalicylic acid on the stimulus-inotropic response curve obtained by increasing the number of field pulses in guinea-pig isolated atria stimulated at 4 Hz. (●) Control ($n = 12$); (○) indomethacin 3 μM ($n = 7$); (□) acetylsalicylic acid 500 μM ($n = 5$). $*P < 0.05$; $**P < 0.01$; $***P < 0.005$. (b) Effect of indomethacin and acetylsalicylic acid in guinea-pig ventricular strips stimulated at 2 Hz. (●) Control ($n = 12$); (○) indomethacin 3 μM ($n = 6$); (□) acetylsalicylic acid 500 μM ($n = 6$).

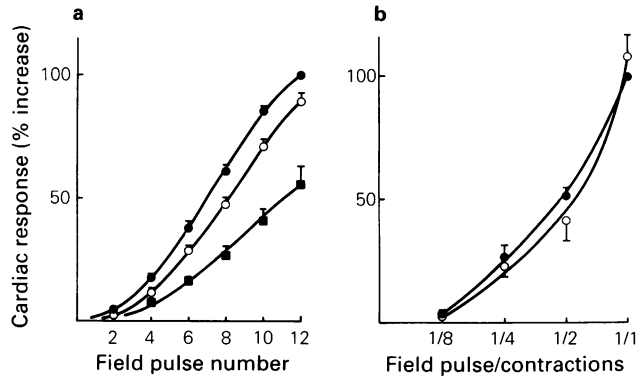


Figure 5 (a) Effect of arachidonic acid on the stimulus-inotropic response curve obtained by increasing the number of field pulses in guinea-pig isolated atria stimulated at 4 Hz ($n = 6$). (●) Control; (○) arachidonic acid 1 μM; (■) arachidonic acid 10 μM. All the points were statistically different from the control according to analysis of variance and Tukey's test. $P < 0.05$ for arachidonic acid 1 μM and $P < 0.01$ for arachidonic acid 10 μM. (b) Effect of arachidonic acid in guinea-pig ventricular strips stimulated at 2 Hz ($n = 4$). (●) Control; (○) arachidonic acid 10 μM.

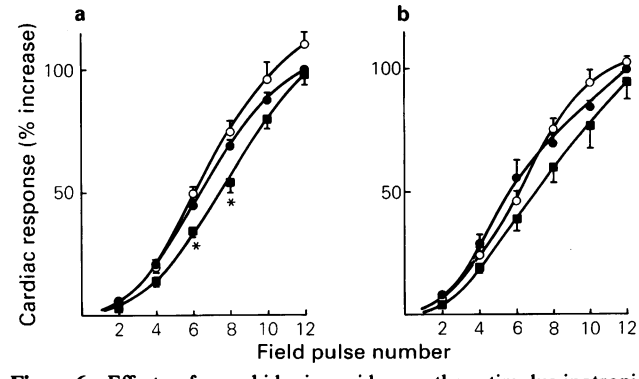


Figure 6 Effect of arachidonic acid on the stimulus-inotropic response curve obtained by increasing the number of field pulses in guinea-pig isolated atria stimulated at 4 Hz, in the presence of indomethacin 3 μM (a, $n = 4$) and acetylsalicylic acid 500 μM (b, $n = 4$). (●) Control; (○) arachidonic acid 1 μM; (■) arachidonic acid 10 μM. $*P < 0.05$.

Effect of arachidonic acid on the sympathetic response to field stimulation

Exposure of guinea-pig atria for 30 min to arachidonic acid (1–10 μM) produced a concentration-dependent and significant depression of the stimulus-response curve (Figure 5a). On the

other hand, in the ventricles 10 μM arachidonic acid was completely ineffective (Figure 5b). Preincubation with indomethacin 3 μM or acetylsalicylic acid 500 μM almost completely abolished the inhibitory effect of arachidonic acid in atrial preparations (see Figure 6).

Discussion

The results of the present study demonstrate that both PGE_2 and iloprost, a stable analogue of prostacyclin (PGI_2), concentration-dependently reduce the cardiac response to sympathetic nerve stimulation in guinea-pig atrial and ventricular preparations. These results are in agreement with previous observations demonstrating an inhibitory effect of prostaglandins of the E and I series on noradrenaline release induced by sympathetic nerve stimulation in rabbit and rat heart (Wennmalm, 1971; Hedqvist, 1977; Khan & Malik, 1980; 1982).

PGE_2 appeared to be much more active than iloprost in our study, thus suggesting that PGE_2 may be a more important modulator of sympathetic transmitter release in the heart than PGI_2, as already proposed by Wennmalm (1978). In fact PGE_2 reduced the cardiac response to field stimulation at a 1000 fold lower concentration than iloprost in both atrial and ventricular strips. The inhibitory effect of PGE_2 was completely attributable to a prejunctional action; in fact at the highest concentration tested PGE_2 did not modify the cardiac response to exogenous noradrenaline in either preparation. In the case of iloprost, the possibility of a postjunctional component of the inhibitory effect in ventricular preparations alone was ruled out. However, in the atrial tissue a small part of the inhibitory effect was attributable to a postsynaptic component, since the highest concentration of iloprost tested slightly reduced the positive inotropic response to exogenous noradrenaline.

An interesting observation arising from this study was the quantitative difference between the prostaglandin effects in the two different cardiac tissues, i.e. atrial and ventricular preparations. The IC_{50} for the inhibitory effect of PGE_2 on the cardiac response to adrenergic stimulation was 0.55 nM in atrial versus 4.06 nM in ventricular tissues; the IC_{50} for iloprost was also lower in atrial (0.7 μM) than in ventricular (1.5 μM) preparations. Moreover, the patterns of cardiac response to sympathetic stimulation by the two prostaglandins were also quite different in the two tissues: the whole stimulus-response curve was shifted to the right by prostaglandins in the atrial section, whilst the prostaglandin-induced inhibitory effect was evident only at low or intermediate degrees of stimulation, in the ventricular tissue. The possibility that these differences were due to the different methods of field stimulation used for atria and ventricles is unlikely, as the response to clonidine, a prejunctional inhibitory agonist, was similar in the two tissues. Also, we have previously observed that dynorphin, an opioid agonist able to inhibit the adrenergic response to field stimulation, was active in ventricles at lower concentrations than in atria (Ledda *et al.*, 1985; Mantelli *et al.*, 1987). Thus, it seems reasonable to conclude that the inhibitory effect of prostaglandins observed in the present study was stronger in atrial than in ventricular preparations. Moreover, inhibition of prostaglandin synthesis, induced by indomethacin and acetylsalicylic acid, enhanced the cardiac response to sympathetic nerve stimulation only in atrial preparations. Enhancement of noradrenaline overflow elicited by nerve stimulation with indomethacin has previously been observed in rabbit (Chanh *et al.*, 1972; Starke & Montel, 1973; Fuder & Muscholl, 1974) and rat heart (Khan & Malik, 1982). Thus our results are in agreement with previous observations and suggest that endogenous prostaglandins may have a modulator role in adrenergic neurotransmission only in the atrial section of the mammalian heart. This last hypothesis is supported by the finding that arachidonic acid reduced the cardiac response to sympathetic nerve stimulation only in atrial preparations, and that indomethacin and acetylsalicylic acid prevented this effect.

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The effects of anticholinesterases on the latencies of action potentials in mouse skeletal muscles

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1 The purpose of this investigation was to determine the long-term effects of a single dose of persistent anticholinesterases on muscle action potentials evoked by nerve stimulation.

2 Action potentials (APs), elicited by stimulation of the phrenic nerve, were recorded intracellularly in muscle fibres of mouse diaphragm. The time between stimulus and AP was measured and the variability of this latency was calculated during trains of APs. At the beginning of trains of APs there was an increase in latency, and this delay was also measured.

3 Within 3 h of subcutaneous injection, a single dose (500 nmol kg^{-1}) of the anticholinesterase, ecothiopate produced about 90% reduction in the acetylcholinesterase activity of homogenates of mouse diaphragm muscle, but five days after injection, this activity was not different from values in untreated animals. The initial delay of APs and the variability of latencies were increased four fold and two fold respectively, remained at these maxima from the 1st to the 5th day after ecothiopate, and returned to the values in untreated animals between 15 and 27 days after ecothiopate.

4 These effects of ecothiopate on AP latency were dose-dependent and were also seen in extensor digitorum longus and soleus muscles.

5 Other anticholinesterases used were BOS (pinacolyl S-(2-trimethylaminoethyl)methylphosphonothioate), a quaternary compound, and diisopropyl fluorophosphate, a tertiary compound, which had effects similar to those of ecothiopate; the greater duration of the effects of this compound may be related to the greater duration of reduction in cholinesterase activity.

6 Ecothiopate had no effect on the delay or variability of latencies of endplate potentials which were recorded in cut-fibre preparations 5 days later.

7 It is concluded that the effects of ecothiopate on the latencies of indirectly-evoked muscle APs are postjunctional, may not be related to the degree of reduction in cholinesterase activity at the time of recording, and are not directly linked to necrosis.

Introduction

Subcutaneous injection of the organophosphate anticholinesterase, ecothiopate, causes necrosis at the endplate of mouse diaphragm (Ferry & Townsend, 1986) which reaches a maximum 6–12 h after injection. However, only a minority of the muscle fibres show necrosis and the question arises as to whether those fibres without structutal damage are more subtly affected. Transmission of excitation from nerve to the muscle and propagation of the action potential along the muscle might be compromised in damaged fibres.

The clinical technique of single fibre electromyography (SFEMG) involves recording extracellularly the current field due to action potentials in single muscle fibres and may be used to measure jitter, the variability in the latency of a series of action potentials (APs). This variability may be in the time between APs of two fibres of the same motor unit in voluntary activity (Stalberg & Trontelj, 1979), or in the time between stimuli and APs in a fibre during a train of evoked responses (Trontelj *et al.*, 1986). Excessive jitter is found in patients with muscular dystrophy or with myasthenia gravis, or after drugs (e.g. (+)-tubocurarine), and is a sensitive indicator of muscular or neuromuscular disease. Jitter was considered by Stalberg & Trontelj (1979) to originate in the interaction of the endplate potential (e.p.p.) and the threshold of the muscle membrane during transmission.

In the experiments *in vitro* described here, a new method of measuring jitter was developed. Action potentials evoked by nerve stimulation were recorded intracellularly which ensured that successive responses would be recorded from the same part of a single fibre. In order that all sources of jitter, presynaptic or postsynaptic, would be included, records were made close to the end of the muscle fibre near the tendon, but in some experiments records were made at the endplate. The membrane potential and overshoot of the AP are sensitive indicators of damage and provided criteria for excluding from further analysis any fibres damaged by the microelectrode during the contraction.

The effect of anticholinesterases (antiChEs) on jitter was studied using several antiChEs to exclude the possibility that effects were other than a consequence of inhibition of acetylcholinesterase (AChE). Attempts were made to assess the role of AChE inhibition in the manifestation of jitter.

Methods

Male albino mice aged 6–7 months were used in all experiments. At various times after subcutaneous injection of drugs, animals were killed by decapitation. The left hemidiaphragm and phrenic nerve were removed, pinned to Sylgard in a Perspex bath and irrigated with a physiological saline of the following composition (mM): NaCl 137, $NaHCO_3$ 12, NaH_2PO_4 1, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, glucose 25 and gassed with 5% CO_2 in O_2 and maintained at 37.0 ± 0.5°C. The phrenic nerve was stimulated via a suction electrode with supramaximal pulses of 0.05 ms duration. To minimize muscle movement many pins were used to fix the preparation to the Sylgard and, if necessary, some muscle fibres were cut. For experiments on extensor digitorum longus (EDL) or soleus (SOL) muscles, mice were anaesthetized with halothane in 50% nitrous oxide in oxygen; the limb muscles and their nerve supply were then removed and recordings were made as with the diaphragm.

Glass capillary intracellular microelectrodes filled with 3 M KCl and of resistance 10–15 MΩ were used to record resting

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membrane potentials (RMPs) and action potentials (APs) of uncut muscle fibres near the tendon. Endplate potentials (e.p.ps) and miniature endplate potentials (m.e.p.ps) were recorded in cut fibres by an electrode inserted focally at the endplate where the risetime of the potentials was less than 1.1 ms mV^{-1}. APs were displayed on an oscilloscope and recorded on an FM tape recorder (Racal Store 4) with a tape speed of 30 inches per second (i.p.s.). These records were then replayed at 15/16 i.p.s. and analysed by a PDP 11/03 minicomputer. The sampling rate of the analogue-to-digital converter was 20 kHz and with a record/replay ratio of 32, the effective sampling rate of the system was 640 kHz. Trains of 30 APs were recorded from each muscle fibre and approximately 10 fibres were sampled from each muscle. The stimulation frequency was usually 30 Hz and if recording conditions were stable also 10 Hz in the same fibre. Occasionally, stimulation was at 1 Hz. Data from a fibre were rejected if, during the train, there was a decrease in the RMP of more than 5 mV or in the AP amplitude of more than 10%.

In experiments with ecothiopate iodide *in vitro*, the preparation was exposed to a 0.5 μM solution for 30–40 min, then to physiological saline for 30 min before the recording was commenced.

Unless the results with stimulation at 10 Hz and 30 Hz were different, only data for 30 Hz are presented.

Analysis of records

Computer programs were devised to measure the amplitude, time course, and latency of each AP. The latency was the interval between the stimulus and a point on the rising phase at 10% of peak amplitude. These data were used to calculate the mean consecutive difference (MCD) of latencies of APs 11–30 (plateau), and the individual latencies of the first 16 APs with respect to the first AP of the train. The formula used to calculate the MCD was:

$$MCD = \{|L_{11} - L_{12}| + |L_{12} - L_{13}| + \cdots + |L_{29} - L_{30}|\}/19$$

Where L_n is the latency of AP number n.

The use of the MCD as a measure of variability may reduce the effects of any long-term drifts in latency, but this technique might hide initial changes at the beginning of trains of APs (Ekstedt *et al.*, 1974). In our experiments, the initial change is measured as the 'delay' of the sixteenth AP relative to the first AP (i.e. latency of AP 16 minus the latency AP 1).

A pulse generator (Digitimer) was used to simulate action potentials at 10 Hz, the value obtained for MCD was 2.4 μs, which represents the intrinsic error of the process of record/replay/analysis.

Measurement of AChE activity

The activity of AChE was assayed in strips of the junctional region of hemidiaphragms. Each hemidiaphragm was cut about 2 mm either side of the intramuscular nerve and the junctional strip of muscle weighted and homogenised in 5 ml of phosphate buffer (0.1 M, pH 8.0). The homogenate was incubated for at least 30 min with 50 μM ethopropazine, a selective inhibitor of butyrylcholinesterase (Bayliss & Todrick, 1953), sonicated and then centrifuged at 1500 g for 15 min at 4°C. The supernatant was assayed for cholinesterase activity by the method of Ellman *et al.* (1961). Cholinesterase activity was expressed as nmol acetylthiocholine hydrolysed per min per mg of wet muscle and is due mainly to the AChE activity of the endplate (Das, 1989).

The anticholinesterases used were:- ecothiopate (*S*-(2-trimethylammoniumethyl)phosphorothioate iodide), pinacolyl *S*-(2-trimethylaminoethyl)methylphosphonothioate (BOS), or diisopropyl fluorophosphate (DFP).

Ecothiopate was made up from Phospholine Eyedrops (Ayerst). This comprised 12.5 mg ecothiopate and 40 mg potassium acetate. When dissolved and diluted in normal saline, the potassium injected was negligible (approximately 6 μmol kg^{-1}). Solutions of BOS and DFP were made up shortly before injection. All mice given anticholinesterases were also given atropine sulphate 0.7 μmol kg^{-1} at the same time. Some mice were given atropine only.

Statistical analysis

Results are expressed as mean $\pm$ 1 s.d. of values from 2 to 5 animals, with the number of muscle fibres in parentheses. The Kolmogorov-Smirnov non-parametric test was used and groups were taken to be significantly different if $P < 0.05$ (2-tail). Where experimental values returned to normal a long time after injection, the one-tail test was used because the direction of the difference from untreated values was predictable.

Results

The electrophysiological characteristics of untreated mice

In untreated animals the RMP recorded at the tendon end of the muscle fibres was -71 ± 5 mV (42). The AP had a time to peak (from 10% peak) of 216 ± 34 μs (35) and an amplitude of 81 ± 8 mV (35). These values were not affected by the frequency of stimulation or by administration of any of the anti-ChEs either *in vivo* or *in vitro*. Records of typical action potentials are shown in Figure 1.

The effect of frequency of stimulation

The effect on jitter of the stimulus frequency was investigated because there is a marked effect of frequency on the amplitude of e.p.ps, which then may affect the latencies of the APs elicited. In untreated preparations, with stimulation at 10 or

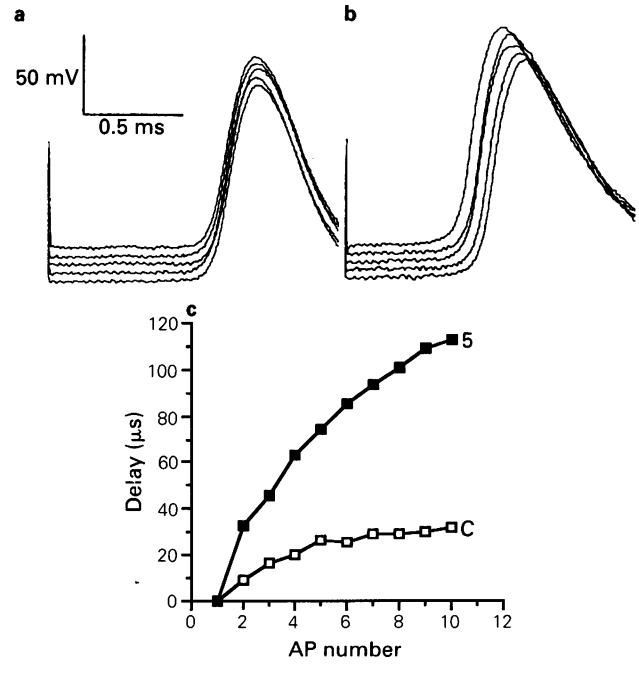


Figure 1 Action potentials (APs) recorded at the tendon end of muscle fibres of diaphragm and elicited by stimulation of the nerve at 30 Hz. (a) Records of APs 1–5 from untreated mice, shown displaced vertically downwards from AP 1. (b) Five days after ecothiopate 0.5 μmol kg^{-1}. Note the increased latencies of APs relative to the first AP which is greater after ecothiopate. (c) Graph showing the mean latency of the first 10 APs relative to the first. (□) Indicate values from untreated mice ($n = 42$) and (■) represent values 5 days after ecothiopate ($n = 40$).

30 Hz the latency of successive APs increased progressively and reached a steady value by about the tenth AP. This increase is quantified as the delay of AP 16 relative to AP 1. The value of the delay at 10 Hz was 34 ± 12 μs (10), which was not different from that at 30 Hz, 32 ± 22 μs (42). With stimulation at 1 Hz, the delay was 1 ± 25 μs (11), which was different from the value at 30 Hz.

The MCD of the APs 11–30 at 10 Hz was 9.7 ± 4.6 μs (44), and at 30 Hz, 9.9 ± 3.6 μs (42). These values were not different from each other but were different from that at 1 Hz, 6.3 ± 1.2 μs (10).

In these untreated mice, the values of MCD and of delay were distributed unimodally (Figure 2a).

It is concluded that in normal diaphragms there was progressive delay of APs at the beginning of a train elicited at 10 or 30 Hz, but not at 1 Hz. There was a non-progressive variation in latency of APs 11–30, the MCD, which was greater at the higher frequencies than at 1 Hz.

The effects of antiChEs in necrotizing doses

Ecothiopate was given subcutaneously to a batch of mice and measurements of MCD and delay made at increasing intervals until the values were no longer different from those of untreated animals. Three hours after a dose of 0.5 μmol kg^{-1} ecothiopate and atropine, which causes necrosis of some muscle fibres, the AChE activity of homogenates of the junctional region was approximately 10% of that of untreated animals (Table 1). One day after ecothiopate 0.5 μmol kg^{-1} the delay and MCD at 30 Hz were increased to maximal values which persisted until day 5 (Table 2). At day 7 the delay and MCD showed smaller increase. Although the delay had returned to normal by 15 days, the MCD was increased, but had returned to normal by 27 days. In mice given only atropine the MCD and delay were never significantly different from normal.

At 5 days after ecothiopate 0.5 μmol kg^{-1} the populations of MCD and delay were not unimodal; many values were greater than those in untreated preparations (Figures 2b and 3b), and a few fibres had very large values for MCD and delay. These fibres excepted, there was no correlation between MCD and delay because muscle fibres with large MCDs did not necessarily have large delays. About 5% of the fibres sampled were not analysed because the train lacked at least one AP. There was repetitive firing of APs in about 20% of fibres 5 days after ecothiopate 0.5 μmol kg^{-1}.

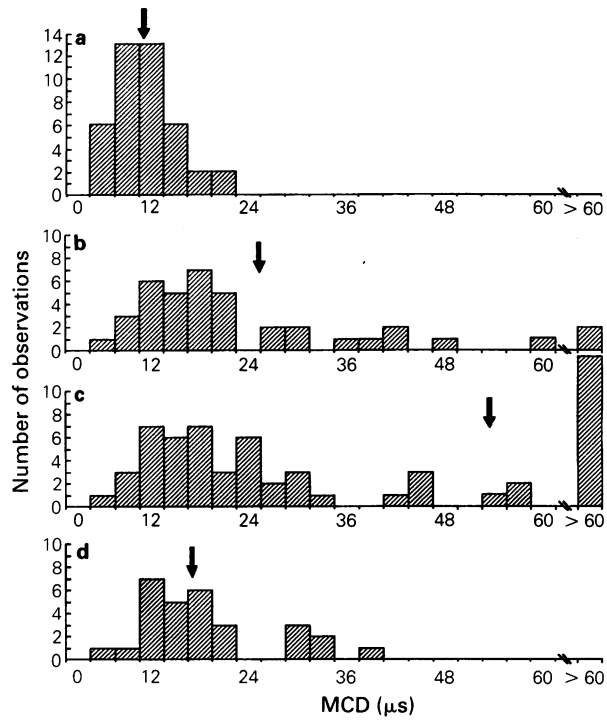


Figure 2 The distribution of values of the mean consecutive difference (MCD) of action potentials (APs) at 30 Hz in (a) untreated muscle, and in muscle 5 days after (b) ecothiopate 0.5 μmol kg^{-1}, (c) pinacolyl *S*-(2-trimethylaminoethyl)methylphosphonothioate (BOS) 8.0 μmol kg^{-1}, (d) BOS 1.0 μmol kg^{-1}. The arrows indicate the mean value of the population.

At 5 days the MCD and delay were still maximally increased, but the AChE was at the untreated normal value (Table 1).

Other antiChEs given in necrotizing doses caused similar changes in AChE and in the delay and MCD of APs (Figure 2c and 3c; Table 3). After BOS 8 μmol kg^{-1} or DFP 14 μmol kg^{-1}, the AChE 3 h later was reduced to similar values found with ecothiopate 0.5 μmol kg^{-1}, but the reduction lasted longer (Table 1).

Five days after BOS (8 μmol kg^{-1}), the increased delay and MCD were not different from those after ecothiopate

Table 1 Acetylcholinesterase activity of homogenates of diaphragm muscle at various times after *in vivo* injection of ecothiopate (ECO), pinacolyl *S*-(2-trimethylaminoethyl)methylphosphonothioate (BOS) or diisopropyl fluorophosphate (DFP)

Dose (μmol kg^{-1})	*Time after injection*	*AChE activity* (nmol min^{-1} mg^{-1})	*Percentage inhibition*
Untreated	–	1.34 ± 0.43 (38)	0
ECO (0.5)	3 h	0.17 ± 0.04 (8)**	87
ECO (0.5)	5 days	1.28 ± 0.23 (11)	4
ECO (0.3)	3 h	0.17 ± 0.05 (8)*[b]	87
ECO (0.1)	3 h	0.57 ± 0.11 (8)*[ab]	57
BOS (8.0)	3 h	0.18 ± 0.05 (11)*	87
BOS (8.0)	5 days	0.79 ± 0.18 (4)*	41
BOS (8.0)	15 days	1.16 ± 0.19 (8)*	13
BOS (1.0)	3 h	0.84 ± 0.07 (4)*[b]	37
BOS (1.0)	5 days	0.96 ± 0.11 (11)**	28
BOS (1.0)	15 days	1.22 ± 0.19 (12)**	9
DFP (14)	3 h	0.12 ± 0.02 (4)*	91
DFP (14)	5 days	1.05 ± 0.15 (5)*	22
DFP (14)	14 days	1.30 ± 0.09 (4)	3
DFP (11)	3 h	0.28 ± 0.05 (8)*	79
DFP (11)	5 days	0.94 ± 0.16 (7)*	30

Values are mean ± s.d. of (*n*) observations. *Indicates significant difference from untreated mice. All values 3 h after drugs are not significantly different from each other except those marked ([a], [b]). **Between values indicates they are significantly different.

Table 2 Mouse phrenic nerve-diaphragm stimulated indirectly at 30 Hz, action potentials (APs) recorded at tendon end of muscle fibres

Time after injection	*Delay of AP16 (μs)*	*MCD (μs)*
Untreated	32 ± 22 (42)	9.9 ± 3.6 (42)
	#	#
Atropine	32 ± 19 (29)	9.4 ± 2.6 (29)
ECO 1 day	103 ± 36 (13)*	23.7 ± 12.1 (13)*
	#	#
3 days	107 ± 50 (14)*	27.9 ± 32.9 (13)*
	#	#
5 days	119 ± 104 (40)*	25.6 ± 28.0 (39)*
7 days	61 ± 37 (18)*	17.8 ± 9.5 (18)*
	#	#
15 days	48 ± 38 (24)	14.7 ± 7.6 (24)*
27 days	25 ± 23 (34)	10.1 ± 3.4 (34)

Delay and the mean consecutive difference (MCD) of APs at various times after ecothiopate 0.5 μmol kg^{-1} (ECO) are shown. Values are mean ± s.d. of (n) observations. Values marked * are significantly different from untreated mice; values either side of # are not different.

(0.5 μmol kg^{-1}). With DFP 14 μmol kg^{-1}, at 5 days the delay was the same as after BOS or ecothiopate; the MCD was not different from that after BOS but greater than after ecothiopate. As with ecothiopate, after BOS and DFP the increase in MCD and delay outlasted the inhibition of AChE. After BOS the AChE was normal at 15 days, when the delay and MCD were still increased. The delay had returned to normal by day 35, and the MCD by day 60 (Table 2). After DFP, the AChE was normal by day 14 and the MCD and the delay, by day 27 (Table 3).

The effect of the acute administration of ecothiopate in vitro

The acute exposure of the phrenic nerve-diaphragm preparation to ecothiopate 0.5 μM *in vitro* caused 90% inhibition of AChE and increased the MCD and delay at 30 Hz to 15.7 ± 6.3 (10) and 138 ± 78 (10), values not different from those 5 days after ecothiopate (0.5 μmol kg^{-1}) *in vivo* (Table 2). At 10 Hz, the delay was 51 ± 24 μs (17) which was greater than that of untreated preparations, whilst the MCD of 11.8 ± 5.4 μs (25) was not different. Thus, the acute effects of inhibition of AChE after ecothiopate *in vitro* are exerted to a greater extent on delay than on MCD and are much more frequency-dependent than the effects of ecothiopate 5 days after administration *in vivo*.

It is concluded that antiChEs in necrotizing doses increased MCD and delay of trains of APs at 30 Hz to a maximum value for 5 days, which thereafter recovered. The period when jitter was increased exceeded the period of recovery of AChE.

Microscopy of endplates after ecothiopate (0.5 μmol kg^{-1})

Phase contrast microscopy of living diaphragms 24 h after a necrotizing dose of ecothiopate (0.5 μmol kg^{-1}) *in vivo* showed in some fibres that the endplate region either appeared swollen with hypercontracted subsynaptic sarcomeres or appeared granular and without cross-striations. Fibres with a granular endplate region or with junctional or extrajunctional contraction clots showed a weak local autofluorescence, when viewed by epifluorescence microscopy with excitation at 450–

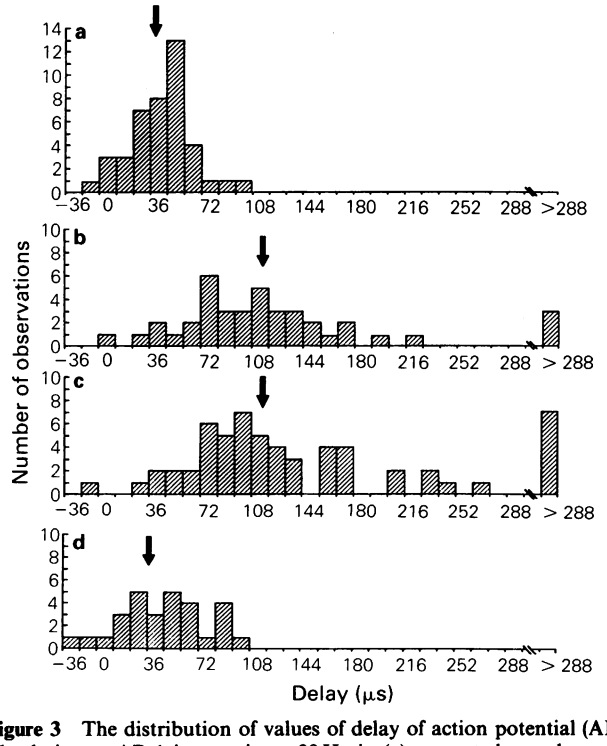


Figure 3 The distribution of values of delay of action potential (AP) 16 relative to AP 1 in a train at 30 Hz in (a) untreated muscle, or 5 days after (b) ecothiopate 0.5 μmol kg^{-1}, or (c) pinacolyl S-(2-trimethylaminoethyl)methylphosphonothioate (BOS) 8.0 μmol kg^{-1} and (d) BOS 1.0 μmol kg^{-1}. The arrows indicate the mean value of the population.

Table 3 The delay of the 16th action potential (30 Hz), with respect to the first action potential, and mean consecutive difference (MCD) of action potentials 11 to 30 after (a) necrotizing and (b) non-necrotizing doses of ecothiopate (ECO), pinacolyl S-(2-trimethylaminoethyl)methylphosphonothioate (BOS) or diisopropyl fluorophosphate (DFP) injected *in vivo*

a	*Necrotizing dose* (μmol kg^{-1})	*Days after injection*	*Delay (μs)*	*MCD (μs)*
	ECO (0.3)	5	52 ± 34 (31)*	12.1 ± 5.2 (31)
	BOS (8.0)	5	162 ± 181 (59)*	53.3 ± 86.2 (59)*
	BOS (8.0)	15	45 ± 28 (28)*	12.0 ± 3.8 (28)*
			#	#
	BOS (8.0)	35	46 ± 20 (42)*	12.3 ± 4.8 (42)
			#	#
	BOS (8.0)	60	37 ± 18 (15)	11.6 ± 4.0 (14)
	DFP (14)	5	153 ± 84 (14)*	60.7 ± 52.6 (14)*
	DFP (14)	14	52 ± 36 (20)*	16.6 ± 11.6 (20)*
			#	#
	DFP (14)	27	33 ± 22 (24)	12.2 ± 4.8 (24)

b	*Non-necrotizing dose* (μmol kg^{-1})	*Days after injection*	*Delay (μs)*	*MCD (μs)*
	ECO (0.1)	5	39 ± 19 (44)	10.8 ± 5.6 (44)
	BOS (1.0)	5	35 ± 32 (29)	17.0 ± 7.9 (29)*
	BOS (1.0)	15	28 ± 20 (21)	10.4 ± 6.6 (21)
	BOS (1.0)	30	23 ± 33 (18)	9.4 ± 3.5 (18)
	DFP (11)	5	48 ± 26 (21)*	10.8 ± 4.1 (21)

Values are mean ± s.d. of (n) observations. Values marked * are significantly different from untreated mice; values either side of # are not different.

Table 4 The delay of the 16th action potential (30 Hz), with respect to the first action potential, and the mean consecutive difference (MCD) of action potentials 11 to 30 in diaphragm, extensor digitorum longus (EDL) and soleus muscles five days after injection of 0.4 or 0.1 μmol kg^{-1} ecothiopate (ECO)

Muscle	*Drug* (μmol kg^{-1})	*Delay* (μs)	*MCD* (μs)
Diaphragm	Untreated	32 ± 22 (42)	9.9 ± 3.6 (42)
	ECO (0.4)	71 ± 77 (31)*	24.7 ± 45.2 (31)*
	ECO (0.1)	39 ± 18.8 (44)	10.8 ± 5.6 (44)
EDL	Untreated	50 ± 27 (32)	10.7 ± 2.9 (32)
	ECO (0.4)	78 ± 45.7 (34)*	19 ± 22.3 (34)*
	ECO (0.1)	56 ± 30.9 (28)	10.6 ± 6.2 (28)
Soleus	Untreated	40 ± 22 (36)	9.2 ± 3.3 (36)
	ECO (0.4)	110 ± 70.4 (30)*	26.3 ± 20.1 (30)*
	ECO (0.1)	64 ± 32.4 (27)*	15.3 ± 10.4 (27)*

Values are mean ± s.d. of (n) observations. *Significantly different from untreated mice.

490 nm and with wavelengths > 525 nm filtered from the emitted beam. Resting membrane potentials and sometimes m.e.p.ps were recorded in undamaged fibres, but convincing RMPs were not recorded from autofluorescing parts of fibres or from endplates which had a granular appearance. It is concluded that such damaged fibres are electrically silent and hence inexcitable and would not have been included in the population showing excessive jitter.

The effect of non-necrotizing doses of antiChEs

Experiments were done with lower doses of antiChEs. After ecothiopate 0.3 μmol kg^{-1} the necrosis was less than after ecothiopate 0.5 μmol kg^{-1} but greater than in untreated animals; thus for ecothiopate, 0.3 μmol kg^{-1} is close to the threshold dose for necrosis, for 0.1 μmol kg^{-1} did not cause necrosis (Das, 1989). The effects of ecothiopate 0.1 and 0.3 μmol kg^{-1} on the delay, MCD and AChE, and the effects of the non-necrotizing doser of BOS (1 μmol kg^{-1}) and DFP (11 μmol kg^{-1}) are shown in Tables 1 and 3b and Figures 2c,d.

Compared with untreated mice, 5 days after ecothiopate 0.3 μmol kg^{-1}, only the delay was increased and to a lesser extent than after ecothiopate 0.5 μmol kg^{-1} or BOS 8 μmol kg^{-1}. After ecothiopate 0.1 μmol kg^{-1}, the delay and MCD were not increased. Five days after BOS 1 μmol kg^{-1} the MCD was increased to a lesser extent than after BOS 8 μmol kg^{-1}, and the effect lasted up to 15 days. After DFP 11 μmol kg^{-1} there was increased delay.

It is concluded that increases in delay and MCD are not necessarily associated with necrosis, and that the extent and the duration of these increases are dose- and drug-dependent.

The effect of ecothiopate on limb muscles

Experiments were done on limb muscles which are less necrotized than diaphragm by ecothiopate (Ferry, unpublished observations). The delay and MCD of diaphragm, extensor digitorum longus (EDL) and, soleus (SOL) muscles were measured 5 days after ecothiopate 0.4 μmol kg^{-1}, which causes necrosis of the diaphragm similar to ecothiopate 0.5 μmol kg^{-1}. Untreated EDL and SOL had values for delay and MCD at 30 Hz similar to diaphragm (Table 4), and after ecothiopate 0.4 μmol kg^{-1} there was an increased MCD, similar in all three muscles, and an increased delay which was greater in SOL than in the others. After ecothiopate 0.1 μmol kg^{-1}, there was increased delay and MCD in SOL but not in EDL or in diaphragm (Tables 3 and 4). Thus SOL was more sensitive to ECO than the other muscles.

Table 5 The delay of the 16th e.p.p. (30 Hz), with respect to the first e.p.p., and MCD of e.p.ps 11 to 30 five days after ecothiopate (ECO), pinacolyl S-(2-trimethylaminoethyl) methylphosphonothiate (BOS) or diisopropyl fluorophosphate (DFP) injected *in vivo*

Drug (μmol kg^{-1})	*Delay* (μs)	*MCD* (μs)
Untreated	38 ± 16 (17)	9.2 ± 3.2 (17)
ECO (0.5)	42 ± 15 (24)	8.9 ± 3.2 (24)
BOS (8.0)	41 ± 13 (14)	10.6 ± 3.2 (14)
DFP (14.0)	24 ± 18 (15)	10.5 ± 5.0 (15)

There was no significant difference between values with ECO, BOS or DFP and those in muscles from untreated mice. Values are mean ± s.d. of (n) observations.

Latencies of e.p.ps in untreated and ecothiopate-treated diaphragms

To investigate if the increased variability in latencies of APs was attributable to increased variability of synaptic events, experiments were done on muscle fibres cut to prevent APs. The electrode was inserted to record m.e.p.ps focally, and the latencies of e.p.ps evoked at 30 Hz were measured. The results were that the delay and MCD of the e.p.ps were not different from those of APs recorded at the tendon end of untreated muscle.

It is concluded that in untreated mice the variability of latencies within the train of e.p.ps is sufficient to account for the variability of latencies of a train of APs recorded near the tendon, and conduction along the muscle fibre causes no further increase in variability.

The MCD and delay of e.p.ps 5 days after ecothiopate 0.5 μmol kg^{-1} were the same as in untreated preparations (Table 5). It is concluded that the increased delay and MCD after ecothiopate cannot be due to increased variation in the timing of release or action of the transmitter.

Discussion

In these experiments, changes in the latency of APs in a train of 30 stimuli were measured by the delay which represents a systematic increase during the change from rest to a steady state of activity, and by the MCD representing a non-systematic variability of latency at this steady state. That the delay and MCD of APs in untreated muscle is frequency-dependent and attributable to the delay and MCD of e.p.ps indicates that this jitter, like the variable quantum content of the e.p.p. may be a normal aspect of neuromuscular transmission (Ferry & Kelly, 1988).

After ecothiopate the MCD and delay of APs at the tendon is increased without increase in the delay and MCD of the e.p.p. Thus the increased jitter is not due to exacerbation of those factors responsible for normal jitter, but to the induction of another factor which appears to be postsynaptic. The mechanism and site of this factor is currently under investigation.

This additional source of jitter after antiChEs results in the distribution of delay and MCD becoming multimodal. After ecothiopate or BOS the distributions contained values indicating that some fibres had apparently normal jitter, some fibres had been moderately affected and some severely affected. In addition to these fibres all of which had carried the train of 30 APs and so had been subject to analysis, there was a fourth population of fibres which was excitable but failed during the train and, thereby, was excluded from the analysis, and a fifth population of fibres which was necrotic and electrically silent. Thus the measurement of jitter underestimates the total damage to muscle.

Regeneration of necrotized fibres begins 2 days after ecothiopate and at 3 days some myoblasts contain regenerated myofibrils (Townsend, 1988). Regenerating necrotized fibres become functionally innervated after 7 days (Grubb & Harris, 1986), and it may be that the few fibres with very large values of MCD and delay could be regenerating.

The extent to which the increased jitter is secondary to the necrosis produced by the antiChEs is questionable. Clearly necrotic cells do not contribute to jitter, for they are electrically silent; and 5 days after non-necrotizing doses of antiChE there is still increased delay and MCD. Thus necrosis is not essential for the appearance of increased jitter. So it may be that the increased jitter in the diaphragm 5 days after a necrotizing dose was in those fibres surviving.

Although there is increased delay and MCD after antiChEs it is likely that these phenomena are not directly related. There is no correlation between the two parameters in the same muscle fibres, untreated or after ecothiopate, except in a few severely affected fibres. Ecothiopate *in vitro*, has a greater effect on delay than on MCD. Delay recovers before MCD after ecothiopate *in vivo*. Furthermore, low doses of BOS increased MCD but not delay, whereas low doses of ecothiopate increased delay more than MCD. Thus it seem that the increased MCD and delay have different causes.

Whether or not the inhibition of AChE at the time of the experiment determines the degree of jitter depends on the interpretation of the biochemical assay of AChE activity of muscle homogenates. It has been found that the jitter after acute exposure to ecothiopate *in vitro*, when inhibition of muscle AChE was maximal, is not different from those peak values of MCD and delay 5 days after injection *in vivo* when AChE was not significantly different from values in untreated diaphragm. This indicates that, although increased jitter is a consequence of AChE inhibition, it does not depend upon the degree of inhibition determined at the same time as the jitter. However, the AChE activity of muscle homogenates measured several days after administration of an antiChE may not represent only the activity of the functional AChE responsible for terminating transmitter action. In our experiments made 5 days after the administration of ecothiopate, about 20% of fibres exhibited repetitive firing after a single stimulus which indicates prolongation of transmitter action, due to inhibition of the functional AChE (Ferry, 1988). Thus at this time, the biochemical determination of AChE activity is not a measure only of the functional AChE responsible for terminating transmitter action; perhaps some other non-functional AChE is also measured.

Whilst it is clear that the increased jitter is a consequence of the inhibition of AChE, the relation between these two remains unclear. The differences in jitter 5 days after ecothiopate 0.5 and 0.3 μmol kg^{-1}, BOS 8 μmol kg^{-1} and DFP 14 μmol kg^{-1}, all of which cause similar inhibition of AChE 3 h after administration, indicate that the maximal extent of inhibition is not sufficient to account for the increase in jitter. Perhaps the rate of onset of inhibition and its persistence may be factors in the aetiology of increased jitter. The relation between AChE activity and jitter thus remains to be determined.

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(+)-Hydrastine, a potent competitive antagonist at mammalian $GABA_A$ receptors

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1 (+)-Hydrastine is a phthalide isoquinoline alkaloid, isolated from *Corydalis stricta*. It has the same 1S,9R configuration as the competitive $GABA_A$ receptor antagonist bicuculline and is the enantiomer of the commercially available (−)-hydrastine.

2 (+)-Hydrastine (CD_{50} 0.16 mg kg^{-1}, i.v.) was twice as potent as bicuculline (CD_{50} 0.32 mg kg^{-1}, i.v.) as a convulsant in mice. This action was stereoselective in that (+)-hydrastine was 180 times as potent as (−)-hydrastine.

3 (+)-Hydrastine was a selective antagonist at bicuculline-sensitive $GABA_A$ receptors in the guinea-pig isolated ileum. It did not influence phaclofen-sensitive $GABA_B$ receptors or acetylcholine receptors in this tissue. (+)-Hydrastine was a competitive antagonist of $GABA_A$ responses (pA_2 6.5) more potent than bicuculline (pA_2 6.1).

4 When tested against the binding of [^{3}H]-muscimol to high affinity $GABA_A$ binding sites in rat brain membranes, (+)-hydrastine (IC_{50} 2.37 μM) was 8 times more potent than bicuculline (IC_{50} 19.7 μM).

5 As an antagonist of the activation of low affinity $GABA_A$ receptors as measured by the stimulation by GABA of [^{3}H]-diazepam binding to rat brain membranes, (+)-hydrastine (IC_{50} 0.4 μM) was more potent than bicuculline (IC_{50} 2.3 μM).

6 (+)-Hydrastine, 10 nM to 1 mM, did not inhibit the binding of [^{3}H]-(−)-baclofen to $GABA_B$ binding sites in rat brain membranes.

Introduction

The discovery of bicuculline as an antagonist of certain inhibitory actions of 4-aminobutanoic acid (GABA) on central neurones provided vital pharmacological evidence for the role of GABA as an inhibitory neurotransmitter in the CNS (Curtis *et al.*, 1970). Mammalian GABA receptors are currently classified into at least two pharmacological classes: $GABA_A$ receptors are antagonized by bicuculline and insensitive to baclofen, whereas $GABA_B$ receptors are activated by baclofen, antagonized by phaclofen and insensitive to bicuculline (Hill & Bowery, 1981; Johnston, 1986; Kerr *et al.*, 1987). Bicuculline is a phthalide isoquinoline alkaloid first isolated from *Dicentra cucullaria* (Manske, 1932) and subsequently from a variety of species of *Corydalis*, *Dicentra* and *Adlumia* (Kametani, 1969). Its convulsant action was reported by Welch & Henderson (1934). Many isoquinoline alkaloids produce convulsions on systemic administration to mammals, but GABA antagonism appears to be restricted to the phthalide isoquinoline alkaloids that have the 1S,9R configuration, i.e. bicuculline and corlumine (Figure 1, Curtis & Johnston, 1974). Thus (−)-bicuculline (1R,9S), (−)-hydrastine (1R,9S), (−)-adlumine (1R,9R) and (+)-adlumine (1S,9S) are inactive as GABA antagonists (Curtis & Johnston, 1974).

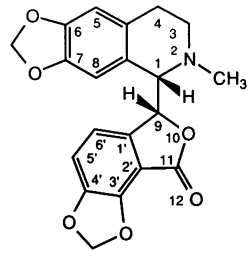
Bicuculline is more potent than the closely structurally related corlumine (Figure 1) as a convulsant and as a selective GABA antagonist (Rice, 1938; Johnston *et al.*, 1972). The present study concerns (+)-hydrastine, a 1S,9R, configured phthalide isoquinoline alkaloid structurally related to bicuculline and corlumine, which has been isolated from *Corydalis stricta* (Fang *et al.*, 1981) and is the enantiomer of the commercially available (−)-hydrastine (Figure 1). (+)-Hydrastine appears to be more potent than bicuculline in 4 test systems using 3 animal species.

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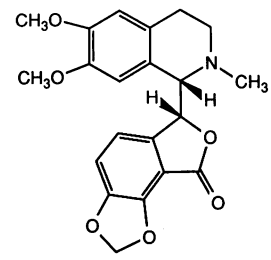
Methods

Convulsant potencies

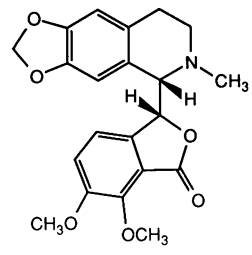
Convulsant potencies were assessed on injection of aqueous solutions of the alkaloids as hydrochlorides into the tail veins of male mice weighing between 18 and 25 g. The CD_{50} value was defined as the dose causing convulsive seizures in 50% of mice within 1 min of injection.



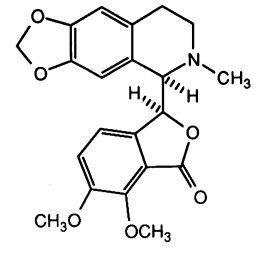
Bicuculline (1S, 9R)



Corlumine (1S, 9R)



(+)-Hydrastine (1S, 9R)



(−)-Hydrastine (1R, 9S)

Figure 1 Structures of the phthalide isoquinoline alkaloids bicuculline, corlumine, (+)-hydrastine and (−)-hydrastine, showing the numbering system and indicating the absolute configurations about carbons 1 and 9.

Guinea-pig isolated ileum preparation

Guinea-pigs of either sex, weighing between 200–400 g, were stunned by a blow on the head, segments of the distal ileum 3–4 cm in length were quickly removed, emptied of their contents and mounted vertically in a 20 ml organ bath containing modified Krebs solution of following composition (mM): Na^+ 151.0, K^+ 4.6, Mg^{2+} 0.6, Ca^{2+} 2.8, Cl^- 134.9, HCO_3^- 24.9, $H_2PO_4^-$ 1.3, SO_4^{2-} 0.6, glucose 7.7 (pH 7.4 at 37°C). The Krebs solution was continuously gassed with a mixture of 95% O_2 and 5% CO_2. The tissues were initially placed under a resting tension of 1 g and were allowed to equilibrate for 60 min in the bath. Isometric contractions of the longitudinal muscle were measured with a Grass FT03 force transducer and recorded on a Grass polygraph. Antagonists were added at least 2 min before agonists were tested at 8–15 min intervals, depending on the recovery of the tissue responses to base line level. Added drug volumes never exceeded 2% of the bath volume (Krantis & Kerr, 1981; Ong *et al.*, 1988).

Ligand binding studies — [^3H]-muscimol, [^3H]-diazepam, and [^3H]-(−)-baclofen

Whole rat brain was homogenized in 1:10 (w/v) volumes of cold 0.32 M sucrose. The homogenate was centrifuged at 1000 *g* for 10 min. The sucrose supernatant was centrifuged at 12,000 *g* for 20 min to yield a crude mitochondrial pellet which was subsequently processed in different ways for the different ligand binding assays.

[^{3}H]-diazepam binding was studied by a filtration assay essentially as described by Skerritt *et al.* (1982a): the well-washed crude mitochondrial pellet was incubated for 20 min at 4°C in 1 ml containing 530 μg membrane protein, 50 mM Tris HCl buffer, 560 pM [^{3}H]-diazepam and test compounds. Nonspecific binding was determined by the addition of 100 μM unlabelled diazepam.

[^{3}H]-muscimol binding was studied in a similar manner using membranes extracted with 0.05% Triton X-100 Tris citrate buffer at 37°C for 30 min. After thorough washing the Triton treated membranes were incubated for 60 min at 4°C in a volume of 1 ml containing 690 μg membrane protein, 50 mM Tris HCl buffer (pH 7.4), 1.47 nM [^{3}H]-muscimol and test compounds. Nonspecific binding was determined by the addition of 100 μM unlabelled GABA.

[^{3}H]-(−)-baclofen binding was studied in a centrifugation assay as described by Drew *et al.* (1984).

Protein for all of the receptor binding assays was measured by the method of Lowry *et al.* (1951).

Materials

(+)-Hydrastine, isolated from *Corydalis stricta*, was the gift of Professor Fang Qi-Cheng in the Institute of Materia Medica, Chinese Academy of Medical Sciences, Beijing. Bicuculline methochloride and phaclofen were synthesized in the Department of Pharmacology at the University of Sydney by Ms Christine Apostopoulos and Dr Ken Mewett respectively. Other substances were obtained from the following sources: (−)-hydrastine, bicuculline, GABA, acetylcholine (Sigma), (−)-baclofen (Ciba Geigy), diazepam (Roche). Radioligands: [^{3}H]-muscimol (New England Nuclear), [^{3}H]-diazepam (Amersham International), and [^{3}H]-(−)-baclofen (New England Nuclear). (+)-Hydrastine, bicuculline, (−)-hydrastine were dissolved in 1 N HCl and neutralised with NaOH to pH 3–4.

Data analyses

Binding data were analysed by the computer programmes EBDA and LIGAND (McPherson, 1983) to obtain K_d (apparent dissociation constant), B_{max} (maximal number of binding sites) and IC_{50} (concentration of inhibitor producing 50% inhibition) values. pA_2 values (a measure of the affinity of a competitive antagonist for its receptor) were calculated from Clark plots (see Krantis & Kerr, 1981) and CD_{50} values (dose producing convulsions in 50% of animals) from log-probit analyses as described by Tallarida & Murray (1981). Values are expressed as means ± standard error (s.e.).

Results

Convulsant potencies

Bicuculline and (+)-hydrastine produced similar convulsions in mice on intravenous injection. (+)-Hydrastine was more potent than bicuculline on the basis of relative CD_{50} values: 0.16 ± 0.01 mg kg^{-1} and 0.32 ± 0.01 mg kg^{-1} respectively (means ± s.e., $n = 10$). The action of (+)-hydrastine was stereoselective in that (−)-hydrastine was some 180 times less potent (CD_{50} 29.8 mg kg^{-1}) than (+)-hydrastine.

Guinea-pig ileal responses to GABA and acetylcholine

When GABA was applied to the guinea-pig isolated ileum, it produced either contractions or biphasic responses (transient contractions followed immediately by relaxation). The GABA-induced contractions, which are known to be mediated by bicuculline-sensitive $GABA_A$ receptors, were antagonized in a dose-dependent manner by (+)-hydrastine, which had no effect upon tissue activity when added to the bath alone. (+)-Hydrastine at doses of 1 and 5 μM elicited a parallel shift to the right in the dose-response curve for GABA-induced contractions (Figure 2). Clark analysis indicated the pA_2 value for (+)-hydrastine was 6.5 ± 0.2 (0.31 μM) while the pA_2 value for bicuculline was 6.12 ± 0.14 (0.75 μM) (Krantis & Kerr, 1981). The pA_2 values showed that (+)-hydrastine is 2.4 times stronger than bicuculline in inhibiting GABA-induced contractile responses in guinea-pig ileum.

(+)-Hydrastine at 1 μM did not show any effect on acetylcholine-induced contractile responses in guinea-pig ileum or on relaxations mediated by $GABA_B$ receptors.

Muscimol binding to high affinity $GABA_A$ binding sites on rat brain membranes

Equilibrium binding assays revealed that (+)-hydrastine and bicuculline inhibited [^{3}H]-muscimol binding to rat brain membranes, whereas (−)-hydrastine was inactive (Figure 3). The following IC_{50} values were determined by computer analyses: (+)-hydrastine 2.37 ± 0.45 μM; bicuculline 19.7 ± 5.2 μM. Scatchard analysis of the results of [^{3}H]-muscimol binding studies indicated binding to a single population of high affinity sites with a K_d of 2.91 ± 0.86 nM and a B_{max} of 1.09 ± 0.18 nmol mg^{-1} protein.

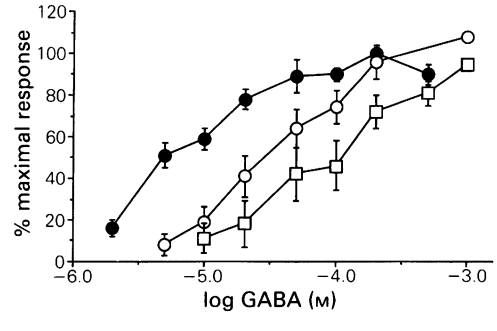


Figure 2 Log dose-response curves of the effects of (+)-hydrastine on GABA-induced contractile responses in the guinea-pig isolated ileum: (●) control response; (○) 1 μM (+)-hydrastine; (□) 5 μM (+)-hydrastine.

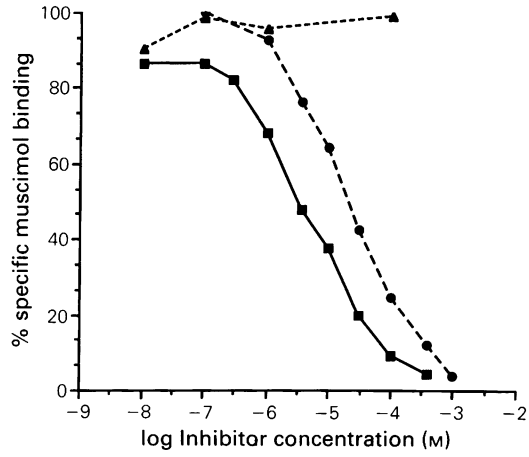


Figure 3 Displacement of [^{3}H]-muscimol binding to rat brain membranes by (+)-hydrastine (■—■), (−)-hydrastine (▲- -▲) and bicuculline (●---●).

GABA-activated diazepam binding to rat brain membranes

GABA at concentrations of 0.1 and 0.2 μM showed no activating effect on [^{3}H]-diazepam binding, but at higher concentrations of 1 μM, especially of 2 μM and 10 μM, GABA had a large activating effect on [^{3}H]-diazepam binding (Figure 4). This action of GABA is known to be mediated via bicuculline-sensitive low affinity GABA receptors. (+)-Hydrastine at a concentration of 10 μM had a strong inhibitory effect on GABA-activated [^{3}H]-diazepam binding. Bicuculline at the same concentration as (+)-hydrastine exhibited a weaker effect on GABA-activated [^{3}H]-diazepam binding. The respective IC_{50} values were (+)-hydrastine 0.4 μM, and bicuculline 2.3 μM, indicating that in this action (+)-hydrastine was some 6 times more potent than bicuculline.

(−)-*Baclofen binding to rat brain membranes*

(+)-Hydrastine, 10 nM to 1 mM, did not inhibit the binding of [^{3}H]-(−)-baclofen to $GABA_B$ binding sites in rat brain membranes.

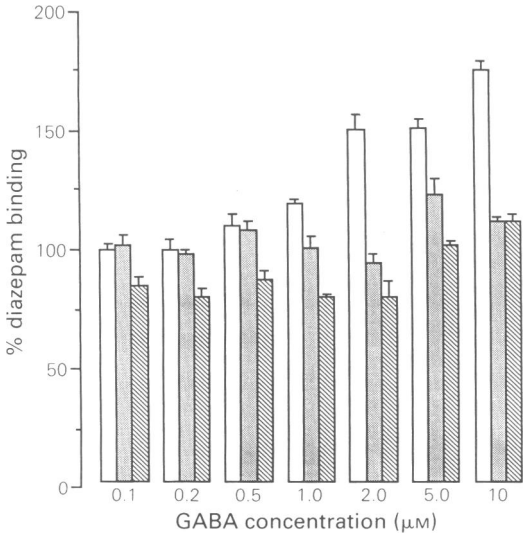


Figure 4 Effects of 10 μM (+)-hydrastine and bicuculline on GABA-stimulated binding of [^{3}H]-diazepam binding to rat brain membranes at various concentrations of GABA: open columns, control; stippled columns, bicuculline; hatched columns, (+)-hydrastine.

Stability of (+)-hydrastine hydrochloride

Olsen *et al.* (1975) found, when they studied several types of assay, that aqueous solutions of bicuculline salts stored at neutral pH values for several hours were less active than fresh solutions due to opening of the lactone ring to give bicucine, which is inactive as a GABA antagonist. A fresh Tris-HCl solution of (+)-hydrastine hydrochloride at pH 7.4 gave an IC_{50} value of 2.4 μM in the muscimol binding assay. The same solution after 3 months storage at 4°C gave IC_{50} value of 5.3 μM in the muscimol binding assay.

Discussion

(+)-Hydrastine is clearly a more potent competitive antagonist at $GABA_A$ receptors than bicuculline. The structural difference between the two alkaloids is that (+)-hydrastine has methoxy substitutions at carbons 3' and 4' whereas bicuculline has a methylenedioxy bridge at these carbons. Binding studies suggest that bicuculline and GABA are able to bind to some common binding sites on the $GABA_A$ receptor complex, since bicuculline is a competitive inhibitor of GABA binding and GABA is a competitive antagonist of bicuculline binding (Andrews & Johnston, 1979). The common binding sites are considered to be the N2-C1-C9-O10-C11-O12 atoms in bicuculline which match the N-C-C-C-C-O sequence of atoms in GABA (Curtis *et al.*, 1970; Andrews & Johnston, 1979). The present studies indicate that the substituents on C4' and C3' on the phthalide system in bicuculline and (+)-hydrastine are important to the GABA antagonist actions of these alkaloids.

GABA binds to 2 kinetically and pharmacologically distinct classes of bicuculline-sensitive $GABA_A$ binding sites on rat brain membranes, with the low affinity binding site being that linked to benzodiazepine receptors (Skerritt *et al.*, 1982b; Johnston, 1986; Allan *et al.*, 1986). The present study indicates that both bicuculline and (+)-hydrastine are more potent antagonists of the low affinity $GABA_A$ binding sites than of the high affinity sites.

Since the discovery of bicuculline as a selective GABA antagonist (Curtis *et al.*, 1970), a number of other competitive GABA antagonists have been described. These include the amidine steroid analogue RU5135 (Hunt & Clements-Jewery, 1981), pitrazepin (Gähwiler *et al.*, 1984), the pyridazinyl-GABA derivative SR95531 (Heaulme *et al.*, 1986) and the alkaloid securinine (Beutler *et al.*, 1985). Studies on the ability of these various classes of $GABA_A$ antagonists to reverse the inhibitory effects of GABA on the binding of [^{35}S]-TBPS (*t*-butylbuyclophosphorothionate) to rat brain membranes suggest that the rank order of potency is RU5135 > pitrazepin > SR95531 > bicuculline > securinine which is similar to that found in studies on antagonism of GABA-induced inhibition of neuronal activity (Squires & Saederup, 1987). The present studies would place (+)-hydrastine as approximately equipotent with SR95531 (2-(carboxy-3'-propyl)-3-amino-6-*p*-methoxyphenylpyradazium chloride). (+)-Hydrastine may offer some advantages over bicuculline in terms of solubility and stability in aqueous solution. The structures of these $GABA_A$ receptor antagonists may aid in the design of further antagonists which may show some selectivity for possible subclasses of $GABA_A$ receptor sites as was proposed by Andrews & Johnston (1979) on the basis of studies of conformationally restricted $GABA_A$ agonists and has been supported by recent cDNA studies indicating a molecular heterogeneity of $GABA_A$ receptor subunit proteins (Ymer *et al.*, 1989).

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Actions of cocaine on rat dopaminergic neurones *in vitro*

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1 Intracellular recordings were made from neurones *in vitro* in the rat substantia nigra zona compacta and ventral tegmental area; the neurones had the characteristic properties of dopamine-containing cells, and fired action potentials spontaneously.

2 Cocaine (1–10 μM) inhibited spontaneous firing, hyperpolarized the membrane and (in neurones voltage-clamped at −60 mV) caused an outward membrane current; the minimally effective concentration was 1 μM. These effects were blocked by sulpiride (30 nM–1 μM).

3 Dopamine (3–100 μM) also inhibited firing, hyperpolarized and caused an outward current. These effects of dopamine were potentiated about five fold by cocaine (10 μM).

4 It is concluded that cocaine (1–10 μM) inhibits the firing and hyperpolarizes substantia nigra zona compacta neurones *in vitro* by blocking the uptake of dopamine which the cells continuously release.

Introduction

Substantial evidence indicates that dopaminergic neurones are critically involved in the rewarding actions of cocaine, and the projection from mesencephalon to nucleus accumbens is clearly implicated (Fibiger, 1978; Roberts & Koob, 1982; Wise, 1984; Koob & Bloom, 1988). There is also a strong correlation between the affinity of cocaine and related substances in binding to dopamine uptake sites, and their propensity for self-administration (Ritz *et al.*, 1987). In turn, there is a good agreement between inhibition of [^{3}H]-cocaine binding and inhibition of dopamine uptake (Schoemaker *et al.*, 1985). It is assumed therefore that an important action of cocaine, and one that contributes to its reinforcing effect, is the inhibition of uptake of dopamine and thus an increase in the extracellular dopamine concentration. Recent studies have measured extracellular dopamine by intracerebral dialysis or *in vivo* voltammetry and provide direct evidence that this is increased by cocaine (Bradberry & Roth, 1988; DiChiara & Imperato, 1988; Pettit *et al.*, 1988; Nicolaysen *et al.*, 1988).

Such an increase in the extracellular concentration of dopamine would be expected to have at least three effects on the overall function of the mesolimbic projections. First, cell bodies and dendrites of the dopamine cells in the ventral tegmental area and substantia nigra will be hyperpolarized and will fire action potentials less frequently or not at all. This effect is expected because dopamine is released from these cell body regions (Cheramy *et al.*, 1981); inhibition by cocaine of the uptake of released dopamine would be expected to increase the extracellular dopamine concentration. Exogenous dopamine is known to inhibit the firing of substantia nigra neurones by acting at D_2 receptors to increase the membrane potassium conductance (Bunney *et al.*, 1973; Groves *et al.*, 1975; Lacey *et al.*, 1987). Second, each action potential that propagates into the terminal regions of the dopamine projections within the nucleus accumbens may release less dopamine, because of increased activation of presynaptic D_2 receptors (Starke, 1981; Tepper *et al.*, 1985). Third, the effects on the nucleus accumbens neurones of any dopamine that is released will be enhanced. This third action stands in contrast to the first two, which would tend to reduce the dopamine concentration around nucleus accumbens neurones.

In the intact animal these three effects would be interdependent. For example, extracellular recording *in vivo* (Woodruff *et al.*, 1976; White & Wang, 1986; White *et al.*, 1987) and intracellular recording *in vitro* (Uchimura *et al.*, 1986; Higashi *et al.*, 1989; Uchimura & North, 1990) indicates that the predominant action of dopamine on nucleus accumbens neurones is inhibition. This could itself alter the firing of mesencephalic dopaminergic neurones, which receive both direct and indirect inputs from the accumbens (reviewed by Paxinos, 1985). Given the likelihood of such complex interactions *in vivo* it was thought of value to isolate the effects experimentally by studying the action of cocaine *in vitro*. Thus, the purpose of the experiments described in this paper was to determine the effects of known concentrations of cocaine on substantia nigral and ventral tegmental neurones; the following paper (Uchimura & North, 1990) presents the results of similar experiments for the nucleus accumbens.

Methods

These have been described previously (Lacey *et al.*, 1987; 1989). In brief, rats were killed following anaesthesia with halothane. The brain was removed and slices (300 μm) of ventral mesencephalon were cut with a vibratome; the slice which contained the medial accessory nucleus of the optic tract was transferred to a recording chamber. It was superfused by a solution containing (mM): NaCl 126, KCl 2.5, NaH_2PO_4 1.2, $MgCl_2$ 1.3, $CaCl_2$ 2.4, glucose 10 and $NaHCO_3$ 26 that was saturated with 95% O_2 and 5% CO_2 and heated so that the temperature within the chamber was 36°C.

Intracellular recordings were made with glass microelectrodes containing potassium chloride (2 M) from the medial third of the substantia nigra zona compacta and ventral tegmental area; these structures could be visualized with a dissecting microscope (Lacey *et al.*, 1989). Membrane potential and membrane current were measured using an Axoclamp 2A single-electrode voltage clamp amplifier; permanent records were made on a Gould 2400 chart recorder. Drugs were applied in known concentrations by changing the superfusing solution to one differing only in its content of the drug; the new solution entered the chamber within 30 s of turning the tap that began its flow, and complete exchange of the contents of the recording chamber required about 2.5 min. When the period of drug application is illustrated in figures, this is the period during which the tap was turned to allow the flow of the drug-containing solution. In some experiments, dopamine was also applied by ejecting a few nl of a solution containing 10 mM dopamine from a glass pipette; the tip of the pipette (diameter about 10 μm) was positioned above the tissue slice but beneath the surface of the superfusing solution, and pressures of 70 kPa for 100 ms were typically used to eject dopamine.

Dopamine hydrochloride (Sigma) was dissolved in 0.9% saline on the day of each experiment, and gassed with N_2 until further dilution immediately prior to application to the tissue. Other drugs used were cocaine hydrochloride, tetrodotoxin

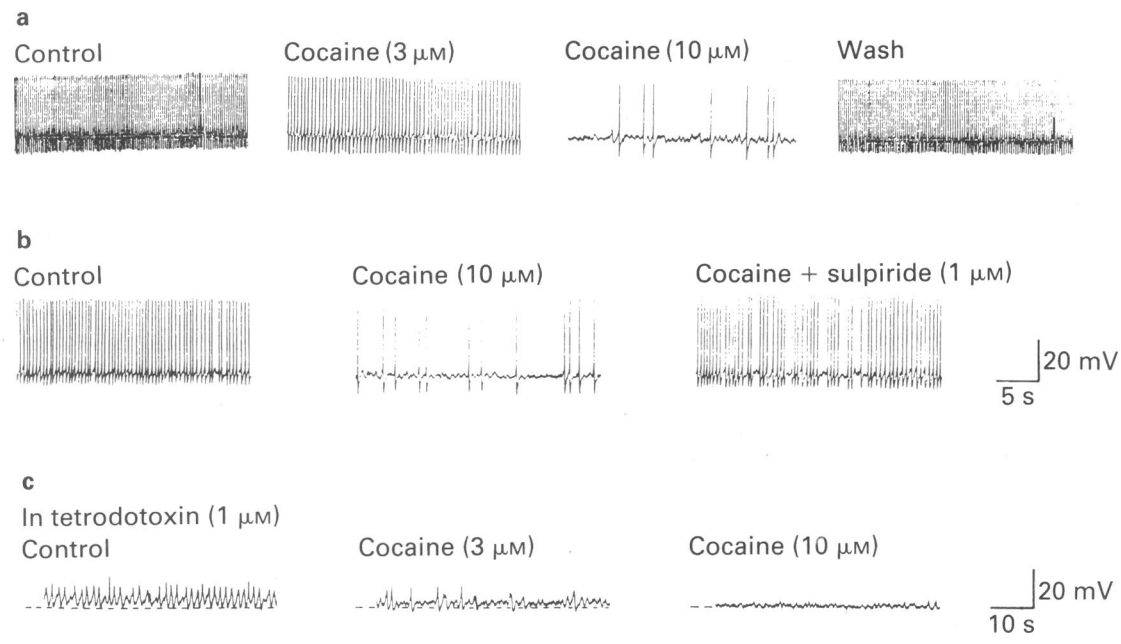


Figure 1 Cocaine inhibits spontaneous firing. (a) Cocaine (3 μM) reduced the rate of action potential firing by 46%, and 10 μM inhibited firing by 96%. After 15 min washing with a cocaine-free solution the control firing rate was restored. (b) The action of cocaine is prevented by sulpiride. Left panel, control. Middle panel, 5 min after changing to cocaine (10 μM). Right side, 3 min after adding sulpiride (1 μM). (c) Cocaine is effective in the presence of tetrodotoxin. Tetrodotoxin blocks the action potentials, revealing low amplitude spontaneous depolarizations (these are reversibly blocked in calcium-free solutions; see Lacey *et al.*, 1987). Cocaine inhibits these spontaneous depolarizations, and slightly hyperpolarizes the membrane. (a) and (b) are from same neurones. Broken line indicates −60 mV. Full amplitude of action potentials are not reproduced due to limited frequency response of pen recorder.

(TTX), 5-hydroxytryptamine creatinine sulphate, noradrenaline bitartrate (all from Sigma) and (−)-sulpiride (gift of Ravizza, Milan, Italy). Numerical data are expressed as mean ± standard error of the mean (s.e.mean).

Results

The results are based on recordings from 75 cells in the medial substantia nigra and 12 in the ventral tegmental area; no significant differences were seen between the two groups of cells. The properties of the cells were as previously described for the principal cells of the substantia nigra zona compacta (Lacey *et al.*, 1987; 1988; 1989); dopamine was applied to 72 neurones and all responded with inhibition of firing and/or hyperpolarization.

Cocaine hyperpolarizes and inhibits firing

Cocaine reduced the frequency of spontaneous action potentials (Figure 1). The inhibition caused by cocaine (1 μM) was 24 ± 10% ($n = 5$), by 3 μM 45 ± 15% ($n = 5$), and by 10 μM 81 ± 8% ($n = 37$, including 14 cells in which firing was completely stopped). Cocaine (10 μM) caused a small hyperpolarization (3.4 ± 0.4 mV, $n = 30$) in cells that were held at −60 mV (by passing a constant current) to prevent action potential discharge. In neurones voltage-clamped at −60 mV, cocaine (10 μM) caused an outward current of 35 ± 4 pA ($n = 21$) (Figure 2b). These actions of cocaine became maximal with applications of 5–10 min duration, did not decline during applications of up to 2 h, and reversed fully within 30 min of washing the tissue with a cocaine-free solution.

The D_2 receptor antagonist (−)-sulpiride (30 nM-1 μM) rapidly and fully reversed the effects of cocaine ($n = 12$) (Figure 1b); (−)-sulpiride had no effect on the properties of the neurones when applied in the absence of cocaine (Lacey *et al.*, 1987). Tetrodotoxin (1 μM), which blocked the spontaneous action potentials (see Lacey *et al.*, 1987), did not prevent the action of cocaine ($n = 3$) (Figure 1c).

Cocaine potentiates the effects of exogenous dopamine

The effect of dopamine was increased by cocaine: this was true for inhibition of firing (Figure 2a), hyperpolarization outward current (Figure 2b). This was particularly marked when dopamine was ejected briefly into the superfusing solution above the brain slice (Figure 2a). Concentration-response curves for applied dopamine were shifted to the left by cocaine (Figure 3a). Where a maximal response to dopamine was clearly obtained, the potentiation could be measured by calculating the dopamine concentration required to cause half the maximal effect (EC_{50}); cocaine (10 μM) caused a reduction in the dopamine EC_{50} by a factor of 4.4 ± 0.7 ($n = 5$).

Such a simple approach to the measurement of the potentiating action of cocaine was complicated by the effect of cocaine itself. Indeed, cocaine (10 μM) alone had an effect that comprised 33 ± 2% of the effect of a maximal concentration of dopamine measured in the presence of cocaine ($n = 10$; the effect was inhibition of firing, hyperpolarization from −60 mV, or outward current at −60 mV). Furthermore, the potentiation of the action of dopamine became less as the concentration of dopamine increased from 3 to 30 μM (Figure 3b); cocaine did not potentiate the effect of 100 μM dopamine ($n = 4$).

Hyperpolarizations caused by noradrenaline (10 μM), which acts through D_2 receptors on these cells (Lacey *et al.*, 1987) were unaffected by cocaine (10 μM, 2 cells). 5-Hydroxytryptamine (10–100 μM) did not hyperpolarize any substantia nigral neurones ($n = 3$).

Discussion

There are two distinct yet interrelated findings. First, cocaine directly inhibits the firing of neurones in the substantia nigra and ventral tegmental area: inhibition has been shown previously using extracellular recording *in vitro* (Brodie & Dunwiddie, 1986) and *in vivo* (Einhorn *et al.*, 1988). Our results indicate that the inhibition results from an outward mem-

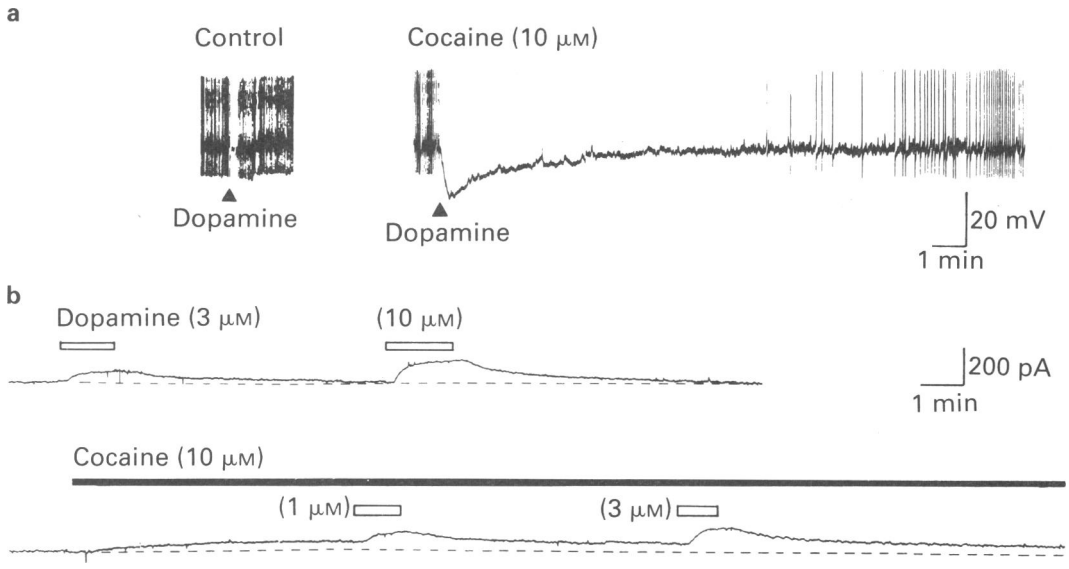


Figure 2 Cocaine potentiates the action of dopamine. (a) Left, dopamine was applied from a pipette tip positioned above the tissue surface (70 kPa pressure pulse for 100 ms). This caused a transient inhibition of firing. Right, the same application of dopamine was repeated when the superfusing solution contained cocaine (10 μM). The effect of dopamine was much prolonged. (b) A similar experiment in which the neurone was voltage-clamped at −58 mV, and both dopamine (open bars) and cocaine (solid bar) were applied by superfusion. The effect of 3 μM dopamine was greater in the presence of cocaine.

brane current similar to that produced by agonists at D_2 receptors (Lacey *et al.*, 1987). Second, cocaine potentiates the inhibitory action of exogenously applied dopamine. These results will be discussed in turn.

Cocaine inhibits cell firing by blocking dopamine uptake

The simplest interpretation of the first result is that cocaine inhibits the firing of the neurones by increasing the concentration of dopamine in the extracellular fluid, and that this results from inhibition of uptake of dopamine that is continuously being released from neurones in the tissue slice. The main evidence for this is the reversal of the effect by sulpiride. We have previously shown that sulpiride competitively antagonizes the inhibitory action of dopamine on substantia nigral neurones, with a dissociation equilibrium constant of about 20 nM (Lacey *et al.*, 1987). Three other kinds of evidence support this interpretation. First, the spontaneous release of dopamine from slices of rat substantia nigra can be directly measured (Cuello & Iversen, 1978), even in the presence of tetrodotoxin (Tagerud & Cuello, 1979). Second, cocaine (1 mg kg^{-1}) has recently been shown directly to elevate the dopamine concentration in the ventral tegmental area measured by *in vivo* dialysis (Bradberry & Roth, 1988). Third, the concentrations of cocaine that were effective (1–10 μM) are similar to those shown to impair dopamine uptake into striatal synaptosomes or tissue slices (Heikkila *et al.*, 1975; Kennedy & Hanbauer, 1983; Hadfield & Nugent, 1983; Missale *et al.*, 1985; Bonnet *et al.*, 1986; Uchimura & North, 1990; see also Table 1 of Nicolaysen *et al.*, 1988). Additionally, these concentrations represent the effective range over which cocaine enhanced the action of exogenous dopamine in the present experiments. Taken together, the present results are consistent with the notion that cocaine is acting by blocking the uptake of dopamine: the large effect of cocaine at a low dopamine concentration (for example, zero or 3 μM) and the lack of effect of cocaine at a very high dopamine concentration (100 μM) would be expected from a saturable uptake process. Unfortunately, more detailed quantification of the results is nigh impossible. The exogenous dopamine adds to the effect of an unknown concentration of endogenous dopamine (e.g. Figure 2b); this unknown concentration may also change as a result of the exogenous dopamine hyperpolarizing the cells.

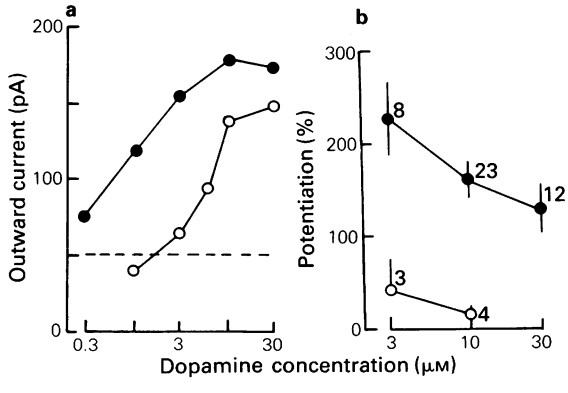


Figure 3 Potentiation of the action of dopamine by cocaine. (a) Outward currents evoked by dopamine recorded from one neurone before (○) and after addition of cocaine (●). Cocaine itself caused an outward current of 50 pA (broken line). (b) Average effect of cocaine in several neurones; response measured was change in firing rate, membrane hyperpolarization or outward current. The potentiation is expressed as {100 × (Increment in response to dopamine caused by cocaine)/Response to dopamine in absence of cocaine}; thus a value of 100 indicates that the response to dopamine in the presence of cocaine (which includes the effect of the cocaine alone, see Figure 2) was twice as large as the response to dopamine in the absence of cocaine. Cocaine 1 μM (○) has little potentiating effect but at 10 μM (●) it causes a large increase in the response to dopamine.

The concentration of cocaine in the plasma of man following an intravenous dose (32 mg) that gives clear subjective effects is about 1 μM (Javaid *et al.*, 1978), which is about the threshold concentration effective in the present experiments. However, if one assumes that cocaine inhibits an uptake process showing typical Michaelis-Menten properties, then the effective concentration of cocaine would depend on the ambient concentration of dopamine. There are no direct measurements that have compared the extracellular dopamine concentration *in vivo* and *in vitro*; all that can be said is that the similar pattern of firing of the neurones in the two circumstances (Lacey *et al.*, 1987; Grace & Bunney, 1983) would be consistent with a similar extracellular dopamine concentration.

Comparison with cocaine actions on other amine neurones

Although the interaction with dopamine cannot be quantified readily, the results nevertheless provide an estimate of the concentrations of cocaine that are effective to potentiate dopamine actions in the cell body region of the substantia nigra and ventral tegmental area. These can be compared with the concentrations effective in other nuclei (noradrenaline cells of the locus coeruleus: Surprenant & Williams, 1987; 5-hydroxytryptamine cells of the dorsal raphe: Williams & Lacey, 1988; Pan & Williams, 1989). In the locus coeruleus, the threshold concentration is about 1 μM and in the dorsal raphe about 300 nM. In both these tissues, cocaine alone causes a hyperpolarization of the membrane which (at 10–30 μM) can approach that caused by a high concentration of exogenous agonist (noradrenaline and 5-hydroxytryptamine, respectively). This implies that in those cell body regions, as in the substantia nigra/ventral tegmental area, an important action of cocaine in the brain slice preparation is to inhibit the uptake of amine that is being continuously released. Given that the experimental conditions are otherwise very similar, cocaine appears to be about 3–10 times more effective in inhibiting the uptake of 5-hydroxytryptamine than that of dopamine or noradrenaline.

Significance for cocaine reinforcement

Adequate concentration of cocaine to reinforce its acute self-administration in rats are about 0.5–1 mg kg^{-1} (e.g. Roberts & Koob, 1982). According to recent measurements of cocaine and dopamine levels in striatal dialysates following an intraperitoneal injection, a dose of 1 mg kg^{-1} in a rat would be expected to give a brain cocaine level considerably less than 1 μM (Church *et al.*, 1987; Nicolaysen *et al.*, 1988). An injection of 10 mg kg^{-1} gave a predicted brain cocaine level of about 3 μM; this is reasonably consonant with the finding that the inhibitory effect of this dose of cocaine on rat ventral tegmental area neurones was small, and partly attributable to descending influences from the nucleus accumbens (Einhorn *et al.*, 1988).

These results are unsurprising if one is to accept the general view that an increase in tone in the mesolimbic pathway is associated with reinforcement (Wise, 1984; see Liebman & Cooper, 1989). If the inhibition of dopamine uptake is indeed the principal cellular mechanism underlying the behavioural effect of cocaine, then the action in the cell body regions would tend to reduce the amount of dopamine released onto limbic target cells. The corollary is that the potentiation of the action of dopamine at the target cells in the nucleus accumbens is more likely to play a role in the reinforcing aspects of the action of cocaine. The following paper (Uchimura & North, 1990) provides an account of the action of cocaine on nucleus accumbens neurones *in vitro*.

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Actions of cocaine on rat nucleus accumbens neurones *in vitro*

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1 Intracellular recordings were made from 103 neurones of the rat nucleus accumbens *in vitro*.

2 Dopamine (3–100 μM; in sulpiride, 1 μM) hyperpolarized neurones (79%) by acting at D_1 receptors: dopamine (3–100 μM; in SCH23390, 1 μM) depolarized neurones (55%) by acting at D_2 receptors. 5-Hydroxytryptamine (1–100 μM) depolarized 86% neurones.

3 Both actions of dopamine as well as the effect of 5-hydroxytryptamine were potentiated by cocaine (0.3–30 μM), which had no effect of its own on membrane potential.

4 Dose-ratio was computed as [(concentration of agonist causing a 4 mV potential change in cocaine)/(concentration of agonist causing a 4 mV potential change without cocaine)]. Cocaine (1–30 μM) caused the same dose-ratio whether dopamine depolarizations (D_2) or hyperpolarizations (D_1) were measured; the dose-ratio ranged from 2 (1 μM) to 50 (30 μM).

5 Responses to 5-hydroxytryptamine were increased more than responses to dopamine; cocaine 1 μM gave a dose-ratio of 13.4 and at 30 μM gave a dose-ratio of 118.

6 It is concluded that cocaine acts to inhibit the uptake of dopamine and 5-hydroxytryptamine in slices of rat nucleus accumbens; lower concentrations of cocaine (0.3 to 1 μM) are particularly effective in potentiating the action of 5-hydroxytryptamine.

Introduction

The nucleus accumbens receives innervation from the dopamine-containing fibres originating in the ventral tegmental area and medial substantia nigra (Björklund & Hökfelt, 1984). Extracellular recording *in vivo* has shown that the main action of dopamine on rat nucleus accumbens neurones is inhibition (Woodruff *et al.*, 1976; White & Wang, 1986). This has been confirmed by *in vitro* experiments in the guinea-pig and rat in which controlled concentrations of agonists and antagonists were used (Higashi *et al.*, 1989; Uchimura *et al.*, 1986): the D_1 receptor is involved in this inhibition, activation of which increases the membrane potassium conductance. However, dopamine has an additional depolarizing action on a smaller group of nucleus accumbens neurones; this results from D_2 receptor activation and closure of potassium channels.

There is good evidence that an important site of action of cocaine is within the nucleus accumbens where it potentiates the action of released dopamine (see Koob & Bloom, 1988; Liebman & Cooper, 1989); the dopamine innervation of the accumbens must be intact for cocaine to be self-administered (Wise, 1984; Koob & Bloom, 1988). However, since cocaine is likely to act at many sites in the nervous system when administered systematically, it was thought of value to determine the concentrations of cocaine needed to potentiate actions of dopamine in isolated nucleus accumbens tissue. This was done in the present experiments by measuring the change in sensitivity to exogenous dopamine caused by various concentrations of cocaine, having first separated the two actions of dopamine (hyperpolarization and depolarization) with selective antagonists.

5-Hydroxytryptamine (5-HT)-containing fibres originating in the dorsal raphe also innervate the nucleus accumbens (Steinbusch, 1981), and 5-HT depolarizes and excites the neurones by reducing the conductance of inwardly rectifying potassium channels (North & Uchimura, 1989). Thus the predominant influences of dopamine and 5-HT on accumbens neurones are opposing; this is consistent with results of experiments which indicate that 5-HT decreases movements when administered into the nucleus (Pijenburg *et al.*, 1976) whereas dopamine agonists stimulate locomotion (Kelly & Roberts, 1983). Because cocaine also inhibits the uptake of 5-HT, it is possible that the potentiation of the actions of 5-HT in the accumbens also contributes to the overall effect of cocaine. A second purpose of the present experiments was to determine the concentrations of cocaine that were effective in potentiating the actions of 5-HT in the nucleus accumbens.

Methods

The methods used were similar to those previously described (Uchimura *et al.*, 1986; Higashi *et al.*, 1989; North & Uchimura, 1989). Rats (200–300 g) were anaesthetized with ether, and the brain was rapidly removed and then sliced frontally with a vibratome. A single slice (about 300 μm thick) containing a section of nucleus accumbens was transferred to a recording chamber (about 500 μl capacity) and submerged in a continuously flowing (3 ml min^{-1}) solution at 36°C. The solution contained (mM): NaCl 126, NaH_2PO_4 1.2, $MgCl_2$ 1.3, $CaCl_2$ 2.4, KCl 2.5, $NaHCO_3$ 26, glucose 10 and was gassed with 95% O_2 and 5% CO_2. Intracellular recordings were made from neurones lying within 500 μm medial to the anterior commissure, at a level of the nucleus accumbens that was about one-third of the distance along its rostral-caudal axis. The electrodes contained potassium chloride (2 M) and had resistances of 40–80 MΩ; both membrane potential and membrane current were measured (Axoclamp 2A amplifier). In voltage-clamp experiments the switching frequency was 2.5–3 kHz, and the head-stage potential was continuously monitored on a separate oscilloscope. The membrane current in response to slow ramp changes in membrane potentials (1 mV s^{-1}) was sometimes plotted directly onto an X–Y plotter; current and potential were plotted on a Gould 2400 pen recorder.

Drugs were applied to the tissue by changing this solution to one which differed only in its content of the drug. Because of the time required for the incoming solution to pass through a heat-exchanger, there was a delay of up to 1 min between the time of selecting the new solution and its first arrival at the tissue; the figures in this paper illustrate the times at which new solutions were selected. Drugs used were cocaine hydrochloride, dopamine hydrochloride, 5-hydroxytryptamine creatinine sulphate (Sigma), quinpirole (Lilly), SCH23390 (7-chloro-2,3,4,5-tetrahydro-3-methyl-5-phenyl-1H-3-benzazepine-7-ol maleate, Schering), SKF38393 (2,3,4,5-tetrahydro-7,8-dihydroxy-1-phenyl-1H-3-benzazepine hydrochloride, Smith Kline French) and (−)-sulpiride (Ravizza).

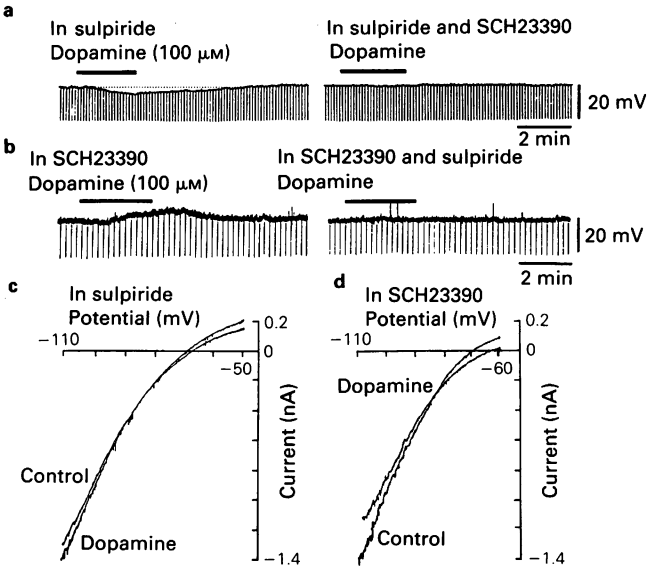


Figure 1 Receptor selective actions of dopamine. (a) In sulpiride (1 μM), dopamine hyperpolarizes a nucleus accumbens neurone (left trace). This is blocked by SCH23390 (1 μM, right trace). Control membrane potential, −75 mV. (b) In SCH23390 (1 μM), dopamine depolarizes a nucleus accumbens neurone (left trace). This is blocked by sulpiride (1 μM). Control membrane potential, −74 mV. In (a) and (b) the downward deflections are electrotonic potentials in response to a fixed current pulse (200 pA: 500 ms in a; 250 pA: 500 ms in b) repeatedly passed across the membrane. (c) Dopamine acts at a D_1 receptor to increase potassium conductance. Sulpiride (1 μM) present. Membrane currents (control and in 100 μM dopamine) were plotted in response to slow ramp depolarizing commands from −110 to −50 mV at 1–2 mV s^{-1}. (d) Dopamine acts at a D_2 receptor to close potassium channels. SCH23390 (1 μM) present. Membrane currents plotted as in (c). In (c) and (d) the extracellular potassium concentration was 6.5 mM.

Results

The present results are based on recordings made from 103 neurones, the properties of which were similar to those previously described for the rat nucleus accumbens (Higashi *et al.*, 1989; North & Uchimura, 1989). The resting potential of the neurones was −82.8 ± 0.9 mV (mean ± s.e.mean).

Actions of dopamine and 5-hydroxytryptamine

Dopamine has two effects on rat nucleus accumbens neurones (Uchimura *et al.*, 1986; Higashi *et al.*, 1989); about 50% of cells show a hyperpolarization followed by a depolarization, about 28% show only a hyperpolarization, and about 10% show only a depolarization. The predominance of the biphasic response to dopamine presented a problem in interpreting any potentiating action of cocaine, since the opposing effects might cancel each other. We therefore first confirmed that the two actions of dopamine could be separated with selective agonists and antagonists, prior to an examination of the effect of cocaine.

In the presence of sulpiride (1 μM), dopamine (3–100 μM) only hyperpolarized nucleus accumben neurones (n = 22 of 28 cells tested) (Figure 1a). In the presence of SCH23390 (1 μM), dopamine (3–100 μM) only depolarized the neurones (n = 25 of 45 cells) (Figure 1b). The antagonists themselves had no effect on the membrane potential. This selectivity was confirmed by experiments with other agonists; quinpirole (0.3–3 μM) caused a concentration-dependent depolarization (n = 7) whereas SKF38393 (0.3–3 μM) caused a concentration-dependent hyperpolarization (n = 8).

These actions of dopamine were studied under voltage clamp (n = 21). In the presence of sulpiride, dopamine caused an outward current at the resting potential which reversed to inward when the potential was hyperpolarized from the resting level (Figure 1c). In the presence of SCH23390, dopamine caused an inward current which reversed to outward when the membrane was hyperpolarized (Figure 1d). The observed reversal potentials conformed to the potassium equilibrium potential, as determined under similar conditions for the reversal of the dopamine-induced potential changes (Uchimura *et al.*, 1986; Higashi *et al.*, 1989) and for the reversal of the current induced by 5-HT (North & Uchimura, 1989).

The depolarization caused by 5-HT (n = 36 of 42 cells) was in all respects similar to that described previously (North & Uchimura, 1989).

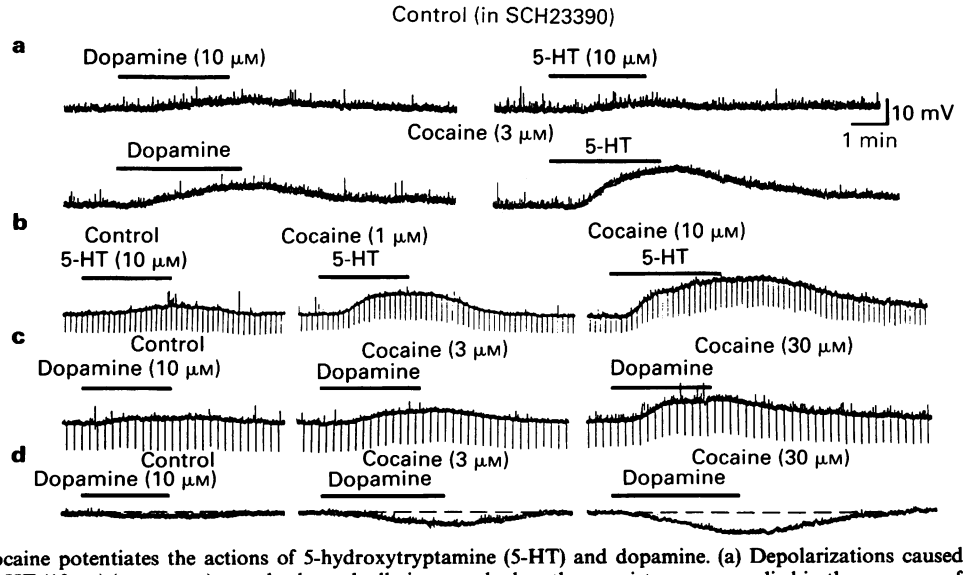


Figure 2 Cocaine potentiates the actions of 5-hydroxytryptamine (5-HT) and dopamine. (a) Depolarizations caused by dopamine (10 μM) and 5-HT (10 μM) (top traces) were both markedly increased when the agonists were reapplied in the presence of cocaine (3 μM) (bottom traces). The potentiation of the response to 5-HT was greater than for the response to dopamine. SCH23390 (1 μM) present throughout. Control membrane potential, −77 mV. (b) Potentiation of response to 5-HT (10 μM) by 1 μM and 10 μM cocaine. Control membrane potential, −76 mV. Downward deflections are electrotonic potentials, current pulse was 100 pA for 500 ms. (c) Potentiation of depolarizing (D_2) response to dopamine (10 μM) by 3 μM and 30 μM cocaine. SCH23390 (1 μM) present. Control membrane, −78 mV. Downward deflections are electrotonic potentials, current pulse was 150 pA for 500 ms. (d) Potentiation of hyperpolarizing (D_1) response to dopamine (10 μM) by 3 μM and 30 μM cocaine. Sulpiride (1 μM) present. Control membrane potential, −76 mV.

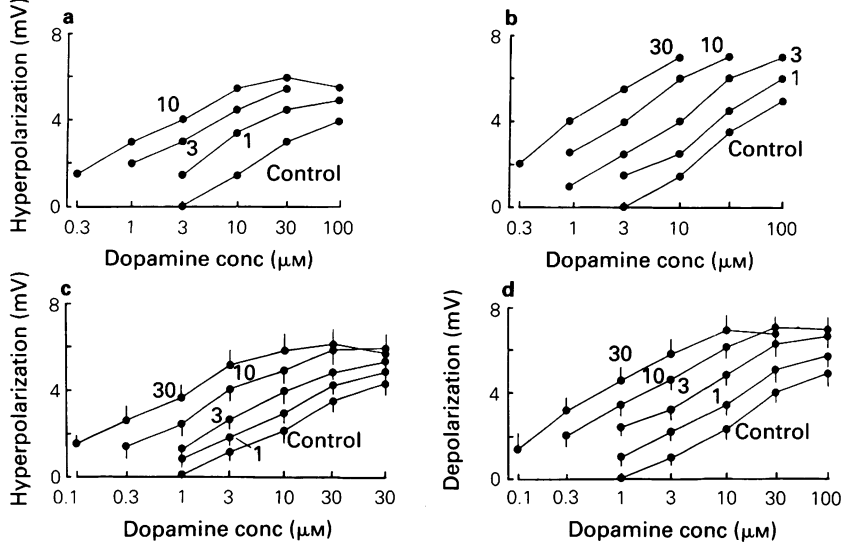


Figure 3 Potentiation of responses to dopamine. (a) and (b) Results from two neurones. (c) and (d) Results from many neurones (points are mean, with s.e.mean shown by vertical lines, 5–19 cells at each point). In each panel, the most righthand curves are the control responses to dopamine and the curves to the left of these were made in the presence of 1, 3, 10 and 30 μM cocaine as indicated. (a and c) Hyperpolarization (D_1) measured in sulpiride (1 μM). (b and d) Depolarization (D_2) measured in SCH23390 (1 μM). Membrane potential of neurones was held at −75 to −78 mV prior to dopamine application.

Cocaine potentiates effects of dopamine and 5-hydroxytryptamine

The actions of cocaine on the responses to dopamine and 5-HT are presented separately. However, some of the same cells were tested with both dopamine and 5-HT, before and after cocaine (e.g. Figure 2a). Cocaine (up to 10 μM) had no effect of its own on membrane potential; at 30 μM it caused a depolarization of up to 2 mV in 3 of 18 cells.

Hyperpolarization: D_1 receptors These experiments were done in the presence of sulpiride (1 μM). The hyperpolarization caused by dopamine was progressively potentiated as the concentration of cocaine was increased; this was evident during the course of recording from a single cell to which several different concentrations were applied (Figures 2 and 3), or by examining the averaged effects of dopamine/cocaine combinations on several cells (Figure 3). The minimum effective concentration of cocaine was 1 μM, and 10 μM caused a potentiation of approximately 21 fold (measured at 4 mV response level; see Figure 3).

Depolarization: D_2 receptors These experiments were done in the presence of SCH23390 (1 μM). The depolarization caused by dopamine was progressively potentiated by cocaine as the concentration was increased. This was clear from studying the action on individual cells (Figures 2 and 3), or from examining the average effect of dopamine in different cocaine concentrations (Figure 3). The threshold concentration of cocaine was 1 μM and a concentration of 10 μM increased the dopamine sensitivity by a factor of 19 (measured at the 4 mV level, Figure 3).

Depolarization: 5-HT_2 receptors Lower concentrations of 5-HT were needed to depolarize the accumbens neurones when cocaine was present. The potentiation of the action of 5-HT was obvious on individual cells (Figures 2 and 4), and was also clear from examination of the average effect of 5-HT in various concentrations of cocaine (Fig. 4). A concentration of 300 nM cocaine had a clear effect on the response to 5-HT, and with 10 μM cocaine the sensitivity was increased by a factor of 100 (measured at the 4 mV response level).

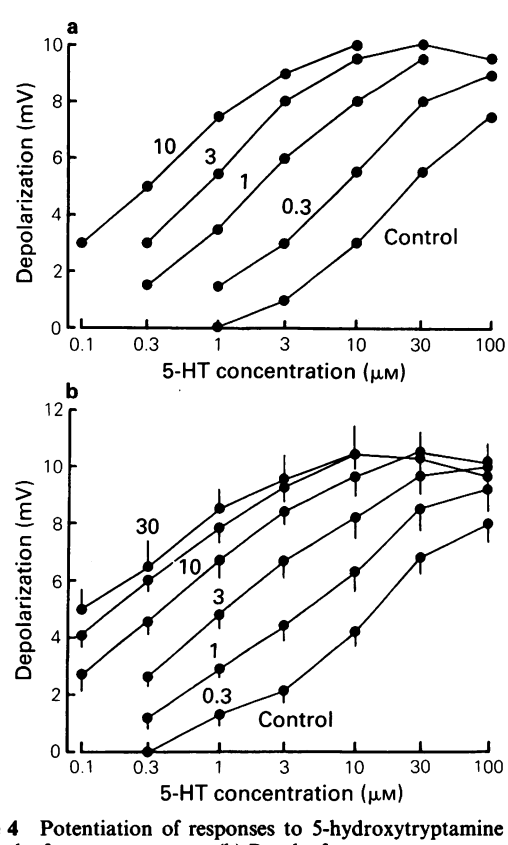


Figure 4 Potentiation of responses to 5-hydroxytryptamine (5-HT). (a) Results from one neurone. (b) Results from many neurones (points are mean with s.e.mean shown by vertical lines, 5–25 cells at each point). In each panel, the most righthand curve is the control response to dopamine and the curves to the left of these were made in the presence of (a) 1, 3, 10 and 30 μM cocaine and (b) 0.3, 1, 3, 10 and 30 μM cocaine as indicated. Membrane potential of neurones was held at −75 to −78 mV prior to 5-HT application.

Discussion

The main result is that cocaine increases the effectiveness of added dopamine or 5-HT in exerting their effects on individual nucleus accumbens neurones situated within a slice of

brain tissue. This is seen whether the effect is hyperpolarization (dopamine acting at D_1 receptors) or depolarization (dopamine acting at D_2 receptors or 5-HT acting at 5-HT_2 receptors; see North & Uchimura, 1989). It is assumed that the action of cocaine results from inhibition of uptake of the added agonists, and the subsequent discussion is within this framework.

Measurement of the ability of cocaine to block uptake

There is a method to estimate the dissociation constant for cocaine as an inhibitor of agonist uptake (K_i; Kenakin, 1987). However, it invokes a number of assumptions that are invalid in the present circumstances. First, it must be assumed that uptake sites and receptor sites are homogeneously distributed throughout the tissue. This is obviously not the case: the receptors under study are localized on a single neurone somewhere within the tissue, but the added agonists are exposed to uptake sites throughout the tissue as they diffuse toward the impaled cell. Thus, unless uptake is blocked one would expect a steep decline of agonist concentration from the surface of the slice to the neurone under study. This seems likely to contribute to the considerable variability in the sensitizing action of cocaine from cell to cell (Figure 5) (see also Surprenant & Williams, 1987).

Further assumptions of the Kenakin method for determining K_i are that the uptake process is described by a Michaelis-Menten formulation and that K_m (the dissociation constant for agonist at its uptake site) considerably exceeds the agonist concentration present. The second condition is not likely to be met. The concentrations of dopamine applied (100 μM) most likely exceed the K_m considerably; estimates of dopamine K_m measured biochemically are typically 100–1000 nM (e.g. Snyder & Coyle, 1969; Snyder *et al.*, 1970). A final difficulty is that the method of Kenakin (1987) requires the demonstration of a maximal sensitization; in most of the present experiments a clear maximal effect was not determined.

Comparison between inhibition of uptake of 5-hydroxytryptamine and of dopamine

Cocaine was no different in its ability to potentiate dopamine at D_1 receptors and dopamine at D_2 receptors; this may be interpreted to mean that the exogenous dopamine added to the slice undergoes a similar fate with respect to diffusion and uptake whether its eventual target is a D_1 or a D_2 receptor. It also implies that cocaine is not exerting any direct effects at one or other of the receptors. However, cocaine was significantly more effective in potentiating the action of 5-HT (Figure 2a and Figure 5). Since it was not possible to estimate a K_i value for cocaine (see above), the sensitization of the dopamine response was directly compared with the sensitization of the 5-HT response for the same four cocaine concentrations (Figure 5). At every concentration tested, cocaine was significantly more effective in increasing the effect of 5-HT. At 1 μM, cocaine was 6.5 times more effective in potentiating 5-HT than dopamine actions; at 30 μM the ratio was 2.5. This reduced differential effect at higher concentrations would be expected for a saturable uptake mechanism. The reasons for the differential effect cannot be determined: obvious possibilities are a different density of uptake sites (5-HT versus dopamine) or different K_i values for cocaine. The latter interpretation is consistent with the finding that the K_i for cocaine is about 4 fold lower when it is measured as an inhibitor of the binding of tritiated ligands at the 5-HT uptake site, than when measured as an inhibitor of binding to the dopamine site (Ritz *et al.*, 1987).

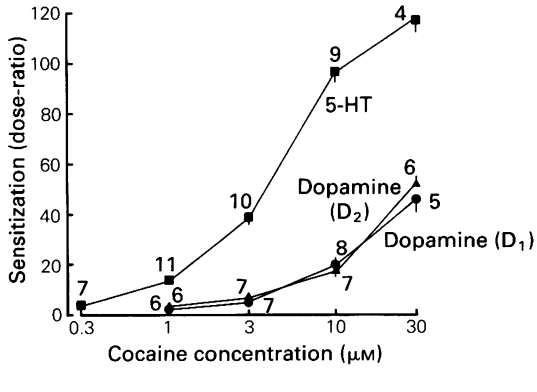


Figure 5 Degree of potentiation of responses to 5-hydroxytryptamine (5-HT) and dopamine caused by different concentrations of cocaine. Ordinate scale is dose-ratio [(concentration of agonist causing a 4 mV potential change in cocaine)/(concentration of agonist causing a 4 mV potential change without cocaine)]. Points show dose-ratio (mean with s.e.mean shown by vertical lines) for a number of individual neurones (indicated beside point): (■) 5-HT; (▲) dopamine at D_1 receptors (1 μM SCH23390 present); (●) dopamine at D_1 receptors (1 μM sulpiride present.)

Comparison with effects of cocaine in other tissues

Because cocaine had no effect when applied alone to the nucleus accumbens neurones, it is concluded that there is insignificant spontaneous release of dopamine (or 5-HT) occurring under these experimental conditions. This situation cannot be directly compared with the effects of cocaine in tissue slices containing cell bodies (noradrenaline in the locus coeruleus: Surprenant & Williams, 1987; 5-HT in the dorsal raphe: Williams & Lacey, 1988; dopamine in the substantia nigra/ventral tegmental area: Lacey *et al.*, 1990) because in those tissues cocaine itself has a strong hyperpolarizing effect through inhibiting the uptake of dopamine (or noradrenaline or 5-HT) that is being continuously released from the spontaneously firing neurones. The effect of cocaine in potentiating the actions of noradrenaline has been studied in a number of peripheral tissues that lack noradrenaline-containing cell bodies; in this case, the cocaine has little effect in the absence of applied noradrenaline. One such preparation is the submucous plexus isolated from the guinea-pig (Surprenant & Williams, 1987). In that tissue, the maximum sensitization by cocaine (measured as the leftward shift of the noradrenaline concentration-response curve) was only about 3.6 fold, compared with 120 fold (for 5-HT) or 50 fold (for dopamine) in the present experiments. The large difference between the tissues seems most likely to arise from the fact that few diffusional barriers exist in the isolated ganglia of the submucous plexus (which contain only 5–12 neurones) whereas agonist added to a brain slice must diffuse through as much as 200 μm of tissue laced with uptake sites before reaching the impaled neurone. This is mentioned as a reminder that the experimental conditions, particularly the opportunity for uptake provided by a long and tortuous diffusional pathway, play a very important role in determining the quantitative effect of an uptake blocker, as stressed by Kenakin (1987).

Those conditions should be equivalent with respect to 5-HT and dopamine in the present experiments and thus do not affect our conclusion that 5-HT uptake is more sensitive to cocaine than is dopamine uptake. However, they also emphasize that the large sensitizations to agonist which were observed are in a sense artifactual because of the long diffusional path which we have provided by recording from a neurone deep within a brain slice. 5-HT and dopamine released into the extracellular space during normal physiological activity would have their effects augmented much less by uptake inhibition. Direct measurements of the concentrations of dopamine (and cocaine) in dialysates of rat striatum extracellular fluid indicate that a change in the cocaine concentra-

tion of 1 μM is associated with a change in dopamine concentration of about 37 nM (Nicolaysen *et al.*, 1988), which is considerably less than the sensitization observed in the present study. The most appropriate way to approach the physiological circumstance may be to examine the effects of cocaine on the synaptic potentials mediated by the amine transmitters (Surprenant & Williams, 1987).

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Regulation of the synthesis and metabolism of striatal dopamine after disruption of nerve conduction in the medial forebrain bundle

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1 After physical (knife-cut) or chemically-mediated (tetrodotoxin 300 nM, 1.5 μl; 1.0 μl min^{-1}) interruption of nerve conduction in the nigrostriatal tract, there was a marked increase in the synthesis and metabolism of dopamine in the isolated dopaminergic nerve terminals of the striatum. The effect peaked at 4 h post-transection, at which time 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) were increased by 300% and 700% respectively (DOPAC: 27 ± 13 vs 80 ± 17 nmol g^{-1}; HVA: 6.66 ± 3.57 vs 54 ± 18 nmol g^{-1}). The increases in dopamine content and metabolism are secondary to an increase in the rate of synthesis on the lesioned side, versus the intact, control side.

2 In both experimental situations, haloperidol (1.0 mg kg^{-1}, i.p.) retained its known ability to induce a significant increase in DOPAC and HVA in the striatum, despite the interruption of nerve conduction in the nigrostriatal tract.

3 Six days after cutting the left nigrostriatal tract, dopamine in the left striatum was reduced to <5% of the control value, and DOPAC and HVA were not detectable. In the denervated, left striatum, the synthesis of dopamine (from injected L-DOPA), and its metabolism to DOPAC and HVA, occurred to the same degree as in the intact right side. In these DOPA-treated rats, haloperidol (1.0 mg kg^{-1}, i.p.) caused a further increase in DOPAC and HVA in the intact striatum, but not in the denervated striatum.

4 Under non-stressful conditions, using a combination of anaesthetic treatments, electrical stimulation (400 μA, 0.4 ms, 15 Hz, 15 min) of the nigrostriatal tract did not increase DOPAC or HVA in the striatum on the stimulated side.

5 It is concluded (a) that there is a significant presynaptic, and/or local circuit mechanism capable of activating the synthesis and metabolism of dopamine in the isolated, striatal, dopaminergic nerve terminals. Furthermore, haloperidol can act directly on the striatal, dopaminergic nerve terminal, to cause an increase in the synthesis and metabolism of striatal dopamine. (b) After degeneration of the striatal dopaminergic nerves, the denervated striatum retains the ability to synthesize (from L-DOPA) and metabolize dopamine, to the same degree as the intact, innervated, contralateral striatum. (c) When stress is minimized, and release of dopamine is induced by electrical stimulation of the medial forebrain bundle, the catabolism of dopamine (to DOPAC and HVA) during the release-uptake cycle may not be a significant factor under physiological conditions. (d) When dopamine synthesis is increased in the striatum, the normal blood concentration of tyrosine is adequate to sustain the increased synthesis, and precursor availability is not a limiting factor. (e) These results suggest that some of the basic concepts about the neurochemical/neurophysiological regulation of monoaminergic neurones may require further re-evaluation.

Introduction

The concepts that now guide our thinking about the regulation of transmitter synthesis and metabolism were fomulated during the past 25 years (Costa & Meek, 1974). For chemical transmission to be a viable means of information transfer in the central nervous system (CNS), mechanisms should exist to prevent transmitter depletion, especially during periods of increased transmitter demand, e.g., during episodes of increased neuronal firing. Two general schemes have been proposed for monoaminergic neurones. The first is reuptake (Iversen, 1971; 1973). According to this view, most of the released neurotransmitter is resequestered into the monoaminergic nerve terminal by an active uptake process. Based on studies of noradrenergic, sympathetic nerve terminals in the heart, Iversen (1971) estimated that the reuptake mechanism is capable of clearing the synaptic cleft of the released noradrenaline within milliseconds. This finding does not of itself prove that resequestered dopamine is conserved, since it has been suggested that dopamine may be significantly catabolized by monoamine oxidase (MAO) following reuptake (Westernik & Kikkert, 1986). The question of whether, in the CNS, the resequestered dopamine re-enters the calcium-dependent pool of releasable transmitter and is re-released, is not fully resolved. The second scheme is related to activation of the synthetic enzymes (particularly tyrosine hydroxylase) during increased nerve firing. It has been reported that short-term (seconds to minutes) activation results in a decrease in the K_m of tyrosine hydroxylase for both the precursor (tyrosine) and the pterin cofactor (Murrin *et al.*, 1976). Longer-term transmitter release induced by stress, results appropriately in V_{max} changes. These latter results have been obtained with the adrenal medulla. The published data indicate that gene activation and the associated transcription and translation mechanisms are involved, and occur prior to the increase in V_{max} (Chuang & Costa, 1974; Guidotti & Costa, 1977; Guidotti *et al.*, 1978; Kumakura *et al.*, 1979). However, a stress-induced increase in the synthesis and metabolism of DA has not so far been equivocally demonstrated in the striatum.

Transmitter levels tend not to change significantly under physiological conditions, even during periods of increased release. Therefore, implicit in the idea of increased synthesis, is the notion of increased transmitter catabolism associated with increased release. This phenomenon has been demonstrated under several experimental conditions. Indeed, so generally

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accepted is the association of increased cell firing, increased release and increased metabolism, that increased metabolism is often used as an index of increased firing and increased release (Commissiong, 1985a). Another corollary of the association of increased firing with increased synthesis is the problem of availability of precursor required for an increase in synthesis. The results obtained suggest that when striatal dopamine synthesis is increased, the normal blood concentration of tyrosine is adequate to accommodate the increased synthesis. Raising blood concentration of tyrosine, by injecting tyrosine, does not cause a further increase in synthesis.

Lastly, there is the question of the exact mechanisms by which firing of the nigrostriatal dopaminergic cells is regulated. One of the proposed mechanisms is blockade of the postsynaptic dopamine receptor, usually induced experimentally by neuroleptic drugs. One of the accepted, current hypotheses is that blockade of the postsynaptic dopamine receptor induces an increase in dopaminergic cell firing by a negative feedback mechanism. However, the exact mechanisms of the negative feedback regulatory processes have not been determined (Kehr *et al.*, 1972; Costa & Meek, 1974; Garcia-Munoz *et al.*, 1977; Rosaria & Gale, 1984). Within the present context, it has been suggested that neuroleptic-induced stimulation of the synthesis and metabolism of striatal dopamine is also partly mediated by blockade of the presynaptic D_2 autoreceptors, activation of which normally inhibits synthesis.

In recent years, some authors have suggested that our understanding of the control processes in monoaminergic neurones has been overstated (Commissiong, 1985a; Kuhn *et al.*, 1986; Westerink & Kikkert, 1986). The present experiments were done to reassess (a) the role of local and/or presynaptic mechanisms, (b) increased neuronal activity and precursor availability in the control of the synthesis and metabolism of dopamine in the striatum. The results obtained suggest that under some conditions, released dopamine may be highly conserved, and that *de novo* synthesis and metabolism may be minimal.

Methods

The experiments were done on female Sprague Dawley rats, 250 g. The left nigrostriatal tract was manipulated in three main ways.

(1) *By physical knife-cut*

A 1.5 mm wide knife, prepared from a single-edge razor blade was mounted in the electrode holder, angled at 70°, of a rat stereotaxic device (SM-15, Narishige Scientific Instrument Company, Tokyo, Japan). The site of insertion was to the left, just lateral (0.5 mm) and caudal to the bregma. The total width of the cut was about 4.5 mm, and it extended to the base of the brain. The hippocampus was avoided by the use of the above coordinates. The procedure resulted in the almost complete severing of the nigrostriatal tract, and a corresponding reduction (>95%) of dopamine in the striatum after 6 days, (Figure 3). The two main metabolities of dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) were reduced to undetactable levels.

(2) *Microinfusion of tetrodotoxin*

The tip of a 10 μl Hamilton syringe was modified to a rounded, tapering, edge. The stereotaxic coordinates used were: AP 2.80 mm; H: 3.0 mm and L: 1.4 mm, and 1.5 μl of a 3.0×10^{-7} M solution of tetrodotoxin (TTX) (Narahashi *et al.*, 1967) was slowly infused (1.0 μl min^{-1}) into the left medial forebrain bundle (MFB). This dose of TTX caused a 50% reduction in the evoked potential measured in the MFB at 4 mm rostral to the infusion site of TTX, for the 4 h duration of the experiment. TTX (1.5 μl, infused at 1.0 μl min^{-1}) at a concentration of 1.0×10^{-6} M was needed to attenuate the evoked potential in the MFB completely, but caused no further, significant increase in the metabolism of dopamine. The MFB was stimulated at 1.2 mm caudal to the infusion site of TTX. The tip of the needle was left in place for a further minute after the infusion, and then was slowly withdrawn incrementally. A test injection of 1.5 μl of a 0.5% Brilliant Blue dye, followed by a transcardiac perfusion, and freezing and cutting of the brain at 48 μm thick sections, indicated that the tip of the syringe could be reproducibly placed in the MFB.

(3) *Electrical stimulation*

A concentric bipolar stimulating electrode (SNEX-100, Korf Instrument Company) was used. The stereotaxic coordinates were: AP: 2.80 mm; H: 3.00 mm; L: 1.4 mm. A grass 48 stimulator was connected to a Grass constant current unit (CCU). The electrode was connected to the output of the CCU which delivered constant current biphasic pulses of 400 μA, and 0.4 ms duration. This arrangement prevented variation in stimulation intensity during the experiment due to variation in the resistance of the tip of the stimulating electrode. Stimulation was at 15 Hz, for 15 min.

Anaesthetics

Brietal (50 mg kg^{-1}, i.p.), Urethane (1.5 g kg^{-1}, i.p.), chloral hydrate (400 mg kg^{-1}, i.p.) and halothane (2.5%) were used in different experiments. In the results illustrated in Table 6, the animals were anaesthetized initially with halothane, and then injected with Brietal, Urethane or chloral hydrate. In this experiment, designed to test the contribution of stress on increased dopamine metabolism in the striatum, each animal was isolated individually in a 1 cubic foot rat box for 15 min, and anaesthesia was induced by a brief exposure to halothane (2.5%). The animals were then quickly removed from the rat box and injected with Brietal or Urethane or chloral hydrate. The animals recovered from halothane very rapidly (within 1 min). Therefore during the electrical stimulation of the MFB to evoke the release of dopamine, anaesthesia was maintained by Brietal or Urethane or chloral hydrate, but never by halothane. In the companion experiment (see Table 7), the animals were injected with Brietal, without prior isolation or without being initially anaesthetized with halothane.

Neurochemical analysis

A Finnigan 4000 gas chromatographic-mass spectrometric system was used. For the analysis of dopamine, DOPAC and HVA the tissues were homogenized in a 100 μM ascorbic acid – 0.1 N HCl solution at a 1:10 w/v ratio, with a Kontes micro ultrasonic cell disrupter (Cole-Parmer, Chicago, ILL.). The homogenate was centrifuged at 35,000 g at 0°C, for 25 min. A more complete precipitation of protein was obtained by adding 6.0 N HCl (1/10 of the homogenizing volume) to the supernatant, allowing the tubes to stand on ice for 15 min, and then centrifuging again at 35,000 g at 0°C, for 30 min. For the analysis of dopamine, 50 μl of the supernatant was transferred to a 1.0 ml microflex vial, and 257 pmol [$^2H_4$]-dopamine in 10 μl of 1.0 N HCl was added as the internal standard. For the analysis of DOPAC and HVA, 100 μl of the supernatant was transferred to a 1.0 ml microflex vial, and 290 pmol [$^2H_5$]-DOPAC and 214 pmol [$^2H_5$]-HVA each in 10 μl 1.0 N HCl were added. The samples were dried in a speed-vac concentrator (Savant instruments Inc., Farmingdale, N.Y.). Dopamine was acylated with pentafluoropropionic anhydride (PFPA), 100 μl, plus 20 μl of ethyl acetate at 60°C for 30 min. DOPAC and HVA were esterified with hexafluoroisopropanol (HFIP), 20 μl, and acylated with PFPA, 100 μl, at 70°C for 90 min. The derivatized samples were dried with ultra pure N_2, reconstituted in 10 μl of dehydrated ethyl acetate prior to being injected into the GC-MS system (Commissiong *et al.*, 1978).

For the analysis of γ-aminobutyric acid (GABA), 2.6 nmol [$^2H_6$]-GABA in 10 μl of 1.0 N HCl was added to 100 μl of the supernatant, and the mixture applied to a 1.0 cm high Dowex

Table 1 Identification of biological dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) from ion ratio and GC retention time data

Criteria	*Dopamine*	*DOPAC*	*HVA*
m/z:	428/387	415/387	478/331
Authentic	22.4 ± 1.94(4)	2.24 ± 0.12(5)	2.19 ± 0.18(5)
Biological	22.0 ± 2.58(29)	2.32 ± 0.15(29)	2.15 ± 0.17(29)
Retention time (min):			
Authentic	1.37 ± 0.02(4)	1.58 ± 0.02(4)	3.14 ± 0.02(4)
Bilogical	1.37 ± 0.03(4)	1.58 ± 0.03(4)	3.13 ± 0.02(4)

Dopamine: O-17 3%; 165°C
DOPAC and HVA: OV-17 3%; 120°C
Dopamine, DOPAC and HVA were identified from the statistical identity of the ratio of two ions from each compound, and from their GC retention times. In each case there was no statistical difference in the values obtained for the authentic compounds vs the putative biological compounds from the striatum. All analyses were done isothermally (dopamine: 165°C, DOPAC and HVA: 120°C) using OV-17 3% (dopamine, DOPAC, HVA) as the stationary phase.

cation exchange resin chromatographic column (Pasteur pipette). GABA was subsequently eluted with 800 μl 3.0 N ammonia solution. The eluate was collected in 1.0 ml microflex vials, dried in a speed-vac concentrator and derivatized with 25 μl HFIP and 100 μl PFPA at 60°C for 60 min. The derivatized samples were dried with N_2 and reconstituted in 10 μl of ethylacetate prior to being injected into the GC-MS system.

The gas chromoatographic (GC) analysis of dopamine was done on a 6 ft glass column, packed with OV-17 3% coated on Gas Chrom Q-HP, 80-100 mesh (chromatographic Specialties, Ontario, Canada). The analysis of dopamine was done isothermally at 165°C. DOPAC and HVA were analyzed on the same GC column used for dopamine, but at 120°C. GABA was analyzed on a 6 ft long glass column, packed with OV-210 3%, at 120°C.

The ions (m/z) monitored were: dopamine: 387, 428, [$^2H_4$]-dopamine: 431; DOPAC: 387, 415; [$^2H_5$]-DOPAC: 420; HVA: 331, 478; [$^2H_5$]-HVA: 483; GABA: 190, 204, 232, [$^2H_6$]-GABA: 238. The identities of the putative biological compounds analyzed were based on (1) ion ratios, (2) GC retention times comparisons, see Table 1.

All deuterated compounds were obtained from Merck Frost, Pointe Claire, Quebec, Canada.

Results

Synthesis in the isolated dopaminergic terminals

The results illustrated in Figure 1 show that after the dopaminergic axons are cut, the isolated dopaminergic nerve terminals can still synthesize and metabolize dopamine for 24 h afterwards. The effect peaked at 4 h, and returned to near control values by 6 h after the lesion. The concentrations of DOPAC and HVA were increased by factors of >3 and >7 respectively at 4 h, in comparison with their control values at 0 h. Blockade of nerve conduction in the nigrostriatal fibres, by the microinfusion of TTX into the MFB, caused a qualitatively similar effect as that obtained with the physical knife-cut procedure (Figure 2). The observed increase in dopamine, DOPAC and HVA after the interruption of nerve conduction by either technique is due to a real increase in the rate of synthesis on the lesioned side, versus the intact, control side, and not merely to an accumulation of dopamine, DOPAC and HVA in the nerve terminal, secondary to a cessation of release, induced by the experimental blockade of neurotransmission. In experiments in which the decarboxylase inhibitor 3-hydroxybenzylhydrazine hydrochloride (NSD-1015, 200 mg kg^{-1} i.p.; 100 mg kg^{-1} given just prior to either the lesion or the infusion of TTX, and a further 100 mg kg^{-1} 2 h later) was used and the striata analyzed at 4 h after the

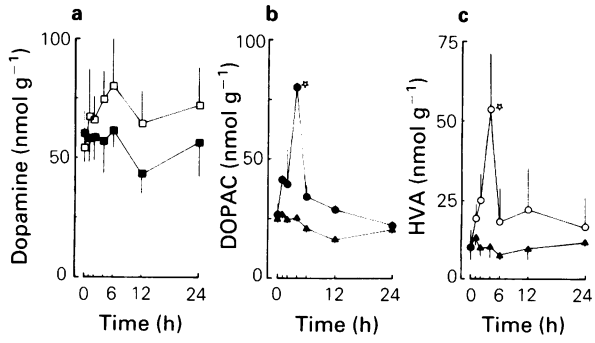


Figure 1 Changes in dopamine (a), 3,4-dihydroxyphenylacetic acid (DOPAC) (b) and homovanillic acid (HVA) (c) at 0-24 h after cutting the left nigrostriatal tract. Rats were anaesthetized with Brietal (50 mg kg^{-1} i.p.), and the left nigrostriatal tract completely cut under stereotaxic guidance. At 0, 1, 2, 4, 6, 12 and 24 h later, the animals were killed by decapitation, the striata quickly removed and frozen, and subsequently analyzed for dopamine, DOPAC and HVA. At 4 h post-lesion, there was a significant increase in DOPAC and HVA (×4 and ×7 times respectively). At 12 h post-lesion there was a significant increase in dopamine but DOPAC and HVA had returned to control values. Values are mean with s.d. shown by vertical lines ($n = 5$). Open symbols, left side; closed symbols, right side. (a) Solid asterisk; $P < 0.05$ vs dopamine concentration at 12 h on the right side (b and c) open asterisk; $P < 0.001$ vs DOPAC and HVA concentrations respectively at 4 h on the right side.

lesion, the concentration of DOPA was 3.5 times higher on the left, lesioned side, compared with the intact, control side (Commissiong & Slimovitch, 1987). This activation of tyrosine hydroxylase in the striatum following blockade of nerve conduction in the nigrostriatal fibres has been demonstrated previously by other authors (Carlsson *et al.*, 1972; Morgenroth *et al.*, 1976; Nowycky & Roth, 1978). It is likely that the increase in synthesis is underestimated, since transport of DOPAC and HVA from the striatum is not taken into account.

The results shown in Table 2, demonstrate that haloperidol retains its known ability to increase the concentration of DOPAC and HVA significantly in the striatal dopaminergic nerve terminals, after they were isolated from the cell bodies.

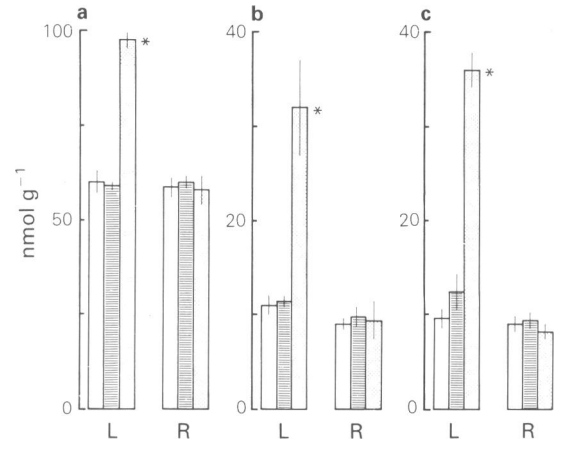


Figure 2 The effect tetrodotoxin (TTX) on the synthesis and metabolism of dopamine in the striatum of the rat. Rats were anaesthetized with Brietal (50 mg kg^{-1}, i.p.) and mounted in the stereotaxic frame. TTX (300 nM; 1.5 μl; 1.0 μl min^{-1}) was infused into the left MFB; 4 h later the animals were decapitated and the striata removed and analyzed for dopamine (a), 3,4-dihydroxyphenylacetic acid (b) and homovanillic acid (c). The results obtained are qualitatively similar to those obtained after a physical interruption of the nigrostriatal axons by a knife-cut technique (see Figure 1). Values are mean with s.d. shown by vertical lines ($n = 5$). Open columns, unoperated controls; lined columns, saline-treated; stippled columns, tetrodotoxin treated. L: left side; R: right side. *$P < 0.01$ vs any of the other five measures in the group.

Table 2 The effect of haloperidol on striatal dopamine metabolism after complete transection of the left nigrostriatal tract

	Unoperated vehicle		*Unoperated haloperidol*		*Lesioned vehicle*		*Lesioned haloperidol*	
	Left	*Right*	*Left*	*Right*	*Left*	*Right*	*Left*	*Right*
Dopamine	56±7	65±9	55±6	51±9	110±9[a]	61±8	98±17	54±5
DOPAC	18±3	19±2	50±6	59±14	20±3	20±3	29±4[b]	70±13[d]
HVA	6.3±1.1	8.0±1.2	19±3	21±4	4.4±1.5[a]	6.8±1.6	10.2±4.8[c]	20±2

The effect of haloperidol on the synthesis and metabolism of dopamine in the striatum of rats at 10 h after transection of the left nigrostriatal tract. Rats were anaesthetized with Brietal (50 mg kg^{-1}, i.p.) and the left nigrostriatal tract was lesioned under stereotaxic guidance; 10 h later, the animals were treated with haloperidol (1.0 mg kg^{-1} i.p., 1 h), and then killed by decapitation. The striata were quickly removed, frozen, and subsequently analyzed for dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), The statistically significant reduction of HVA on the left, lesioned side in the lesioned/vehicle group was unexpected and difficult to explain.

Values are nmol g^{-1} ± s.d. (n = 5)

[a]: P<0.01 vs L: Unoperated/Vehicle
[b]: P<0.01 vs L: Lesioned/Vehicle or L: Unoperated/Vehicle
[c]: P<0.01 vs L: Lesioned/Vehicle or L: Unoperated/Vehicle
[d]: P<0.02 vs R: Unoperated/Haloperidol

For the analysis of variance for the L side:
Dopamine: $F(7, 32) = 35.6$; $P<0.01$
DOPAC: $F(7, 28) = 32.3$; $P<0.01$
HVA: $F(7, 30) = 27.4$; $P<0.01$

This effect of haloperidol is, however, attenuated, versus its normal effect on the intact striatum. It should be noted, that at 10 h after lesioning the nigrostriatal tract, the nerve terminal had previously (0–6 h, peaking at 4 h) been through a cycle of markedly enhanced synthesis and metabolism of dopamine. Whether this is responsible for the observed, attenuated effect of haloperidol is not certain. The experiment was designed in this way, so that the analyses could be done when the concentrations of DOPAC and HVA were in a steady state, or changing at a very low rate. When nerve conduction in the nigrostriatal pathway was blocked by the microinjection of TTX into the MFB, haloperidol still caused a statistically significant increase in both DOPAC and HVA in the striatum (Table 3). In this case haloperidol was injected at 20 min after the infusion of TTX, when the animals had recovered from the anaesthetic. This allowed enough time for the diffusion of TTX and blockade of the voltage-dependent sodium channels (Koizumi *et al.*, 1967). The rats were killed 1 h later, by which time the TTX-induced increase in dopamine, DOPAC and HVA was negligible (see Figure 1). Therefore, the results for the left striatum shown in Table 3 are due to the action of haloperidol on striatal dopaminergic terminals, with nerve conduction blocked in the left MFB. The results show that the effect of haloperidol in these recently-anaesthetized animals is attenuated, versus the effect normally obtained in unoperated, control animals. More importantly, in this case, unlike the knife-cut experiments (see Table 2), the effects on the left and right sides are identical, suggesting that the effect of haloperidol on the synthesis and metabolism of dopamine in the striatum, may be entirely presynaptic.

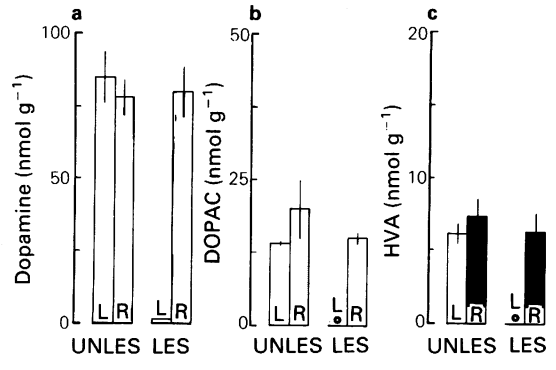


Figure 3 The depletion of dopamine (a), 3,4-dihydroxyphenylacetic acid (DOPAC) (b) and homovanillic acid (HVA) (c) in the chronically-denervated left striatum of the rat. Rats were anaesthetized with Brietal (50 mg kg^{-1}, i.p.) and the left nigrostriatal tract was cut under stereotaxic guidance. Six days later the animals were killed by decapitation, the left and right striatum were quickly removed and frozen, and subsequently analyzed for dopamine, DOPAC and HVA. Dopamine was just detectable on the lesioned (L) side, and DOPAC and HVA (a, b and c respectively) were below the limits of detection. Values are mean with s.d. shown by vertical lines (n = 5). L: left striatum; R: right striatum; LES: lesioned; UNLES: unlesioned.

Chronic denervation

The effect of chronic denervation (cutting the nigrostriatal dopaminergic fibres six days previously) on dopamine,

Table 3 The effect of haloperidol on striatal dopamine metabolism after the microinfusion of tetrodotoxin (TTX) into the left nigrostriatal tract

	Unoperated vehicle		*Unoperated haloperidol*		*TTX vehicle*		*TTX haloperidol*	
	Left	*Right*	*Left*	*Right*	*Left*	*Right*	*Left*	*Right*
Dopamine	63±10	66±6	48±6	46±6	75±20	75±9	61±15	61±8
DOPAC	31±7	28±8	118±49	108±25	29±14	33±7	59±14[b]	61±13[e]
HVA	7.0±1.3	6.9±1.2	23±2	22±4	6.2±2.6	6.0±2.0	14±3[e]	15±1.7[f]

The effect of TTX and haloperidol on the synthesis and metabolism of dopamine in the striatum of the rat. Rats were anaesthetized with Brietal (50 mg kg^{-1} i.p.) and TTX (1.5 µl: 300 nM) was microfused (1.0 µl min^{-1}) into the left MFB under stereotaxic guidance. After recovery from the anaesthetic (about 20 min) the animals were injected with haloperidol (1.0 mg kg^{-1}, i.p.; 1 h), and then decapitated. The striata were quickly removed and frozen, and subsequently analyzed for dopamine 3,4-dihydroxphenylacetic acid (DOPAC) and homovanillic acid (HVA). There was a significant (P < 0.05) increase in both DOPAC and HVA on the left (L) side in the TTX/Hal group vs the concentration in the L: TTX/Vehicle group. The dose of TTX used (1.5 µl, 3.0 × 10^{-7} M), reduced the evoked potential in the MFB to <50% within a few minutes of being applied. This degree of suppression of nerve conduction remained effective for up to 4 h.

Values are nmol g^{-1} ± s.d. (n = 5)

[b]: P < 0.01 vs L: TTX/Vehicle or L: Unoperated/Vehicle
[e]: P < 0.01 vs R: TTX/Vehicle or R: Unoperated/Vehicle
[f]: P < 0.01 vs R: TTX/Vehicle or R: Unoperated/Vehicle

For the analysis of variance for the L side:
Dopamine: $F(7, 31) = 3.38$; $P < 0.05$
DOPAC: $F(7, 29) = 6.89$; $P < 0.01$
HVA: $F(7, 32) = 6.19$; $P < 0.01$

Table 4 The effect of haloperidol on the metabolism of dopamine (synthesized from injected L-DOPA) in the chronically-denervated rat striatum

Conditions		Dopamine Left	Dopamine Right	DOPAC Left	DOPAC Right	HVA Left	HVA Right
Unles	HAL^- $DOPA^-$	35±4	44±12	21±5	23±9	7.7±1.6	7.7±1.4
Les	HAL^- $DOPA^-$	2.5±0.8	51±11	Det	24±5	Det	4.7±1.2
Les	HAL^+ $DOPA^-$	1.2±0.1	44±3	3.1 ± 1.3	80±18	Det	22±1
Les	HAL^- $DOPA^+$	10±4	79±9	218±45	176±43	45±14	37±8
Les	HAL^+ $DOPA^+$	10 ± 7	49±5*	199±50	350±111*	43±4	50±1*

In lesioned (Les) animals, the left nigrostriatal tract (L-NST) was cut as described in the Methods section. The experiment was done 6 days later, after the striatal dopaminergic nerves had degenerated (Figure 3). Haloperidol (1.0 mg kg^{-1}, i.p.) was injected at 0 min, L-DOPA (100 mg kg^{-1}, i.p.) at 15 min, and the animals killed at 75 min. In the lesioned animals treated with L-DOPA (Les/Hal^-/$DOPA^+$ and Les/Hal^+/$DOPA^+$ groups), the production of DOPAC and HVA in the denervated left striatum was as great as in the intact right striatum. However, haloperidol induced a further increase in DOPAC and HVA in the intact right striatum, but not in the denervated left striatum.

Det: detectable only. Values are nmol g^{-1} ± s.d. ($n = 5$).
* $P < 0.05$ vs the right side of the Les/Hal^-/$DOPA^+$ group
Unles: unlesioned; Les: lesioned.
Hal^-: not treated with haloperidol; Hal^+: treated with haloperidol.
$DOPA^-$: not treated with DOPA; $DOPA^+$: treated with DOPA.

Table 5 The effect of tyrosine on the lesion-induced enhanced synthesis and metabolism of dopamine in the striatum of the rat

Condition		Dopamine	DOPAC	HVA
Unles + Veh	L	60±8	20±7	6.7±1.4
	R	62±3	18±2	6.9±1.0
Unles + Tyr	L	61±4	22±6	6.3±0.4
	R	67±3	23±4	6.6±1.2
Les + Veh	L	117±8	57±12	22±2
	R	68±2	24±7	7.3±1.1
Les + Tyr	L	105±4*	51±6	21±2
	R	69±4	19±3	6.5±0.8
Left side ANOVA	$F(3, 16) =$	9.64	15.7	23.6
	P	0.01	0.01	0.01

Rats were anaesthetized with Brietal (50 mg kg^{-1}, i.p.) and the left MFB cut under stereotaxic guidance; 2.5 h later, the animals were injected with tyrosine (150 mg kg^{-1} i.p.), and killed 1.5 h later, i.e. at 4 h after the lesion, when the lesion-induced metabolism of dopamine is at its peak (see Figure 1). The striata were rapidly removed, frozen, and subsequently analyzed for dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA). The injected tyrosine caused a significant ($P<0.05$) reduction in the lesion-induced synthesis of dopamine, and also caused a small but non-significant reduction in the lesion-induced increase in DOPAC and HVA.
Values are nmol g^{-1} ± s.d. ($n = 5$)
* $P < 0.05$ vs L: Les + Veh group. Student's unpaired t test.

DOPAC and HVA in the striatum is shown in Figure 3. Dopamine was reduced by >95%, and DOPAC and HVA were reduced to nondetectable levels. However, after the injection of L-DOPA (100 mg kg^{-1} i.p., 1 h), there was a striking increase in the synthesis and metabolism of dopamine in the chronically denervated striatum (Table 4). The concentrations of DOPAC and HVA increased from undetectable levels to over 200 and 45 nmol g^{-1} respectively. On the innervated right side, the magnitude of the effect was similar to that on the denervated left side (Table 4). Haloperidol caused a further, statistically significant increase in the metabolism of dopamine in the intact right striatum, but not in the denervated, left striatum (Table 4). Therefore, haloperidol does exert a specific effect on the dopaminergic neurone. The experimental design used does not permit a differentiation between a purely presynaptic/local effect, and an effect based on postsynaptic receptor blockade and subsequent activation of the dopaminergic neurone by a negative feedback mechanism.

Precursor considerations

Experiments were done in acutely denervated and in normal rats to test the effect of administering tyrosine (150 mg kg^{-1} i.p., 1.5 h) on the synthesis and metabolism of dopamine in the striatum. The results are shown in Table 5. Tyrosine had no effect on the synthesis of dopamine in the intact control right striatum but caused a significant ($P < 0.05$) reduction in the concentration of dopamine in the denervated, left striatum. An

Table 6 The effect of electrical stimulation on the left negrostriatal tract, under non-stressful conditions on metabolism of dopamine in the striatum

Conditions		Dopamine Left	Dopamine Right	DOPAC Left	DOPAC Right	HVA Left	HVA Right
Control (B)		90±2	90±3	21±4	22±4	7.2±1.1	8.3±1.6
Sham Stim (B)		87±7	89±4	26±9	23±7	8.0±2.1	7.4±2.0
Stimulated (CH)		88±9	86±24	18±3	18 ± 3	8.8±2.0	8.4±2.4
Stimulated (B)		92±8	86±12	22±2	19±4	7.9±0.7	7.5±1.4
Stimulated (U)		97±12	102±5	27±6	23±4	7.7±1.4	7.4±1.0
	F	0.80	0.06	1.99	0.59	0.48	0.33
	P	NS	NS	NS	NS	NS	NS

Values are nmol g^{-1} ± s.d. ($n = 5$)
After randomization, each animal was isolated in a 1.0 cubic ft. rat box for 15 min, and anaesthetized with halothane. Anaesthesia was then continued with either chloral hydrate (CH, 400 mg kg^{-1}, i.p.); or Brietal (B, 50 mg kg^{-1}, i.p.) or Urethane (U, 1.5 g kg^{-1}, i.p.). The left nigrostriatal tract was stimulated electrically (400 μA; 15 Hz) for 15 min. The animals were decapitated and the left and right striata removed and analyzed for dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA). Under these experimental conditions, using ANOVA analysis, there was no significant difference among the members of any group. There was also no significant difference between the left vs right sides for any of the three anaesthetics in any of the four conditions.

Table 7 The effect of electrical stimulation of the left negrostriatal tract on the synthesis and metabolism of dopamine in the striatum

Conditions		Dopamine		DOPAC		HVA	
		Left	*Right*	*Left*	*Right*	*Left*	*Right*
Control (B)		64±10	66±8	18±3	22±5	5.9±1.1	6.5±1.3
Sham Stim (B)		69±3	73±10	18±3	21±1	5.6±0.5	6.7±0.8
Stimulated (B)		101±39	77±6	43±3*	26±9	8.5±1.2	8.5±1.2
	F	2.27	2.88	6.12	0.71	7.12	4.43
	P	NS	NS	0.05	NS	0.01	0.05

*$P < 0.05$ vs the stimulated, right side Values are nmol g^{-1} ±s.d. ($n = 5$)
The animals were randomized, and then anaesthetized with Brietal. The left nigrostriatal tract was stimulated electrically (400 μA; 15 Hz) for 15 min. The left and right striata were then removed, and analyzed for dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA). ANOVA analysis showed that there was a significant difference among the members of the following groups: DOPAC, left side; HVA: left and right sides. However, in the left vs right comparisons, the only significant difference found was for DOPAC.

ANOVA statistical analysis was done initially ($F(3, 16) = 9.64$; ($P < 0.01$)) followed by an unpaired Student's t test.

Electical stimulation

Electrical stimulation (of the MFB) experiments were done to test the relationship between release and metabolism. The left nigrostriatal fibres were stimulated in three groups of rats anaesthetized respectively with chloral hydrate (400 mg kg^{-1}, i.p.), Brietal (50 mg kg^{-1}, i.p.) or Urethane (1.5 g kg^{-1}, i.p). The unstimulated control and sham-stimulated control groups were anaesthetized with Brietal (50 mg kg^{-1}, i.p.). Each animal used in this experiment was isolated in a rat box for 15 min, and anaesthesia was induced with halothane. Subsequently, the animals were injected with the anaesthetic agents described above. Under these relatively non-stressful conditions, neither chloral hydrate nor electrical stimulation caused an increase in DOPAC and/or HVA (Table 6). However, when the experiment was repeated in the traditional manner, without pre-anaesthetizing the animals with halothane, the more usual increases in DOPAC and HVA were observed (Table 7).

Discussion

Two techniques have been used to interrupt nerve conduction in the nigrostriatal, dopaminergic fibres: physical knife cut of the axons, and specific chemical blockade of the voltage-dependent sodium channels with TTX. In both cases there was a marked increase in dopamine, DOPAC and HVA on the experimental, lesioned side, versus the intact, control side (Figures 1 and 2). The increases in the content of dopamine, DOPAC and HVA are due to an increase in the rate of synthesis of dopamine on the experimental side, versus the intact, control, side. In experiments in which the decarboxylase inhibitor NSD-1015 was used, the accumulation of DOPA, measured at 4 h after the lesion, was 3.5 times greater on the lesioned left side than on the control right side. Therefore, tyrosine hydroxylase (TH) was activated in the striatum on the lesioned, left side, as compared with the control, right side, as has been shown previously (Carlsson *et al.*, 1972; Roth *et al.*, 1974; Morgenroth *et al.*, 1976; Walters and Roth, 1976a; Nowycky & Roth, 1978; Kehr *et al.*, 1982; Wood, 1982; Wood & Richard, 1982). The present results reaffirm the interesting paradox that both increased impulse flow and cessation of impulse flow in the nigrostriatal fibres can cause an activation of TH in the striatum, and an associated increased synthesis/metabolism of dopamine (Nowycky & Roth, 1978). It will be argued (see later) however, that increased impulse flow induced by electrical stimulation may not, in fact, always cause an activation of TH, as has been proposed (Murrin *et al.*, 1976). A question of general concern is the neurochemical viability of the striatum during the long duration of some of these experiments (Table 2). GABA is a relatively labile transmitter with a fast turnover rate, and can be used to give a useful neurochemical/physiological index of the state of a brain region in which it is found. The results in Figure 4 were obtained from an analysis of the same supernatant used to generate the data in Table 2. GABA was unchanged throughout the 10 h duration of the experiment, suggesting that the striatum may not have been compromised in any significant way during the course of the experiment.

In the subsequent experiments, the two methods of increasing striatal dopamine synthesis were used to study some of the factors that are thought to modulate the synthesis of dopamine. In this regard, the mechanism of action of antipsychotic drugs is one of the most interesting problems. Haloperidol caused a classical dose-dependent increase in DOPAC and HVA in the striatium (Figure 5), and a consistently small, usually non-significant fall in dopamine. The results in Table 2 and 3 show that haloperidol was still effective in increasing the concentration of DOPAC and HVA in the striatum after

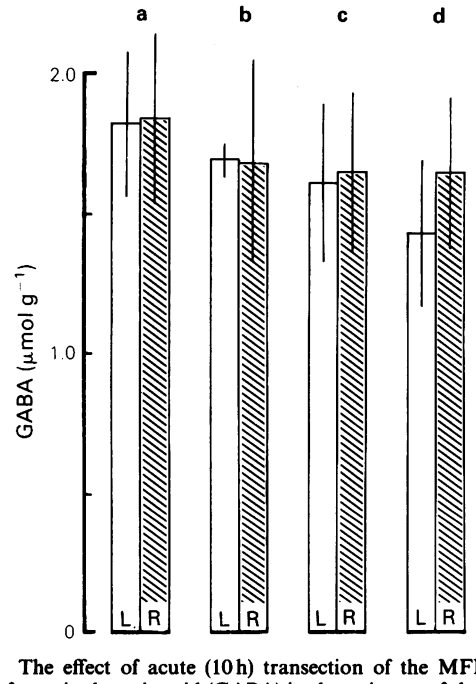


Figure 4 The effect of acute (10 h) transection of the MFB on the content of γ-aminobutyric acid (GABA) in the striatum of the rat. The supernatant is from the tissue used to obtain the data illustrated in Table 2. The results show that there was no significant change in the concentration of GABA in any of the 8 groups of tissue analyzed. Values are mean with s.d. shown by vertical lines ($n = 5$). (a) unoperated-vehicle; (b) unoperated-haloperidol; (c) lesioned-vehicle; (d) lesioned-haloperidol. L: left, lesioned side; R: right, intact, control side.

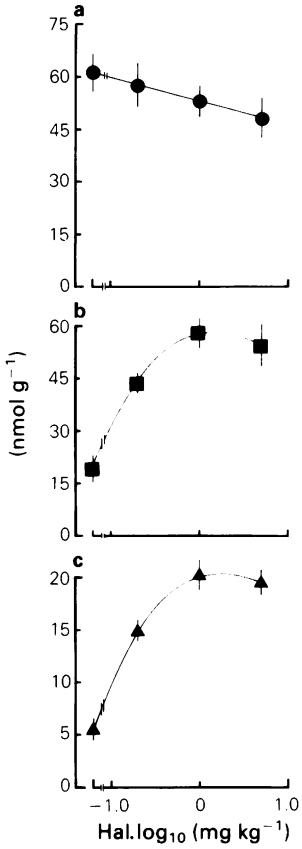


Figure 5 A semi-logarithmic plot of the dose-response effect of the action of haloperidol (Hal) on the concentration of (a) dopamine, (b) 3,4-dihydroxyphenylacetic acid (DOPAC) and (c) homovanillic acid (HVA) in the striatum of the rat. Rats were injected with haloperidol at 0, 0.2, 1.0 and 5.0 mg kg^{-1} i.p. One hour later, the animals were killed by decapitation, the striata quickly removed, frozen, and subsequently analyzed for dopamine, DOPAC and HVA. Haloperidol consistently caused a reduction (usually non-significant) in the concentration of dopamine and a significant increase in DOPAC and HVA. Values are mean with s.d. shown by vertical lines ($n = 5$). In (a) $F = 2.17$, NS; (b) $F = 29.5$, $P < 0.001$; (c) $F = 52.1$, $P < 0.001$.

blockade of nerve conduction in the MFB. Taken together, these results siggest that haloperidol can act directly on the dopaminergic nerve terminal. Therefore, the results may be unrelated to a strionigral, long-loop, negative feedback activation of the dopaminergic neurone, as is usually suggested (Carlsson *et al.*, 1972; Iwatsubo & Clouet, 1977; Kilpatrick *et al.*, 1985; Johnson *et al.*, 1986). An alternative explanation is that haloperidol may increase striatal DOPAC and HVA by blocking their transport from the striatum (Westerink & Kikkert, 1986). However, this explanation is unlikely, in view of the results illustrated in Table 4, in which haloperidol increased DOPAC and HVA in the striatum only on the innervated side.

In Table 4, it is also shown that in the denervated striatum (see Figure 3) the synthesis and metabolism of dopamine from injected DOPA were the same as in the innervated control striatum. Therefore, provided the precursor (DOPA) is presented beyond the TH regulatory point, the striatum, minus its dopaminergic nerves, can still synthesize and metabolize dopamine as efficiently as an intact striatum. The decarboxylase enzyme, however, is known to be present in both non-neural (glia, endothelium) and other monoaminergic neurones (Langelier *et al.*, 1972; Jaeger *et al.*, 1973; Hefti *et al.*, 1981; Commissiong, 1985b; El Gemayel *et al.*, 1986). Thus considerable caution should be exercised in the interpretation of dopamine metabolism data, since it is likely that a large fraction of DOPAC and HVA may be non-neural in origin, and therefore may not be indicative of events in the dopaminergic neurone. Moreover, despite the magnitude of the synthesis/metabolism that ensued after DOPA injection, haloperidol still enhanced the effect, but only in the innervated striatum. One plausible explanation of this effect is a direct action of haloperidol on the synthesis-inhibiting D_2 autoreceptors on the dopaminergic nerve terminal, since haloperidol has been shown to increase tyrosine hydroxylase activity directly, *in vitro*, in a synaptosomal preparation (Christiansen & Squires, 1974). It is surprising that this regulatory mechanism remains active even under conditions of an ongoing excessive synthesis and metabolism of DA (Table 4). This result suggests that there may be stringent, inhibitory mechanisms in the dopaminergic nerve terminal which normally limit the synthesis and metabolisms of DA to a small fraction of the maximum capacity. These results are also of general significance with regard to the therapeutic use of L-DOPA to treat Parkinson's disease. Evidently, the entire CNS must be flooded with high concentrations of dopamine in the hours immediately after ingestion of L-DOPA (see Commissiong, 1985b). Looked at from this perspective, it is surprising that L-DOPA has proven to be a beneficial therapeutic agent and not surprising that it ceases to be effective in a significant fraction of cases after a long period of use.

The increased post-lesion synthesis of dopamine afforded a good experimental test of the idea that when dopamine synthesis is activated, precursor (blood tyrosine concentration) may become a limiting factor. This hypothesis was tested directly (Table 5). Other authors have addressed this question previously (Murrin *et al.*, 1976; Milner & Wurtman, 1986). The results obtained (Table 5), suggests that when striatal dopamine synthesis is increased, blood tyrosine concentration is not a limiting factor. Indeed, the data suggest that *in vivo*, increasing tyrosine concentration may actually inhibit the synthesis (though curiously not the metabolism) of dopamine.

The last and most important aspect of the results described in this paper relate to the fate of released dopamine. The results shown in Table 6 indicate that when stress is minimized, transmitter catabolism (increased DOPAC and HVA) may not be a necessary accompaniment of increased dopamine release following electrical stimulation. These data were obtained from rats that were subjected initially to an additional anaesthetic (halothane) exposure, but the halothane was used only to induce anaesthesia, and the animals recovered rapidly (usually within a minute), if anaesthesia was not maintained by Brietal or Urethane or chloral hydrate. This experimental design was based on the previous evidence (Buhler *et al.* 1978) that plasma catecholamines increase rapidly in response to the stress of gentle handling. It has been demonstrated that halothane does not interfere with the evoked release of dopamine (Besson *et al.*, 1971; Nieoullon *et al.*, 1977; Zetterstrom *et al.*, 1983). Nevertheless, general anaesthetics have been shown to suppress and enhance synaptic transmission, presumably by an effect on transmitter release (Richards, 1974). On balance, it is unlikely that the present results can be accounted for solely by a halothane-mediated suppression of dopamine release, since at the time the MFB was stimulated electrically, anaesthesia was being maintained by Brietal or Urethane or chloral hydrate. The results also suggest that reuptake is a robust mechanism, and may be adequate to replenish transmitter stores even in cases of greatly enhance release. This conclusion is supported by the observation that when the experiment was done without preventing the stress caused by handling, by the initial use of halothane, some evidence for catabolism was obtained (Table 7).

The general thrust of this paper has been to show that some of the basic ideas that guide our thinking in the interpretation of monoaminergic neurochemical/neurophysiological data may be equivocal. In particular, the use of metabolite measurements as an index of release and by implication, as an index of nerve activity is in all probability an unsound procedure. Recent evidence, based on *in vivo* release data, using the intracerebral dialysis technique, suggests that dopamine

release and metabolism (indicated by DOPAC and HVA) can be varied independently under some experimental conditions (Imperato & Dichiara, 1985). It has been shown that different mechanisms underlie the effects of neuroleptics on dopamine release and metabolism. Intraneuronal synthesis and metabolism, unrelated to release, could be another important, confounding factor in the interpretation of data. Presynaptic and heterosynaptic mechanisms appear to play significant roles in the regulation of the synthesis/metabolism of dopamine in the striatum (Walters & Roth, 1976b; Leviel *et al.*, 1979; Ganon & Buda, 1985; Kalser, 1985; Laduron, 1985; Nissbrandt *et al.*, 1985). Several reports have shown that the mesotelencephalic dopaminergic neurones constitute a variety of heterogeneous subgroups, and that they are regulated differently (Demarest & Moore, 1979; Bannon *et al.*, 1982; Bannon & Roth, 1983; Roth, 1984; DiChiara & Imperato, 1985). One significant recent advance is the ability to monitor dopaminergic neuronal activity in transplants with apparently no afferent connections, and therefore to test the effects of drugs directly on isolated dopaminergic neurones. The preliminary results to date suggest that the electrophysiological profile of these isolated neurones, their response to drugs, and transmitter release control mechanisms are quite similar to the mechanims in the CNS *in situ*. For example haloperidol causes an increase in DOPAC in these isolated neurones (Seiger *et al.*, 1976; Wuerthele *et al.*, 1984). These data and the results of Trulson (1985) support some of the conclusions drawn in this study, but many basic questions remain unresolved.

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Effect of cyclo-oxygenase blockade on the renal actions of vasopressin and SK&F 105494 in the rhesus monkey

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1 Vasopressin administration to pentobarbitone-anaesthetized, hydrated female rhesus monkeys resulted in dose-dependent increases in urine osmolality and decreases in urine flow. Treatment with indomethacin at a dose (5 mg kg^{-1}, i.v.) that reduced urinary prostaglandin E_2 (PGE_2) excretion by at least 70% did not alter these responses.

2 The vasopressin antagonist, SK&F 105494 (Pas^{1,6}D-Tyr(Et)^{2}Val^{4}Arg^{7}D-Arg^{8}desGly^9 arginine vasopressin; 10 μg kg^{-1}), caused significant rightward shifts ($P < 0.05$) of both the vasopressin-urine osmolality and the vasopressin-urine flow dose-response curves. Treatment with indomethacin did not alter these responses.

3 SK&F 105494 alone or after indomethacin treatment had minimal effects on urine osmolality and urine flow.

4 The data indicate that indomethacin does not alter the antidiuretic activity of vasopressin in the rhesus monkey and that SK&F 105494 is a potent antagonist of exogenous vasopressin with minimal agonist activity.

Introduction

Inhibition of cyclo-oxygenase activity has been shown to augment the antidiuretic activity of vasopressin in the rat (Berl *et al.*, 1977) and the dog (Anderson *et al.*, 1975; Fejes-Toth *et al.*, 1977; Albrightson-Winslow *et al.*, 1989). Indomethacin and meclofenamate unmask the antidiuretic partial agonist activity of the vasopressin antagonist, SK&F 101926, in the dog (Albrightson-Winslow *et al.*, 1989). On the other hand, indomethacin appears not to enhance vasopressin antidiuretic activity in man (Padfield & Grekin, 1981) and SK&F 101926 demonstrates full agonist activity as an antidiuretic in man without prior cyclo-oxygenase inhibition (Allison *et al.*, 1988). SK&F 101926 is also a full antidiuretic agonist in the absence of cyclo-oxygenase blockade in the rhesus monkey (Brooks *et al.*, 1988a). Another vasopressin antagonist, SK&F 105494, had minimal antidiuretic agonist activity in the rhesus monkey and produced a water diuresis in hydropenic animals (Brooks *et al.*, 1988a). One of the implications of these findings is that in the rhesus monkey, as in man, prostaglandins may not modulate vasopressin antidiuretic activity. In the present study, therefore, we have tested the ability of SK&F 105494 to inhibit exogenous vasopressin activity in the rhesus monkey and evaluated the effect of cyclo-oxygenase inhibition on the renal actions of both vasopressin and SK&F 105494.

Methods

Vascular-Access-Ports (Norfolk Medical Products) were inserted into a femoral vein of 3 female rhesus monkeys (*Macaca mulatta*; 5–8 kg). Ports were flushed twice a week and filled with a heparin (500 u ml^{-1}) saline (0.9%) solution. Before each experiment, animals were deprived of food but not water for 16 h. On the morning of the experiment, catheters were placed in the bladder (Foley Pediatriac 08 FR) and the animals were lightly anaesthetized with sodium pentobarbitone. Animals were placed on circulating water heating pads (Aquamatic model K-20), an infusion of hypotonic dextrose (3%) was initiated at 0.5 ml kg^{-1} min^{-1} and urine collected at 10 min intervals. When urine osmolality had decreased to 50 mOsm kg^{-1} H_2O either indomethacin (5 mg kg^{-1}, i.v.) or vehicle was administered followed 30 min later by either SK&F 105494 (Pas^{1,6}D-Tyr(Et)^{2}Val^{4}Arg^{7}D-Arg^{8}desGly^9 arginine vasopressin; 10 μg kg^{-1}, i.v.) or vehicle. Ten min urine collections were taken for the next 90 min. A second dose of vehicle or SK&F 105494 was administered and then an arginine vasopressin dose-response curve was generated. Arginine vasopressin (0.3 to 100 ng kg^{-1}) was administered in a cumulative manner and urine collections made at 5 min intervals. The infusion rate of dextrose was adjusted to equal urine output throughout the experiment. Blood pressure was monitored indirectly at 5 min intervals using a Dynamap (Criterikon). Each animal underwent all of the 4 protocols with at least a 2 week interval between each procedure.

Urinary prostaglandin E_2 (PGE_2) excretion was determined, before and after indomethacin treatment, by radioimmunoassay after extraction of PGE_2 from acidified (pH 3.4–4) urine by methods described previously (Albrightson *et al.*, 1989). Data are expressed as mean ± s.e.mean. Dose-response curves were compared by calculating either the ED_{200} (dose of vasopressin that increased urine osmolality to 200 mOsm kg^{-1} H_2O) or $ED_{1.0}$ (dose of vasopressin that decreased urine flow to 1.0 ml min^{-1}). The ED_{200} and $ED_{1.0}$ values were transformed logarithmically because of non-homogeneity of variance and compared statistically by a paired t test. Values are given as mean ± s.e.mean.

Results

Arginine vasopressin administration resulted in dose-dependent increases in urine osmolality and decreases in urine volume (Figure 1). Indomethacin pretreatment did not significantly alter urine osmolality or volume responses (Figure 1); $ED_{200} = 6.3 \pm 2.4$ and 6.3 ± 3.1 ng kg^{-1} and $ED_{1.0} = 1.5 \pm 0.2$ and 1.3 ± 0.6 ng kg^{-1} in vehicle- and indomethacin-treated monkeys respectively ($n = 3$ per group). SK&F 105494 produced a significant ($P < 0.05$) rightward shift in the urine osmolality and urine flow responses to vasopressin and these shifts were not altered by indomethacin (Figure 1); $ED_{200} = 61.7 \pm 25.9$ and 36.3 ± 12.3 ng kg^{-1} and $ED_{1.0} = 8.7 \pm 3.2$

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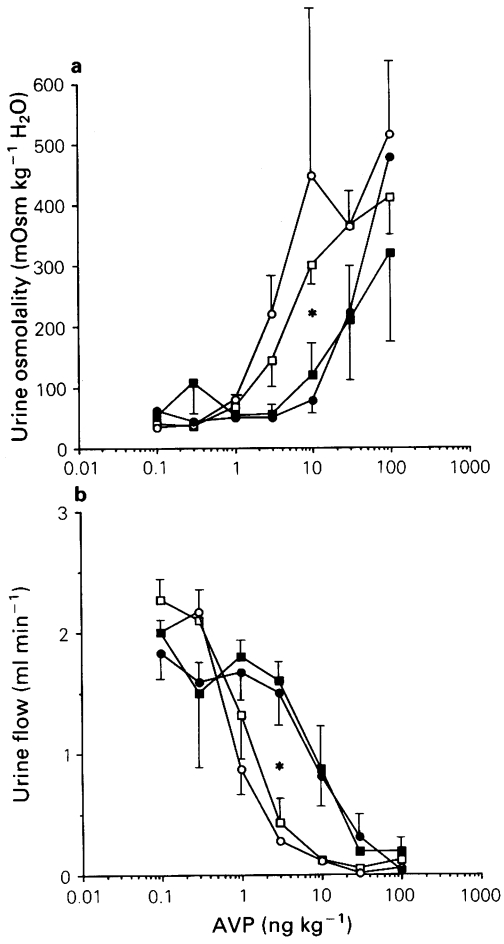


Figure 1 The effects of vehicle (□), indomethacin (○, 5 mg kg^{-1}), SK&F 105494 (■; 10 μg kg^{-1}) or a combination of indomethacin and SK&F 105494 (●) on changes in urine osmolality (a) and urine flow (b) induced by increasing doses of vasopressin (AVP). * $P < 0.05$, SK&F 105494 vs. vehicle.

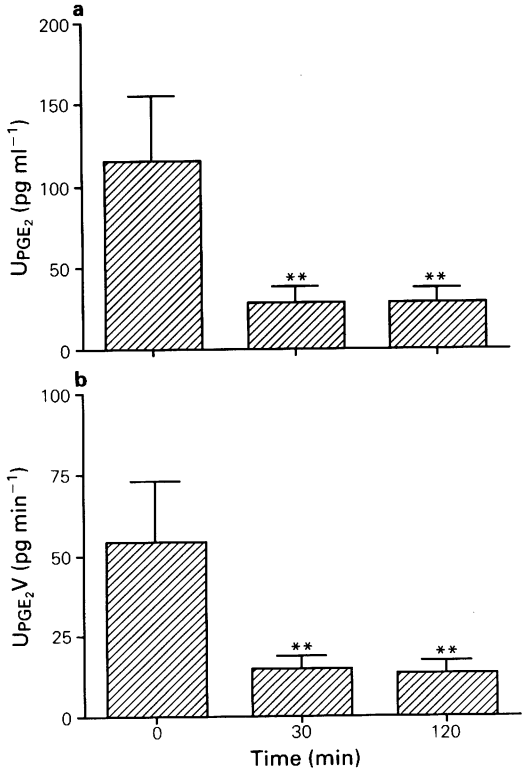


Figure 2 (a) Urinary prostaglandin E_2 (PGE_2) concentration (U_{PGE_2}) and (b) urinary PGE_2 excretion ($U_{PGE_2}V$) before (Time 0) and after administration of indomethacin (5 mg kg^{-1}). ** $P < 0.01$ vs. Time 0.

and 9.0 ± 2.7 ng kg^{-1} in vehicle- and indomethacin-treated monkeys respectively ($n = 3$ per group).

SK&F 105494 administration, in either the presence or absence of indomethacin, did not alter significantly either urine osmolality (44 ± 4 and 42 ± 5 mOsm kg^{-1} H_2O at 60 min in vehicle- and indomethacin-treated groups, respectively) or urine flow (2.2 ± 0.2 and 2.2 ± 0.3 ml min^{-1} at 60 min, respectively). In 1 animal, however, there was a transient reduction in urine flow within 20 min of administration of SK&F 105494 which was associated with a transient reduction in blood pressure (62 to 42 mmHg). However, both parameters returned to control levels at 60 min.

Urinary excretion of prostaglandin E_2 (PGE_2) was 54 ± 19 pg min^{-1} before indomethacin treatment (5 samples from 3 monkeys). At 30 and 120 min following indomethacin administration, the PGE_2 concentration in 8 of 10 urine samples fell below the detection limit of the assay (2.5 pg 100 μl^{-1}), indicating that the urinary PGE_2 excretion had been reduced by at least 60 and 70%, respectively (Figure 2).

Discussion

In the present study, we demonstrated for the first time that in the rhesus monkey SK&F 105494 is a potent inhibitor of exogenous vasopressin activity. Furthermore, indomethacin, in a dose that reduced urinary prostaglandin excretion by at least 70%, failed to alter vasopressin antidiuretic activity either in the presence or absence of receptor blockade. This is in contrast to observations made in rats and dogs where cyclo-oxygenase inhibition enhances vasopressin antidiuretic activity (Anderson *et al.*, 1975; Berl *et al.*, 1977; Fejes-Toth *et al.*, 1977; Albrightson-Winslow *et al.*, 1989) but is similar to what is observed in man (Padfield & Grekin, 1981). Our assay system did not permit us to observe a greater than 70% reduction in urinary prostaglandin excretion, suggesting that some residual production may be present. However, we do not believe that this is likely, since this dose of indomethacin reduced the PGE_2 concentration in 8 of 10 urine samples to below the detection limit of the assay and, in dogs, a smaller dose of indomethacin reduced PGE_2 levels in the urine by 80–90% and shifted the vasopressin dose-response curve (Albrightson-Winslow *et al.*, 1989). The results suggest that in the rhesus monkey, as in man, renal prostaglandin production may not modulate the antidiuretic activity of vasopressin. These observations are consistent with the observation that indomethacin can unmask the agonist activity of the vasopressin antagonist SK&F 101926 in the dog (Albrightson-Winslow *et al.*, 1989); whereas, in both man (Allison *et al.*, 1988) and rhesus monkey (Brooks *et al.*, 1988a), this agent demonstrated full agonist activity without prior cyclo-oxygenase inhibition. SK&F 105494 demonstrated minimal agonist activity in the rhesus monkey, even in the presence of indomethacin. Furthermore, the ability of SK&F 105494 in the rhesus monkey to inhibit the antidiuretic activity of exogenous vasopressin was also unaffected by indomethacin treatment. We have previously shown that SK&F 105494 can cause a water diuresis in the hydropenic rhesus monkey (Brooks *et al.*, 1988a).

This study further indicates the possible utility of the rhesus monkey as a model for renal vasopressin agonist and antagonist activity in man. Previously, we have also shown that the stereochemical requirements of vasopressin antagonism are similar for *in vitro* activity in human renal tissue and both *in vitro* and *in vivo* activity in rhesus monkey (Brooks *et al.*, 1989). The rhesus monkey also appears to be a good model for the flushing activity associated with vasopressin V_2 agonists (Brooks *et al.*, 1988b).

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Rat hippocampal muscarinic autoreceptors are similar to the M_2 (cardiac) subtype: comparison with hippocampal M_1, atrial M_2 and ileal M_3 receptors

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1 Affinity constants for 15 non-selective or putatively selective muscarinic antagonists were determined at muscarinic autoreceptors and postsynaptic receptors (linked to phosphatidylinositol (PI) hydrolysis) in rat hippocampal slices, at muscarinic receptors mediating contractility in guinea-pig atria or ileal smooth muscle and at binding sites in rat cerebral cortical membranes labelled with [^{3}H]-1-quinuclidinyl benzilate or [^{3}H]-pirenzepine.

2 Comparison of the affinities of these antagonists at central M_1 receptors (inositol-monophosphate formation in rat hippocampal slices) with their affinities at peripheral M_1 receptors (inhibition by McN-A-343 of electrically stimulated twitches in rabbit vas deferens) provides support for the suggestion that these receptors may differ pharmacologically.

3 Comparison of affinity constants obtained by displacement of specifically bound [^{3}H]-pirenzepine from rat cerebral cortical membranes with those obtained in functional tests showed poor correlations between affinities for binding sites and for functional atrial receptors or for hippocampal autoreceptors. A significant correlation was found between affinities for [^{3}H]-pirenzepine binding and those determined at muscarinic receptors linked to PI turnover in rat hippocampus. A significant correlation was also obtained between the affinities for specific [^{3}H]-pirenzepine binding sites in cortical membranes and the affinities at ileal receptors.

4 Comparison of the affinity values for muscarinic autoreceptors in rat hippocampus with affinity values obtained from *in vitro* models of muscarinic receptor subtypes showed no significant correlations between these autoreceptors and either M_1 or M_3 receptors. A significant correlation was found between antagonist affinities for hippocampal autoreceptors and muscarinic receptors in the heart. Therefore, muscarinic autoreceptors in rat hippocampus are pharmacologically similar to the M_2 (cardiac) muscarinic receptor subtype.

Introduction

Late-onset Alzheimer's dementia appears to be a relatively pure cholinergic lesion confined to the hippocampus and temporal cortex (Rosser *et al.*, 1984). It is thus possible that antagonists of the acetylcholine autoreceptors in these brain regions may be therapeutically useful in the early stages of this disease. The synthesis of compounds to test this hypothesis, however, first requires a clear characterization of these autoreceptors.

Early studies found that atropine enhanced the stimulated release of tritium from cortical (Richardson & Szerb, 1974) or hippocampal (Nordstrom & Bartfai, 1980) tissues prelabelled with tritiated choline, suggesting that the autoreceptors in these tissues were muscarinic in character.

It is now evident that there are multiple types of muscarinic receptors. The non-classical antagonist, pirenzepine (PZ), differentiates muscarinic receptors in various tissues (Hammer *et al.*, 1980) and receptor subtypes were initially defined as M_1 with a high affinity for PZ, (pA_2 or pK_b between 7.5 and 8.0; see Eglen & Whiting, 1986) and 'M_2' (or non-M_1) with a lower affinity for PZ. Other compounds shown to display some selectivity between muscarinic receptors in different tissues included dicyclomine (M_1-selective; Potter *et al.*, 1984) and gallamine ('M_2'-selective; Mitchelson, 1984). All three antagonists have been used to characterize muscarinic autoreceptors in rat brain. PZ (Marchi & Raiteri, 1985; Schoffelmeer *et al.*, 1986; Roberts & Tutty, 1986) and dicyclomine (Marchi & Raiteri, 1985) were found to display low affinities for muscarinic autoreceptors, indicating that these receptors were not M_1. However, further efforts to characterize these autoreceptors produced ambiguous results. Gallamine blocked muscarinic autoreceptors in rat striatum at lower concentrations ($pEC_{50} = 5.5$; Schoffelmeer *et al.*, 1986) than were required to antagonize these autoreceptors in rat cerebral cortex ($pK_b < 4$; Roberts & Tutty, 1986). These observations are consistent with the findings of Ladinsky *et al.* (1987) that muscarinic autoreceptors in different brain regions may be differentially regulated and emphasize the importance of tissue variations within the same species.

More recently, data from the characterization of 'M_2' receptors in heart or smooth muscles suggests that they are not homogeneous. Himbacine (Anwar-ul *et al.*, 1986) and AF-DX 116 (Giachetti *et al.*, 1986a) were found to be more potent as antagonists of muscarinic receptors in heart than in smooth muscles. Conversely, hexahydrosila-diphenidol (HHSiD) was more potent in smooth muscles than in heart (Mutschler & Lambrecht, 1984). Results obtained with these and other putatively selective antagonists have led to proposals (see Eglen & Whiting, 1986; Birdsall *et al.*, 1987) that muscarinic receptors be divided into at least three categories: M_1 receptors, found in neuronal tissues, are selectively antagonized by PZ; M_2 receptors in heart are selectively blocked by himbacine and AF-DX 116; and M_3 receptors, located in smooth muscles or glandular tissues, are selectively antagonized by HHSiD.

The evidence for the existence of muscarinic receptor subtypes is based not only on pharmacological studies but also on results obtained from the application of molecular biology techniques to their study. There are currently at least five genes which encode for muscarinic receptor proteins (Bonner *et al.*, 1988). Although up to five tissue-dependent types of muscarinic receptor have been proposed (Mitchelson, 1988), the relationship between gene products and these putative receptor subtypes remains unclear. Moreover, the lack of selective antagonists for some of these gene products impedes

definitive subdivision of tissue receptors. Until this deficit is filled, characterization of muscarinic receptors in tissues is confined to the above three categories.

By use of muscarinic antagonists that have been shown to display selectivity among the three pharmacologically defined receptor subtypes, a more refined characterization of muscarinic autoreceptors in rat hippocampus was undertaken. The formation of inositol-monophosphate (IP_1) in rat hippocampal slices in response to cholinoceptor agonists, which is blocked with low concentrations of PZ (Gil & Wolfe, 1985), was used as a model for postsynaptic (Fisher *et al.*, 1980; Smith *et al.*, 1989) muscarinic receptors. The affinities of these same compounds were also determined in a model of M_2 receptors (electrically contracted guinea-pig atria) and in a model of M_3 receptors (electrically contracted guinea-pig ileum).

In addition to the four *in vitro* assays, binding assays were set up using rat cerebral cortical membranes labelled with either [^{3}H]-QNB (l-quinuclidinyl [phenyl-4-^{3}H]-benzilate) or [^{3}H]-PZ. The former is a potent, non-selective, lipophilic antagonist that labels muscarinic receptors on or in the plasma membrane (El-Fakahany, 1985). [^{3}H]-PZ, a hydrophilic M_1-selective antagonist, labels a portion of the receptors that are selectively labelled by [^{3}H]-QNB (Watson *et al.*, 1986). The ratios of affinity values at the two binding sites obtained with the 15 compounds used in this study were calculated to determine if this information would be useful in predicting selectivity at M_1 or non-M_1 sites in the CNS.

Thus, there were three objectives in the present study: to compare central muscarinic autoreceptors and muscarinic postsynaptic receptors in the same tissue, to attempt to place these autoreceptors in the current scheme of three pharmacologically-defined subtypes, and to investigate the usefulness of [^{3}H]-PZ binding and [^{3}H]-QNB binding to define selective ligands. In addition, determination of the affinities of several antagonists of different putative selectivities in a model of central M_1 receptors made possible a comparison with their affinity values at peripheral (Eltze, 1988) M_1 receptors.

These results were presented, in part, at the European Neuroscience Association meeting in Zurich, Switzerland, September, 1988.

Methods

Tissue preparation

Male Sprague-Dawley rats (Charles River France), 220–300 g, were stunned, decapitated and the brain quickly removed onto a glass plate. Transverse hippocampus slices (0.4 mm thick) were prepared as described by Teyler (1980). The slices weighed approximately 4 mg (wet weight) and the protein content was 0.21 ± 0.05 mg per slice ($n = 6$). The slices were kept in Krebs-Henseleit buffer (see below) which was continuously gassed with a mixture of 95% O_2 and 5% O_2 (carbogen) during all steps in the assays for muscarinic autoreceptors and muscarinic receptors linked to formation of inositol-monophosphate (IP_1).

Autoreceptor assay Hippocampus slices from one rat were preincubated in 10 ml of buffer containing 0.1 μM methyl-[^{3}H]-choline chloride for 15 min in a shaking bath at 37°C. The slices were then rinsed and placed in glass superfusion units fitted with platinum electrodes (for details, see Richards, 1985), two slices per unit. After 30 min of superfusion at 1.2 ml min^{-1}, the perfusion rate was reduced to 0.9 ml min^{-1} and a fraction collector, set to collect 2 min (1.8 ml) fractions, was started (time 0). At the start of fraction 2, all slices were stimulated (S1) for 2 min at 1 Hz with monophasic pulses of 2 ms duration. The tritium released by stimulation returned to basal values within 12 min (fraction 8). Test compounds were added to the superfusion media during the collection of fractions 8 and 20 and the slices were stimulated again during the collection of fractions 14 (S2) and 26 (S3). The superfusion was stopped after fraction 32 had been collected. The quantities of tritium remaining in the slices and that contained in the fractions were determined by liquid scintillation counting. Efflux coefficient values (% of tritium in a fraction as % of tritium in the slices at the start of collection of that fraction) were calculated. The areas under the curves (minus the basal level extrapolated from the first to last fraction under the curve) were determined by integration using the trapezium method. In each experiment the effects of the various treatments were determined in two slices. In most experiments, four concentrations of the agonist were tested at S2 and a given concentration of antagonist, sufficient to shift the concentration-response curve to carbachol to the right by 2 fold or more, was added to the buffer and to agonist-containing media before S3. The S2/S1 and S3/S1 ratios were calculated, plotted against the log of the concentration of agonist and the apparent pA_2 value calculated (Schlicker & Gothert, 1981). Values are expressed as means $\pm$ s.e.mean for n experiments.

Formation of inositol monophosphate The assay procedure of Brown *et al.* (1984) was used. Hippocampus slices from six rats were suspended in Krebs-Henseleit buffer continuously gassed with carbogen. During the 60 min preincubation period, the slices were in a container set in a shaking bath at 37°C and gently agitated to keep them from settling; the buffer was changed every 15 min. Individual slices were then placed in flat-bottomed 5 ml vials containing 5 mM lithium chloride in buffer. An aliquot of [^{3}H]-myo-inositol (purified just before use by passing through a small column of Dowex 1-X8 formate form) was added to each vial to a final concentration of 0.1 μM and the vials incubated for 30 min. Antagonists, when used, were added to the appropriate vials 25 min before addition of the agonist; an equal volume of buffer was added to the other vials. Different concentrations of carbachol were mixed with the contents of the vials to complete the incubation volume to 300 μl. After each addition, the vials were gently vortexed, flushed with carbogen, capped and returned in the shaking bath at 37°C. The reaction was stopped after 30 min incubation with carbachol by the addition of 940 μl chloroform/methanol (1:2 v:v) and the samples were vigorously vortexed. Additional chloroform and water (310 μl each) were added to each vial and the phases separated by centrifugation. An aliquot of 750 μl of the aqueous phase was taken to determine the quantity of [^{3}H]-inositol-monophosphate ([^{3}H]-IP_1) formed in each slice. [^{3}H]-IP_1 was separated from [^{3}H]-myo-inositol by ion exchange column chromatography. Radiolabelled standards were used to verify that the fraction eluted with 8 ml of 0.2 M ammonium formate in 0.1 M formic acid was inositol monophosphate. In each experiment, basal and blank values were determined in triplicate at the same time as the responses to agonists and antagonists. D.p.m. from blanks (no slice present) were subtracted from all samples (see below). The results were then expressed as % above basal and plotted vs log concentration. Three to seven concentrations of an antagonist, in the range that inhibited the reponse to 1 mM carbachol by 20 to 80%, were tested and the IC_{50} values determined graphically. pK_i values were calculated as described by Hawcock *et al.* (1986).

It became evident during the course of this work that a contaminant (or contaminants) in the stock solutions of [^{3}H]-myo-inositol passed through the purification column and was changed by incubation in buffer at 37°C (but not at 4°C) to another contaminant that co-eluted with IP_1. This gave rise to blank values ranging from less than 100 to more than 1000 d.p.m. depending on the specific activity and source of the [^{3}H]-myo-inositol. It was also noted that, although the stock solutions were stored as recommended by the manufac-

turers, blank values from a given solution increased with time. Moreover, solutions containing products to absorb radiodegradation contaminants (Amersham TRK.911 with PT6–271) gave blank values (after passage through a small purification column) of less than 100 to more than 250 d.p.m. Therefore, blank values were determined in each experiment and subtracted from experimental values before the effects of different treatments on IP_1 formation were calculated.

Atria

Left atrial preparations from male guinea-pigs (120–200 g) were set up in 10 ml isolated organ baths containing Tyrode solution (mM: NaCl 137, KCl 5, $CaCl_2$ 1, $MgCl_2$ 1, $NaHCO_3$ 11.9, NaH_2PO_4 0.4, glucose 11) which was gassed with carbogen and maintained at 31°C. Resting tension was adjusted to 1 g and contractions measured under isometric conditions by Grass FTO3 transducers connected to a Grass 79D polygraph. The atrial preparations were stimulated via punctate electrodes at 0.2 Hz with square wave pulses of 3 ms duration. Voltage was adjusted to be just above threshold (1–3 V). These stimulation conditions did not appear to release catecholamines because the mechanical responses were unaffected by (±)-propranolol (1 μm). Compounds were tested for their ability to antagonise acetylcholine-induced responses. After construction of an initial cumulative concentration-effect curve to acetylcholine, atria were incubated in the presence or absence of the compound under test for 60 min before a second concentration-effect curve to acetylcholine was elicited. Further concentration-effect curves to acetylcholine were elicited using the same protocol in the presence of higher concentrations of antagonist. The pA_2 values were calculated by the method of Arunlakshana & Schild (1959).

Electrically stimulated guinea-pig ileum

Pieces of proximal ileum between 2 and 3 cm in length were removed from guinea-pigs (250–400 g) and suspended in Tyrode solution for isometric recording. Electrical field stimulation of the enteric cholinergic nerves was carried out by means of ring electrodes using the following parameters: 0.05 Hz, 2 ms, supramaximal voltage. Antagonist effects were quantified as the maximum inhibitory effect on electrically-induced contractions achieved during a 2 min contact period. Concentration-response curves were obtained, non-cumulatively, with a 30 min interval between each drug addition. Antagonist concentrations producing half-maximal inhibition (IC_{50}) were determined graphically. Mean pIC_{50} values ± s.e.mean were calculated from 4 to 6 experiments.

Binding assays

Membranes were prepared from cerebral cortices of male Sprague-Dawley rats. The tissues were homogenized at 4°C with a Polytron (Brinkman, setting 9) for 15 s in 50 mM sodium potassium phosphate buffer, pH 7.4. The homogenates were centrifuged for 10 min at 1000 *g* at 4°C, the resulting supernatant recentrifuged for 30 min at 48000 *g* and the pellet (P2) resuspended in the homogenizing buffer to a dilution of 70 times tissue wet weight for [^{3}H]-PZ binding and 280 times wet weight for [^{3}H]-QNB binding. The homogenates were incubated with 0.1 nM [^{3}H]-PZ or 0.2 nM [^{3}H]-QNB for 90 min at room temperature and the reactions terminated by filtration. Non-specific binding was defined as that in the presence of 1 μM atropine. IC_{50} values were determined graphically and corrected for the concentration of the tritiated ligand (Cheng & Prushoff, 1973). For [^{3}H]-PZ, the correction was $IC_{50}/1.05$; for [^{3}H]-QNB, the correction was $IC_{50}/2.05$. The corrected IC_{50} values were then expressed as the negative log (pIC_{50}).

Drugs and chemicals

Characterization of receptors depends a great deal on the ready availability of selective compounds and the generosity of individuals and enterprises in their exchange. Of the 15 compounds tested in these assays, 8 were gifts. I would like to thank the following: Dr R.B. Barlow and Dr M. Shepherd, University of Bristol, Bristol, U.K. for 4-diphenyl-acetoxy-N-piperidine methiodide (4-DAMP); Dr M. Eltze, Byk Gulden Lomberg Chemische Fabrik GmbH, Konstanz, F.R.G. (telenzepine); Dr R. Hammer, Boehringer Ingelheim Zentrale GmbH, Ingelheim, F.R.G. (pirenzepine and 11[[2-[(diethylamino)methyl] - 1 - piperidinyl]acetyl]5,11 - dihydro - 6H - pyrido[2,3-b][1,4]benzodiazepine-6-one (AF-DX 116)); Dr M. Laduron, Rhone-Poulenc Sante, Vitry-sur-Seine, France (thiazinamium); Dr G. Lambrecht, Johann-Wolfgang Goethe Universität, Frankfurt-am-Rhein, F.R.G. (HHSiD); Dr W.C. Taylor, University of Sydney, Sydney, Australia (himbacine); Dr I. van Wijngaarden, Duphar B.V., Weesp, Holland (secoverine).

The following products were purchased from Sigma Chemical Co., St. Louis, MO. U.S.A.: atropine methyl nitrate, carbamylcholine (carbachol), gallamine triethiodide, hemicholinium-3 and (D, L)-trihexyphenidyl hydrochloride (THP; benzhexol). Amitriptyline was obtained from Produits Roche s.a., Neuilly, France. N-methyl scopolamine bromide (NMS) was from E. Merck, Darmstadt, F.R.G. Dicyclomine hydrochloride (DC) was obtained from Merrell-Dow, Cincinnati, OH, U.S.A.

Krebs-Henseleit buffer was prepared from analytical grade compounds and contained (in mM): NaCl 118, KCl 5.0, $CaCl_2$ 1.3, KH_2PO_4 1.0, $MgSO_4$ 1.2, $NaHCO_3$ 25 and glucose, 10. The pH was maintained at 7.4 with carbogen.

Dowex AG 1 X8 resin, formate form (100–200 mesh) was purchased from BioRad, Richmond, CA U.S.A. The resin was purified according to the procedure of Kakimoto & Armstrong (1962) except that 2 M formic acid was substituted for the 2 M HCl called for in the procedure. After the last wash (with acetone), the excess fluid was removed by filtration under vacuum. The resin was then weighed and a volume of 1 M formic acid equal to this weight was used to resuspend the resin. Aliquots of 1 ml suspension were used per column. One hundred columns (Econo-columns, 0.7 × 15 cm, BioRad, Richmond, CA, U.S.A.) were set up in a perspex holder. Before use, the resin in the columns was treated with 2 times 10 ml of 10 mM myo-inositol (Sigma Chemical Co., St. Louis, MO, U.S.A.) and one cycle of washing/eluting buffers to reduce variable recovery off the columns. The eluting mixtures were delivered to the columns with an Ismatec peristaltic pump set up to wash 20 columns simultaneously. The resin in the columns was regenerated after each experiment with 25 ml 1 M formic acid. The resin was renewed after approximately 20 experiments.

The following products were from New England Nuclear-Dupont, Dreieich, F.R.G.: methyl-[^{3}H]-choline chloride, 80 Ci mmol^{-1}; N-methyl-[^{3}H]-pirenzepine, 84.1 Ci mmol^{-1}; l-myo-1,2-[^{3}H]-(N) inositol, 47.1–61.2 Ci mmol^{-1}; myo-2-[^{3}H]-(N) inositol, 14 Ci mmol^{-1}; Aquasol-2 universal LSC cocktail. Amersham, International, Amersham, U.K., supplied l-quinuclidinyl(phenyl-4-[^{3}H]-benzilate, 39 Ci mmol^{-1}; myo-(2-[^{3}H])inositol, 16.6–17.9 Ci mmol^{-1} and l-myo-(U-[^{14}C]-inositol-1-phosphate, 55 mCi mmol^{-1}. American Radiolabelled Chemicals, Inc., St. Louis, MO, U.S.A., supplied myo-(2-[^{3}H]-(N))-inositol, 15 Ci mmol^{-1}.

Results

Binding studies with [^{3}H]-QNB and [^{3}H]-pirenzepine

Comparison of [^{3}H]-*QNB and* [^{3}H]-*pirenzepine binding* In this study, all the compounds tested in both binding assays

Table 1 Effects of muscarinic antagonists on binding in rat cerebral cortex, carbachol-induced formation of inositol monophosphate (IP_1) and carbachol-activated autoreceptors in rat hippocampus slices and electrically-induced contractions of guinea-pig atria and ileum

	Binding (corr. pIC_{50})			*IP_1*		*Atria*	*Ileum*	*Autorec.*
	[^{3}H]-*QNB* (n = 3–4)	[^{3}H]-*PZ* (n = 3–4)	n_H	*App. pK_i* (n = 3–7)	n_H	*pA_2* (n = 3–5)	*pIC_{50}* (n = 4–6)	*app. pA_2* (n = 3–6)
Non-selective antagonists								
Atropine	8.73	9.42	0.99	8.84	1.02	9.11	8.53	8.55
	±0.23	±0.07	±0.39	±0.03	±0.08	±0.23	±0.08	±0.10
NMS	ND	9.35	1.28	9.39	0.82	ND	ND	8.86
		±0.12	±0.06	±0.06	±0.04			±0.05
Putative M_1 selective antagonists								
THP	7.94	9.32	0.94	8.01	0.81	6.91	8.45	8.10
	±0.03	±0.12	±0.19	±0.14	±0.02	±0.20	±0.22	±0.12
Thiazinamium	7.96	8.50*	0.78	7.95	1.12	ND	8.20	7.82
	±0.14	±0.18	±0.15	±0.08	±0.09		±0.10	±0.18
DC	7.76	8.92	1.14	7.45*	0.72	6.22	8.05	6.54
	±0.06	±0.08	±0.09	±0.06	±0.03	±0.24	±0.31	±0.10
PZ	6.69	8.19	0.99	7.63*	0.56	6.94	6.79	6.63
	±0.07	±0.06	±0.07	±0.08	±0.06	±0.33	±0.06	±0.18
Telenzepine	ND	8.76	1.11	8.51	1.03	ND	ND	7.42
		±0.14	±0.08	±0.11	±0.12			±0.08
Putative ileal-selective (M_3) antagonists								
4-DAMP	8.54	9.17	1.09	8.13	0.91	7.80	8.89	8.18
	±0.20	±0.10	±0.03	±0.11	±0.06	±0.08	±0.04	±0.07
HHSiD	7.63	8.22	0.95	6.99*	0.72	ND	7.79	6.30
	±0.14	±0.03	±0.04	±0.06	±0.01		±0.15	±0.09
Secoverine	ND	8.71	1.02	7.83	ND	7.93	7.66	7.89
		±0.08	±0.06	±0.16		±0.28	±0.11	±0.13
Amitriptyline	7.52	8.28	1.09	6.97	1.23	6.74	7.66	5.94
	±0.35	±0.03	±0.09	±0.14	±0.06	±0.27	±0.09	±0.06
Putative atrial-selective (M_2) antagonists								
Himbacine	7.04	7.19	0.95	7.18*	0.71	7.42	6.94	8.28
	±0.19	±0.02	±0.06	±0.21	±0.09	±0.21	±0.04	±0.10
AF-DX 116	5.90	6.56	1.02	6.03	0.91	6.70	5.87	6.84
	±0.12	±0.03	±0.20	±0.17	±0.15	±0.16	±0.08	±0.05
Gallamine	ND	6.50*	0.77	<5	ND	5.01	4.62	5.35
		±0.15	±0.01			±0.42	±0.12	±0.20
Bret.tos	ND	5.16	0.92	<5	ND	ND	ND	5.66
		±0.03	±0.03					±0.13

Values are mean ± s.e.mean. ND—not determined.
* Indicates values which may not be true affinity constants because the compounds display low Hill coefficients (n_H).

had higher affinities for the binding sites labelled with [^{3}H]-PZ than for those labelled with [^{3}H]-QNB (Table 1). Atropine was 5 times more active in displacing specifically bound [^{3}H]-PZ than [^{3}H]-QNB. The putative M_2- and M_3-selective antagonists had affinity ratios (concentration displacing 50% of the specifically bound [^{3}H]-QNB divided by the concentrations displacing 50% of the specifically bound [^{3}H]-PZ; Table 2) similar to that of atropine. Of the four putative M_1-selective

Table 2 Ratios of affinity values of antagonists at different muscarinic receptors determined in binding or *in vitro* functional tests

Compound	[^{3}H]-*QNB* / [^{3}H]-*PZ*	*IP_1* / [^{3}H]-*PZ*	*A/M_1*	*A/M_2*	*A/M_3*	*M_2/M_1*	*M_3/M_1*	*M_2/M_3*
Atropine	4.90	3.80	1.95	3.63	0.91	0.53	2.14	0.26
NMS	—	0.91	3.39	—	—	—	—	—
THP	23.98	20.42	0.81	0.06	1.86	12.59	0.44	34.67
Thiazinamium	3.47	3.55	1.35	—	2.14	—	0.63	—
DC	14.45	29.51	8.13	0.48	70.79	16.98	0.11	67.61
PZ	31.62	3.63	10.00	2.04	1.62	4.90	6.17	0.71
Telenzepine	—	1.78	12.30	—	—	—	—	—
4-DAMP	4.27	10.96	0.89	0.42	4.90	2.14	0.18	12.30
HHSiD	3.89	16.98	4.90	—	35.48	—	0.14	—
Secoverine	—	7.59	0.87	1.10	0.63	0.79	1.38	0.54
Amitriptyline	5.75	20.42	10.72	6.31	54.95	1.70	0.19	8.32
Himbacine	1.41	1.02	0.08	0.14	0.05	0.58	1.70	0.33
AF-DX 116	4.57	3.39	0.15	0.72	0.11	0.21	1.38	0.15
Gallamine	—	>32	>0.45	0.46	0.19	—	—	0.41
Bret. tos.	—	>0.7	>0.22	—	—	—	—	—

A = autoreceptor, M_1 = IP_1 response, both in rat hippocampus slices. M_2 = atrial response, M_3 = ileal contractions, both in guinea-pig tissues. — = not determined.

antagonists tested at the two binding sites, three- THP, DC and PZ were at least 14 fold more active in displacing [^{3}H]-PZ than [^{3}H]-QNB. The fourth compound, thiazinamium, had a ratio of affinities for the sites labelled by the two ligands similar to those of atropine. However, the Hill coefficient for this compound calculated from displacement experiments was less than unity when [^{3}H]-PZ was the ligand (Table 1), which may indicate a non-competitive interaction between displacer and ligand at the binding site.

Comparison of Hill coefficients between [^{3}H]-pirenzepine binding and (inositol monophosphate) formation It was of interest to compare not only affinity values but also Hill coefficients of compounds antagonizing IP_1 formation and specifically displacing [^{3}H]-PZ (Table 1). All compounds except thiazinamium and gallamine appeared to intereact competitively with [^{3}H]-PZ binding sites. In contrast to its low Hill slope determined from displacement studies of [^{3}H]-PZ, thiazinimium appeared to be a competitive antagonist at muscarinic receptors linked to IP_1 formation. There were four compounds that displaced [^{3}H]-PZ in an apparent competitive manner but displayed low Hill coefficients (<0.8) when tested for their ability to inhibit IP_1 formation -PZ, DC, HHSiD and himbacine.

Formation of inositol monophosphate in hippocampal slices induced by carbachol in the absence or presence of muscarinic antagonists

The number of d.p.m. isolated as IP_1 from hippocampal slices under basal or stimulated conditions varied depending on the specific activity and source of [^{3}H]-myo-inositol. In several series of experiments ($n = 9$ to 17 experiments per series for a total of 64 independent observations), accumulation of IP_1 under basal conditions ranged from 386 ± 53 d.p.m. ($n = 14$) to 677 ± 80 d.p.m. ($n = 11$). In this same series of experiments, the maximum stimulation of IP_1 formation by 1 mM carbachol ranged from $1636 \pm 99\%$ ($n = 17$) to $2102 \pm 86\%$ ($n = 11$) above basal. The average of the means from these series was 1852% above basal. The pD_2 value (negative log of the EC_{50}) for carbachol ranged from 4.53 ± 0.05 ($n = 11$) to 5.00 ± 0.05 ($n = 14$). The average pD_2 value from these five series of experiments was 4.90.

The effects on basal accumulation of IP_1 were determined for the highest concentration of each antagonist used in this test. None except bretyllium tosylate and gallamine influenced basal IP_1 formation.

The fifteen antagonists used in this study inhibited, in a concentration-dependent manner, the formation of IP_1 induced by 1 mM carbachol. The pK_i values are listed in Table 1. The non-selective antagonists, atropine and NMS, displayed the highest affinities followed by the putative M_1-selective antagonists, telenzepine, THP, thiazinamium, PZ and DC. 4-DAMP, secoverine, and to a lesser degree, himbacine also antagonized IP_1 formation and had moderate to high affinities for the receptors. Compounds which displayed lower affinities ($pK_i < 7$) at this site included HHSiD, amitriptyline and AF-DX 116. Gallamine and bretyllium tosylate had very low affinities ($pK_i < 5$). However, in the presence of the high concentrations of these compounds required to block the carbachol response, basal formation of IP_1 was doubled, complicating the interpretation of these results.

The Hill coefficients were calculated for some of the antagonists that inhibited carbachol-induced IP_1 formation. Telenzepine and amitriptyline had Hill coefficients significantly greater than one. Increasing the incubation time with these antagonists to 60 min decreased the Hill coefficient to unity for telenzepine while that obtained with amitriptyline decreased from 1.44 ± 0.04 ($n = 3$) to 1.23 ± 0.06 ($n = 3$). Atropine, NMS, telenzepine, 4-DAMP, thiazinamium, amitriptyline and AF-DX 116 had Hill coefficients between 0.8 and 1.2, consistent with competitive antagonism at this receptor. Compounds with Hill coefficients of less than 0.8 included HHSiD, himbacine, DC and PZ. It is of interest to note the difference between PZ and its M_1-selective analogue, telenzepine.

Effects of antagonists on muscarinic receptors in guinea-pig atria

Only atropine antagonized with high affinity the inhibition of atrial contractions induced by acetylcholine (ACh). Antagonists exhibiting moderate affinities (pA_2 between 6 and 7) at this site included secoverine, 4-DAMP and himbacine (Table 1). In the present studies, himbacine was less active than reported by Anwar-ul *et al.* (1986) due, perhaps, to different concentrations of calcium in the media and/or differences in the electrical stimulation parameters. Compounds with pA_2 values less than 7 included THP, PZ, amitriptyline, AF-DX 116 and DC. Gallamine had the lowest pA_2 value.

Effects of antagonists on guinea-pig ileal muscarinic receptors

In this test, 4-DAMP displayed the highest affinity followed by atropine, THP, thiazinamium and DC. Compounds with pIC_{50} values of between 7 and 8 were HHSiD, secoverine and amitriptyline. Antagonists with affinity values of less than 7 included PZ, himbacine, AF-DX 116 and gallamine.

Muscarinic autoreceptors in rat hippocampal slices

Rat hippocampal slices, stimulated three times at 1 Hz for 2 min, released tritium in a reproducible manner. For example, in one series of experiments, the areas under the curves (X 100) were: S1 = 1.02 ± 0.05, S2 = 1.00 ± 0.11, S3 = 1.00 ± 0.12 ($n = 6$).

Carbachol 0.3 to 10 μM, concentration-dependently inhibited stimulated tritium efflux with no effect on basal tritium overflow. In ten series of experiments (to test antagonists, $n = 3$–6 experiments per series), the pD_2 value for carbachol was 6.03 ± 0.02 (range = 5.90 to 6.16) and the maximum inhibition, obtained with 10 μM carbachol, was $83.5 \pm 1.3\%$.

The antagonists tested in this study antagonized the carbachol-induced inhibition of stimulated release (apparent pA_2 values are listed in Table 1) with no effect on basal efflux of tritium. It is of interest to note that, at concentrations sufficient to antagonize activation of autoreceptors by the exogenous agonist, carbachol, none of the antagonists enhanced the stimulated overflow of tritium.

Antagonists with high affinity (apparent $pA_2 > 8$) in this test included NMS, atropine, THP, 4-DAMP and himbacine. Moderate affinities (apparent pA_2 values between 7 and 8) were displayed by thiazinamium, telenzepine and secoverine. Apparent pA_2 values between 6 and 7 were found with DC, PZ, HHSiD and AP-DX 116. Compounds with affinities of less than 6 included amitriptyline, gallamine and bretyllium tosylate.

Because himbacine exhibited the greatest selectivity for muscarinic autoreceptors compared to postsynaptic receptors linked to PI turnover (Table 2), additional concentrations of the antagonist were tested against carbachol-induced inhibition of stimulated tritium release and the data were plotted as a Schild regression. The pA_2 value was 7.94 and the slope was 0.8.

Regression analysis of correlations

Affinity values obtained with antagonists that did not display selectivity among the four *in vitro* functional tests were omitted from calculations of correlation coefficients. Defining selective compounds as those with ratios of less than 0.2 or greater than 5.0 in the *in vitro* tests in Table 2 eliminated atropine, NMS, thiazinamium, secoverine and bretylium tosylate.

Table 3 Correlation between affinity values of muscarinic antagonists, obtained by displacement of specifically bound [^{3}H]-pirenzepine ([^{3}H]-PZ) in rat cerebral cortical membranes or by antagonism of autoreceptors in rat hippocampus slices, and affinity values obtained from *in vitro* functional tests

	[^{3}H]-*PZ binding*			*Autoreceptor*		
	*df**	r	P	*df**	r	P
Functional Test						
Autoreceptor	9	0.420	0.226	—	—	—
Atria	7	0.469	0.241	7	0.827	0.011
IP_1 formation	8	0.815	0.007	8	0.480	0.191
Ileum	8	0.927	0.0003	8	0.566	0.113

* degrees of freedom

The significance of the correlations between affinity values obtained from any two tests was determined by regression analysis.

The level of significance of correlations between affinity values displayed by the ten selective antagonists for the displacement of [^{3}H]-PZ and their affinities in the four functional tests (Table 3) indicated which subtype model best described the binding data. There were poor correlations between affinities in the binding test with either those at atrial or those at hippocampal autoreceptors. As expected, a significant correlation was obtained between IP_1 formation, a model for M_1-receptors, and [^{3}H]-PZ binding in cerebral cortical membranes. However, the strongest correlation was observed between the affinities of these antagonists at [^{3}H]-PZ binding sites and their affinities at ileal M_3 receptors.

A comparison of affinities for the inhibition of carbachol-stimulated IP_1 formation in hippocampus with affinities at peripheral receptors showed a low correlation between atrial receptors and IP_1 formation ($r = 0.406$, $p = 0.37$, degrees of freedom (d.f.) = 6) while the correlation between affinities at ileal receptors and IP_1 formation was significant ($r = 0.799$, $P = 0.0175$, d.f. = 7).

The correlation between affinities at atrial receptors and those at ileal receptors was only slightly greater than the level accepted as significant ($r = 0.699$, $P = 0.0537$, d.f. = 7).

Regression analysis was also used to determine the significance of correlations between affinity values of these selective antagonists at muscarinic autoreceptors in rat hippocampus with their affinity values measured in functional models of M_1, M_2 or M_3 receptors. As shown in Table 3, the only correlation that attained significance was between muscarinic autoreceptors in rat hippocampus and muscarinic receptors in the heart.

Discussion

Although the affinities of many of these putatively selective antagonists have been previously obtained in two of the three models used in the present study, few have been determined for all three receptor subtypes tested in the same laboratory. Moreover, central M_1 receptors may differ from peripheral M_1 receptors (Lambrecht *et al.*, 1987; Bloom *et al.*, 1987). These considerations as well as the variable results presented in the literature (see, for example, Mitchelson, 1988) for some of these standard antagonists led us to set up these models in house.

A comparison of the ratios of affinity values of putatively selective antagonists for displacing [^{3}H]-PZ and [^{3}H]-QNB was of limited value for the prediction of M_1 versus non-M_1 selectivity in functional tests. Putative M_1 selective antagonists displayed affinity ratios of 3 to 32. If thiazinamium was eliminated from the comparison because it displayed no selectivity in functional tests (Table 2), the remaining putative M_1-selective compounds produced ratios of 14 or greater. The ratios of both M_2- and M_3-selective antagonists were less than 6. M_3 antagonists displaced both ligands and had high affinities at both binding sites whereas M_2 antagonists displaced both ligands but had low affinities. The highly significant correlation between affinities of M_3 antagonists for displacing [^{3}H]-PZ from binding sites in the cerebral cortex with their affinities for antagonizing ileal contractions could be a reflection of the homology demonstrated between m_1 and m_3 gene products (Bonner *et al.*, 1987) and suggests caution should be applied in interpreting binding data obtained by displacement of specifically bound [^{3}H]-PZ from rat cerebral cortical membranes (50% M_1, 15% M_2, 35% M_3; Giraldo *et al.*, 1987). The apparent lack of displacement of [^{3}H]-QNB by M_2 antagonists could be due to the small percentage of M_2 sites in this tissue.

The results obtained with eleven of the antagonists used in this study allowed another question to be addressed, that of possible differences between central and peripheral M_1 receptors. Affinities of compounds determined at M_1 receptors of rat hippocampus were compared with their affinities at M_1 receptors in rabbit vas deferens obtained by Eltze (1988), which were found to be similar to the ganglionic M_1 receptor (Eltze *et al.*, 1988). Himbacine, THP and AF-DX 116 were about 6 times more active at antagonizing the latter than the former while both 4-DAMP and HHSiD were 8 tmes more active. Thus, three antagonists in addition to HHSiD (Lambrecht *et al.*, 1987) and AF-DX 116 (Bloom *et al.*, 1988) appear to present some selectivity between central and peripheral M_1 receptors.

Of the 15 antagonists tested in this study against carbachol-induced IP_1 formation, 11 had Hill coefficients of about 1, compatible with competitive antagonism at this site. Low Hill coefficients were found with himbacine, HHSiD, DC and PZ. This has been previously observed for PZ on IP_1 formation in rat CNS (Rooney & Nahorski, 1986) and in guinea-pig cortex (Kunyzs *et al.*, 1988; but see Ek & Nahorski, 1988). Thus, these four compounds may not be competitive antagonists or there may be multiple muscarinic sites in hippocampal slices linked to PI turnover, possibly on different cell types. *In situ* hybridization experiments have demonstrated the presence of m1 and m3 mRNA in rat hippocampus (Buckley *et al.*, 1988) whose corresponding receptors have both been found to be linked to phosphatidylinositol turnover (Peralta *et al.*, 1988; where HM4 = m3). Recently, the affinity values and Hill coefficients for PZ, HHSiD and DC were determined in binding experiments using CHO-K1 cells expressing m1 or m3 genes (Buckley *et al.*, 1989). The former two antagonists displayed n_H values of approximately unity for their interactions with both gene products whereas the n_H values obtained from the interaction of DC with either m1 or m3 gene products was less than 0.7. These data suggest, therefore, that the low Hill coefficients calculated from data obtained with PZ and HHSiD for antagonizing carbachol-induced IP_1 formation in rat hippocampus may be due to interactions with multiple receptors linked to this second messenger, while the low n_H value calculated from data obtained with DC may be due to an allosteric interaction of this latter antagonist with one or both receptors.

In peripheral tissues DC potently inhibited electrically-induced contractions of guinea-pig ileum, being 70 times more active in this tissue than in blocking atrial receptors. Doods *et al.*, (1987) demonstrated that the antagonist was 28 times more active in inhibiting salivation (M_3) than it was in heart (M_2). In rat frontal cortex DC was 250 times more active than PZ in blocking muscarinic receptors modulating [^{3}H]-dopamine release (March & Raiteri, 1985), implicating M_3 receptors in this response. The lack of antagonism by gallamine on striatal muscarinic receptors influencing dopamine release (Schoffelmeer *et al.*, 1986) would indirectly support this suggestion. It would be of interest to determine the effects of other M_3-selective antagonists on muscarinic modulation of dopamine release.

While the determination of affinity values of muscarinic antagonists in four *in vitro* functional receptor systems was to provide a basis for the characterization of hippocampal muscarinic autoreceptors, comparison of the affinities of these antagonists among the other tests was also of interest. For example, the significant correlation between the affinities of the selective antagonists for receptors mediating hippocampal IP_1 formation with those at ileal receptors mediating contractility lends support to the suggestion that this second messenger may play a role in ileal smooth muscle contractions (see Goyal, 1988, and references therein). The low correlation between affinity values of these antagonists at IP_1 formation and muscarinic receptors in atria is in accord with findings that muscarinic receptors inducing negative inotropic responses are not linked to PI hydrolysis (Eglen *et al.*, 1988). The relatively high correlation coefficient between affinities at heart receptors with affinities at ileal receptors is consistent with the lack of a high degree of selectivity of the antagonists used in these models.

The primary goal of this work was to determine affinity values of putatively selective or non-selective antagonists at blocking activation of muscarinic autoreceptors in electrically stimulated rat hippocampal slices. The activities of these antagonists were also determined in different *in vitro* models of muscarinic receptor subtypes and the affinity values in each test were then correlated with their affinity values for antagonizing muscarinic autoreceptors. In the four *in vitro* functional tests, the classical muscarinic antagonist, atropine, displayed high but differential affinities, being 3.8 times less active in atrium than ileum. If atropine is assumed to be a non-selective antagonist, then differences of greater than 0.6 log units will be necessary (but perhaps not sufficient) to distinguish receptor subtypes.

In binding studies, THP (Tien & Wallace, 1985; Nivelbrant & Sparf, 1986) and thiazinamium choride (Muth *et al.*, 1985) were shown to display M_1 selectivity. Functional tests *in vivo* and *in vitro* have shown that THP was more active at blocking peripheral M_1 than M_2 receptors (Giachetti *et al.*, 1986*b*; Eltze, 1988) whereas we and others (Eglen & Whiting, 1987) found THP to be most active at antagonizing M_3 receptors. In the present study, thiazinamium also displayed high affinity at ileal receptors. This may explain, in part, its bronchodilator activity (Muth *et al.*, 1985) which could be due to antagonism of M_3 receptors on airway smooth muscle (Barnes *et al.*, 1988). However, both THP and thiazinamium were as active at central M_1 sites as at ileal M_3 receptors and both compounds displayed high affinity for the muscarinic autoreceptors in rat hippocampus. These results are inconsistent with these compounds acting as selective antagonists.

Muscarinic antagonists other than PZ that have been shown to be M_1-selective include telenzepine (Eltze *et al.*, 1985) and DC (Potter *et al.*, 1984). In hippocampus slices both of these compounds, as well as PZ, were 8 to 10 times more active in inhibiting the PI response than in blocking activation of the autoreceptor. This supports the conclusion of Raiteri *et al.* (1984), that muscarinic autoreceptors in rat hippocampus are not likely to be M_1 and accords with similar conclusions based on data obtained with muscarinic autoreceptors in rat striatum (Schoffelmeer *et al.*, 1986) and cerebral cortex (Marchi & Raiteri, 1985; Roberts & Tutty, 1986).

Antagonists suggested to be selective for the M_3 ileal muscarinic receptor include 4-DAMP (Barlow & Shepherd, 1985), HHSiD (Mutschler & Lambrecht, 1984) and secoverine (Zwagemakers & Claassen, 1980). Amitriptyline was shown to be 29 times more active in blocking striatal muscarinic receptors negatively linked to adenylate cyclase than cortical muscarinic receptors linked to PI turnover (Nomura *et al.*, 1987). In the present study, secoverine showed no selectivity in any of the functional tests. Amitriptyline was more active on ileal than atrial receptors indicating some M_3 selectivity. Consistent with their M_3 selectivity, both 4-DAMP and HHSiD were most active in antagonizing muscarinically-mediated ileal contractions. Comparison of the affinities of these three compounds on ileal receptors versus muscarinic autoreceptors in hippocampus showed greater activity at the former. 4-DAMP displayed the smallest degree of selectivity (5 fold) while HHSiD and amitriptyline were 35 and 55 times, respectively, more active at blocking ileal receptors than hippocampal autoreceptors. These data indicate that the muscarinic autoreceptors in rat hippocampus are not likely to be of the M_3 subtype.

Putative cardioselective agents bretylium tosylate (Schreiber & Sokolovsky, 1985), gallamine (Mitchelson, 1984), himbacine (Anwar-ul *et al.*, 1986) and AF-DX 116 (Giachetti *et al.*, 1986a) were also tested in the four *in vitro* assays. These compounds were more active as antagonists at hippocampal autoreceptors than at postsynaptic receptors and all four antagonized muscarinic autoreceptor activation at similar concentrations to those required to antagonize cardiac receptors. The selectivity ratios between pre- and postsynaptic hippocampal receptors were small for gallamine and bretylium tosylate whereas the ratios for AF-DX 116 and himbacine were 6 and 13, respectively. Moreover, the analysis of correlation coefficients between autoreceptors and the three *in vitro* models of muscarinic receptor subtypes showed a significant correlation only between autoreceptors and cardiac receptors. These data indicate that muscarinic autoreceptors in rat hippocampus are pharmacologically similar to muscarinic receptors in the heart.

Thus the evidence presented does not support classification of muscarinic autoreceptors in rat hippocampus as M_1 or M_3 but does suggest that these autoreceptors are similar to the M_2 subtype. However, the homology between m2 and m4 gene products (Bonner, 1989) and the lack of selective antagonists for the latter do not allow a firm conclusion, based on pharmacological evidence alone, to be drawn. Again, supporting evidence may be obtained from *in situ* hydridization results. The presence of m2 mRNA and the apparent absence of mRNA for other muscarinic receptors, including m4 mRNA, in medial septal nuclei (Buckley *et al.*, 1988) is consistent with hippocampal autoreceptors being of the M_2 subtype. This also suggests that muscarinic autoreceptors in rat hippocampus may be a relatively pure population and that the variability obtained with muscarinic antagonists is indicative of possible multiple receptors being recognized by these compounds in the other *in vitro* tests.

In conclusion, comparison of 15 muscarinic antagonists in four *in vitro* functional tests and two binding assays provided additional evidence for at least three pharmacologically defined muscarinic receptor subtypes. There may be pharmacological differences between peripheral and central M_1 receptors. Muscarinic autoreceptors in rat hippocampus appear to be pharmacologically similar to the M_2 (cardiac) receptor subtype.

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The role of the epithelium in modulating the responses of guinea-pig trachea induced by bradykinin *in vitro*

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1 The effect of removing the epithelium on the responses of the guinea-pig isolated trachea (GPT) to bradykinin (BK) and prostaglandin E_2 (PGE_2) was investigated.

2 BK (3 pmol–10 nmol) induced dose-related relaxations of the intact (with epithelium), and contracted the rubbed (without epithelium) preparation of GPT. Similar responses were also obtained with PGE_2 (0.3–3.0 nmol).

3 Indomethacin (1.4 μM) modified the BK-induced response of intact GPT, from a relaxation to a contraction, but inhibited the BK-induced contraction of the rubbed GPT.

4 There was a significant increase in PGE_2 release from the intact GPT following stimulation with BK.

5 Removal of the epithelium from the GPT significantly reduced both basal and BK-induced generation of PGE_2.

6 The induction of tone in the rubbed GPT by addition of acetylcholine (ACh) caused BK and PGE_2 (0.3 nmol–3 nmol) to produce relaxations of the tissue.

7 Salbutamol (10^{-8} M–10^{-6} M) reduced the relaxations induced by BK on intact GPT, in a concentration-dependent manner.

8 These results suggest that both tone and an epithelial-dependent cyclo-oxygenase mechanism are important in modulating BK-induced responses of GPT.

Introduction

Non-specific hyperreactivity of the airways is a characteristic sign of bronchial asthma and is associated with damage to, or loss of, the epithelial cells lining the airways (Laitinen *et al.*, 1985). There is substantial evidence showing that the epithelium influences responses of many bronchomotor agonists (Goldie *et al.*, 1986; Hay *et al.*, 1987a). Several studies in the literature describe the potentiation of constrictor agonists in the absence of epithelium (Hay *et al.*, 1987a). Theories advanced to account for this potentiation include the suggestion that intact epithelium acts as a protective barrier, reducing contact between agonists and airway smooth muscle (Holroyde, 1986). Another hypothesis suggests the release of an epithelial-derived relaxing factor (EpDRF) which acts to modulate the tone and reactivity of airways (Vanhoutte, 1988).

The effect of removing the epithelium from trachea on responses to many agents (predominantly constrictor) has been studied, but the most profound effects are those associated with the use of arachidonic acid (AA). Arachidonic acid causes the relaxation of guinea-pig trachea (GPT) *in vitro*, but following epithelial removal the response is changed from a relaxation to a contraction (Nijkamp & Folkerts, 1987; Farmer *et al.*, 1987).

Bradykinin (BK) has also been shown to elicit relaxations of GPT *in vitro*, when the tissue was pre-contracted with histamine (Mizrahi *et al.*, 1982). More recently, Bewley *et al.* (1987) have demonstrated that both BK and kallidin induce biphasic responses on the GPT. Mizrahi and colleagues suggested that BK exerts its action on the GPT via an indirect mechanism, which is entirely dependent on generation of prostaglandin E_2 (PGE_2) (Mizrahi *et al.*, 1982). It has recently been suggested that the response of GPT due to PGE_2 is dependent on the tone of the preparation (Braunstein *et al.*, 1988). Thus endogenously generated PGE_2 may have a relaxant or a constrictor effect on tracheal smooth muscle, depending on the tone of GPT.

In this study we show that epithelial removal alters the response of GPT to BK from a relaxation to a contraction in both conventional organ bath and superfusion cascade systems. The importance of the tone on responses of GPT due to BK, and the possible contribution of cyclo-oxygenase products to these responses were also investigated.

A preliminary account of this work was presented to the British Pharmacological Society (Bramley *et al.*, 1988).

Methods

Male Dunkin-Hartley guinea-pigs (400–500 g) were killed by a blow to the head, followed by exsanguination. The tracheae were then removed and placed in Tyrode solution containing (mM): NaCl 138, KCl 2.7, $CaCl_2 \cdot 6H_2O$ 1.8, glucose 5.5, $NaHCO_3$ 11.9, $MgCl_26H_2O$ 0.1 and NaH_2PO_4 0.4.

Tracheal strips

Connective tissue and fat were removed and the trachea cut longitudinally opposite the band of smooth muscle. It was then cut in half, one half was kept intact while the other was stripped of epithelium by gently rubbing the lumen with a cotton wool-tipped applicator. Proximal and distal halves were always randomly selected for epithelial removal.

The tracheal strips were cut in a zig-zag fashion according to the method of Emmerson & Mackay (1979). Histology was performed on selected samples to test for the presence or absence of epithelium.

The tissues were superfused with Tyrode solution at a flow rate of 5 ml min^{-1} and a temperature of 37°C. Changes in length of the tracheal strips were measured with Harvard smooth muscle transducers, using auxotonic levers (Paton, 1957), and the responses displayed on a Graphtec Linearcorder MK VII.

Administration of drugs

The following drugs were used: acetylcholine (ACh), bradykinin (BK), salbutamol, PGE_2 and the thromboxane A_2 (TXA_2)-mimetic U-44069. All agonists were given as bolus injections directly into the superfusing buffer. Salbutamol and U-44069 were used as standard agonists on the intact and rubbed preparations respectively.

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In the first series of experiments the effect of indomethacin (1.4 μM), administered as a continuous infusion into the superfusing buffer, was investigated on both the intact and rubbed preparations of GPT. In order to study the effects of tone on responses due to BK and PGE_2, intact and rubbed preparations were also treated with salbutamol (10^{-6} M–10^{-8} M) or ACh (10^{-4} M), which reduced and increased tone respectively. These were given as a continuous infusion into the superfusing buffer for twenty minutes before and then continuously during re-testing of BK and PGE_2.

A variety of antagonists was also used to investigate the possible mediators involved in the responses. Combined antagonists to histamine H_1, 5-hydroxytryptamine (5-HT), α- and β-adrenoceptors, and muscarinic receptors (Piper & Vane, 1969). In addition, the substance P antagonist spantide (Folkers *et al.*, 1984), TXA_2 antagonist GR 3219B (Lumley *et al.*, 1989) and the leukotriene antagonist SK&F 104353 (Hay *et al.*, 1987b) were also used in some of the experiments.

Radioimmunoassay

In order to detect the release of prostaglandins by radioimmunoassay, experiments were also carried out in 10 ml organ baths. In these experiments the tracheae were prepared in the same way. However, tracheal zig-zag strips were cut from quarter tracheae and the buffer was Krebs-Henseleit solution of the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2 \cdot 6H_2O$ 2.5, KH_2PO_4 1.2, $MgSO_4 \cdot 7H_2O$ 1.2, $NaHCO_3$ 2.5 and glucose 5.5. Again preparations of both intact and rubbed tracheae were used.

Radioimmunoassay was performed on samples collected from organ bath experiments, where cumulative additions of BK (3 nM–10 μM) were used. These samples were partially purified using Sep-Pak C_{18} cartridges and then assayed for the presence of immunoreactive PGE_2 and immunoreactive 6-keto $PGF_{1\alpha}$, the stable metabolite of PGI_2, according to the method of Jose *et al.* (1976). In these experiments four organ baths were set up each containing a zig-zag preparation obtained from the same guinea-pig trachea. Tissues were allowed to equilibrate for an hour, with wash-outs carried out at 15 min intervals. Following a 20 min period, the fluid was collected from each bath to determine the basal levels of PGE_2 and 6-keto $PGF_{1\alpha}$. The tissues were then washed again and allowed to re-equilibrate before administration of drugs.

Each of the four preparations were treated differently: cumulative doses of BK alone were administered to the first bath (A), whereas cumulative doses of BK were added to the second bath in the presence of indomethacin (B). The remaining two organ paths (C,D) served as controls. One of them was treated with indomethacin alone (C), while the other received no drug treatment (D) and served as a time-matched control. Where appropriate, indomethacin (2.8 μM) was administered 15 min before the commencement of the experiments.

Materials

The following were used: ACh, BK, PGE_2 and salbutamol, (Sigma Chemicals Ltd); indomethacin (Merck, Sharp and Dohme) and U-44069 ((15S)-hydroxy-9α,11α-(epoxymethano) prosta-S2,13E-dienoic acid, The Upjohn Company); hyoscine hydrobromide (BDH); mepyramine maleate (May and Baker); methysergide maleate (Sandoz); phenoxybenzamine hydrochloride; propranolol hydrochloride (ICI); spantide (Peninsula Ltd); GR 3219B ([1R-[1 $\propto$ (z),2β,3β,5α]]-(+)-7-[S-[(1,1-biphenyl]-4-ylmethoxy)-3-hydroxy-2-(1-piperidinyl)cyclopentyl]-4-heptanoic acid. Glaxo) and SK&F 104353 (2(S)-hydroxy-3(R)-[(2-carboxyethyl)thiol]-3-[2-(8-phenylodyl)-phenyl]-propanoic acid, Smith Kline and French), [^{3}H]-PGE_2 and [^{3}H]-6 keto $PGF_{1\alpha}$ (New England Nuclear), 6-keto $PGF_{1\alpha}$ antiserum (Wellcome Research Laboratories) and PGE_2 antiserum, developed at the Royal College of Surgeons.

Statistical analysis

The results are expressed as means of a number of experiments, n (where n is the number of animals used) $\pm$ s.e.mean. Data were analysed by Student's t test for unpaired data where appropriate, values being considered as significant when $P < 0.05$.

Results

The intact preparations of GPT gained appreciable tone during the equilibration period, whereas the rubbed preparations had little or no visible tone.

Dose-response curves to bradykinin

In intact GPT, BK (3 pmol–10 nmol) induced dose-related relaxations, each relaxation being preceded by a small transitory peak. BK was approximately equipotent with salbutamol in causing relaxations of the tissue. However, when the epithelium was removed, BK induced dose-related contractions and was approximately 3–10 times more potent than U-44069 (Figure 1).

Effects of antagonists

When the combined antagonists to histamine H_1, 5-HT, α- and β-adrenoceptors and muscarinic receptors were added, no changes in the responses of either the intact or rubbed preparations to BK were observed (n = 3–4).

The addition of spantide, 1.0 and 3.0 μM, had no significant effect on the responses of the intact and rubbed preparations of GPT to BK. The addition of GR 3219B reversibly inhibited responses to U-44069, but did not affect the responses of either the intact or rubbed preparations of GPT to BK. However, upon addition of SK&F 104353 some reduction in the response induced by BK on the indomethacin-treated trachea was observed, but the response to U-44069 was also reduced (n = 4 in all cases).

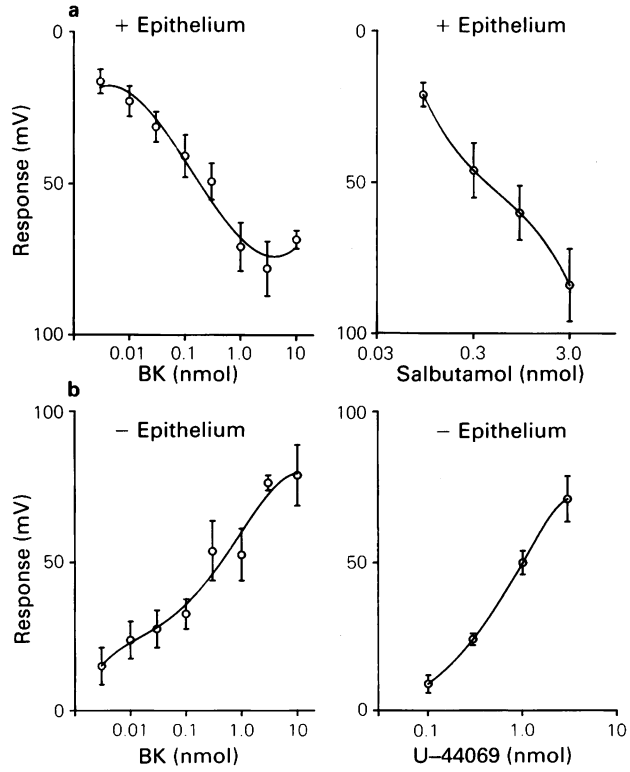


Figure 1 Dose-related responses of the superfused guinea-pig trachea (GPT). (a) Relaxations of the intact GPT induced by bradykinin (BK, 0.003–10 nmol) and salbutamol (0.1–3 nmol). (b) Contractions of the rubbed GPT induced by bradykinin (0.003–10 nmol) and U-44069 (0.1–3 nmol). Each point shows the mean response (n = 4–7) and vertical lines indicate s.e.mean.

Indomethacin

In the presence of indomethacin the response of the intact preparation of GPT to BK was changed from a relaxation to a contraction (Figure 2).

However, in the rubbed preparation, indomethacin inhibited the contractions induced by BK. At 100 pmol and 1 nmol BK there was 93 ± 5 and $79 \pm 1\%$ inhibition, respectively (Figure 3).

Effect of epithelial removal on responses of guinea-pig trachea to prostaglandin E_2

The responses of the GPT to PGE_2 were similar to those observed with BK. PGE_2 (0.3–3.0 nmol) caused dose-related relaxations of the intact GPT, but produced dose-related contractions of the rubbed GPT.

Effect of increased tone on the rubbed guinea-pig trachea

After the induction of tone in the rubbed GPT, the contractile responses elicited by BK (1.0 nmol and 3.0 nmol) were changed to protracted relaxations, without the initial transient peak which was observed in the intact GPT (Figure 4).

This reversal from a contraction to a relaxation was also observed with bolus administration of PGE_2 to the rubbed preparation, when the tone of the GPT was raised with ACh.

Effect of reduced tone on the intact guinea-pig trachea

Salbutamol at concentrations from 10^{-8} M to 10^{-6} M was infused to reduce the tone of the intact preparations of GPT.

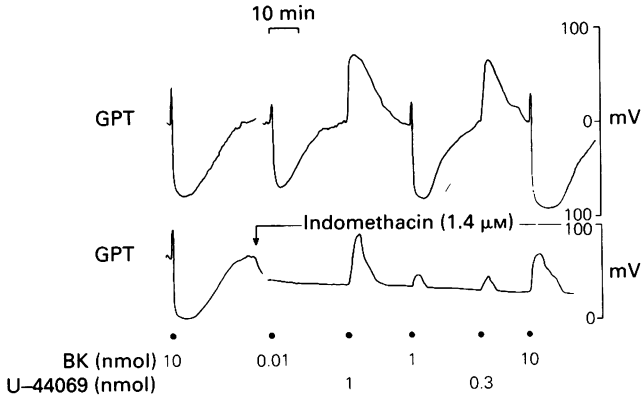


Figure 2 The effect of indomethacin (1.4 μM) on bradykinin (BK)-induced relaxations of superfused intact guinea-pig tracheae (GPT). Indomethacin was infused over the lower GPT only, the top GPT acting as a control ($n = 4$–7).

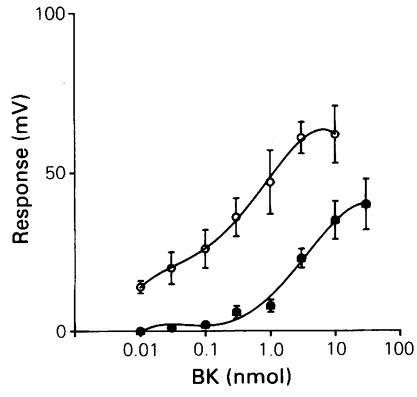


Figure 3 Dose-related contractions to bradykinin (BK, 0.01–30 nmol) of guinea-pig superfused rubbed tracheae before (○) and during (●) treatment with indomethacin (1.4 μM). Each point represents the mean ($n = 4$–7) and vertical lines show s.e.mean.

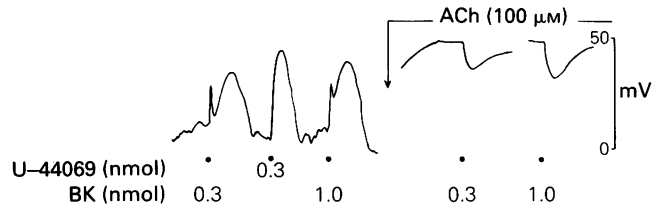


Figure 4 The effect of the induction of tone, on a guinea-pig rubbed (without epithelium), superfused trachea, with acetylcholine (ACh, 100 μM) on responses to bradykinin (BK) ($n = 4$–5).

All concentrations of salbutamol reduced the tone of the intact GPT, but in the presence of 10^{-8} M salbutamol, BK still caused a relaxation of the tissue. However, in the presence of higher concentrations of salbutamol (10^{-7} M–10^{-6} M), the BK-induced relaxation was reduced and no relaxant effect was observed when 10^{-6} M salbutamol was infused; at this concentration of salbutamol no contraction to U-44069 (up to 30 nmol) could be obtained.

Bradykinin-induced release of prostaglandins in intact and rubbed guinea-pig trachea

When the epithelium was removed from the GPT the basal production of PGE_2 was significantly reduced compared with that of the intact GPT. There was also a significant increase in the amount of PGE_2 released from the intact GPT ($P < 0.01$) compared with the time-matched control, following stimulation with BK (Figure 5a).

In the rubbed tissues there was also an increase in the amount of PGE_2 released following stimulation with BK and although this was significant ($P < 0.05$), the amount of PGE_2 generated was considerably less than that produced in the intact preparation following administration of BK.

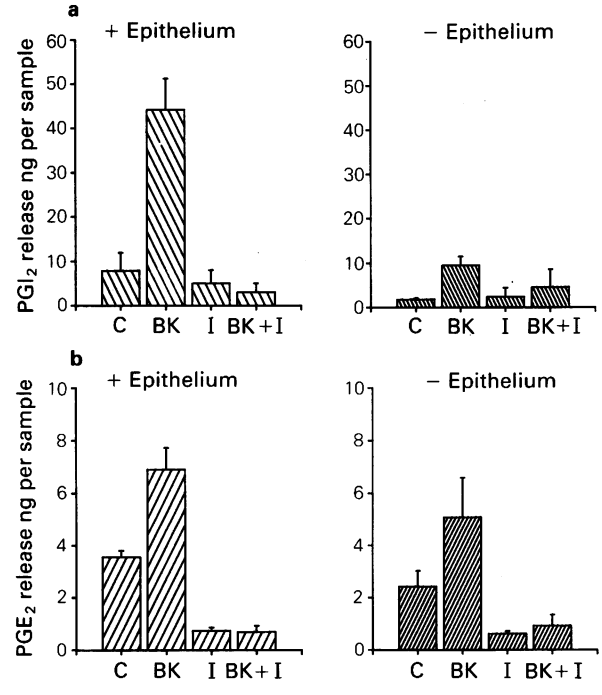


Figure 5 The production of prostaglandin E_2 (PGE_2) (a) and prostaglandin I_2 (PGI_2) (b) from intact (left) and rubbed (right) preparations of guinea-pig isolated tracheae ($n = 5$–6 in all cases). Measurement of the prostaglandins was in ng per sample (where sample = 10 ml of buffer). All treated tissues were compared to the time-matched control and values considered significant where $P < 0.05$. C = control, BK = bradykinin-treated, I = indomethacin-treated, BK + I = treated with both BK and indomethacin.

The amount of PGE_2 released by the rubbed tissue after stimulation with BK was approximately the same as that produced by the intact control tissue after the same contact time (Figure 5).

Indomethacin (2.8 μM) did not significantly alter the basal release of PGE_2 from either the intact or rubbed GPT. However, in the presence of indomethacin, the BK-induced release of PGE_2 from the intact GPT was significantly inhibited. In contrast indomethacin had no significant effect on BK-stimulated PGE_2 release from the rubbed GPT.

The basal release of PGI_2 was not significantly altered by removal of the epithelium. Following stimulation with BK, there was a significant increase in PGI_2 release compared with the control, from both the intact and rubbed preparations of GPT ($P < 0.05$). There was no significant difference in PGI_2 release between the intact and rubbed preparations of GPT when stimulated with BK (Figure 5b).

Both the basal release of PGI_2 and BK-stimulated production of PGI_2 were significantly inhibited by indomethacin in the intact and rubbed preparations of GPT.

Discussion

The removal of the epithelium from GPT has pronounced effects on the tone of the tissue and on the responses to various agonists. In canine bronchial tissue, it has previously been demonstrated that removal of the epithelium attenuates relaxations induced by isoprenaline (Flavahan *et al.*, 1985) and, more recently, that removal of the epithelium from GPT can alter the response to AA from a relaxation to a contraction (Nijkamp & Folkerts, 1987; Farmer *et al.*, 1987). This evidence suggests that when the epithelium is removed there is a loss of an inhibitory factor rather than a loss of a protective barrier which modulates agonist responses (Hay *et al.*, 1987c).

Our data show that in the intact GPT, BK elicits dose-related relaxations, each being preceded by a small transitory peak. However, following epithelial removal, this partially biphasic response is altered to a dose-related contraction, with the relaxant part of the biphasic response being absent.

The observation that the response of the intact GPT seems to be comprised of two phases could suggest that two different BK receptors are involved. Although there is evidence for the existence of BK_2 and BK_3 bradykinin receptors in GPT (Farmer *et al.*, 1989; Bramley *et al.*, 1990), so far there is no evidence to support the existence of a BK_1 receptor. However, we have observed similar responses on the GPT with kallidin (Lys-Bk), which is thought to be more selective for BK_1 receptors (unpublished observation). Further investigation, by use of selective BK antagonists may help to establish the nature of the response. So far in this study, using the combined antagonists of muscarinic, α- and β-adrenoceptors, 5-HT and histamine H_1-receptors we have been unable to affect the response of either the intact or rubbed GPT to BK. Furthermore, the lack of effect of the substance P antagonist and the TXA_2 antagonist suggests that neither substance P nor thromboxane A_2 are important in mediating the responses of the GPT to BK. However, there was some inhibition of the BK responses on the indomethacin-treated intact GPT in the presence of SK&F 104353, but as the response to U-44069 was also reduced these effects may have been non-specific. It is therefore difficult to assess whether leukotriene D_4 (LTD_4) or LTE_4 may be important in the response of the indomethacin-treated intact GPT to BK.

Previously, it had been suggested that the BK-induced relaxation of intact GPT was dependent on PGE_2 (Mizrahi *et al.*, 1982), therefore, we also investigated the effects of epithelial removal on responses of GPT to exogenous addition of PGE_2. The responses obtained with PGE_2 were similar to those observed with BK, which suggests that PGE_2 may be involved in the response of GPT to BK.

The treatment of intact GPT with indomethacin causes changes similar to those obtained when the epithelium is removed, i.e. a loss of tone and a change in the response of the GPT to BK from a relaxation to a contraction. However, the effect of indomethacin on the rubbed GPT is to inhibit the contractions induced by BK. This suggests that a cyclo-oxygenase product from the epithelium is important for the relaxation of the intact GPT, but that the contraction observed on the rubbed trachea is due to a cyclo-oxygenase product released from some other site. This could possibly be the tracheal cartilage, as generation of prostaglandins from the cartilage has been demonstrated (Raeburn *et al.*, 1987). The fact that indomethacin alters the response of the intact GPT to BK from a relaxation to a contraction suggests that the latter is caused by a different mechanism from that causing the contraction in the rubbed trachea.

PGE_2 probably contributes to the relaxation of intact GPT induced by BK, since the levels of PGE_2 generated from the intact GPT were significantly increased following stimulation with BK. The finding that removal of the epithelium significantly reduced both basal and BK-induced release of PGE_2 also demonstrates the importance of the epithelium for the generation of PGE_2.

These results indicate that PGI_2 is less important in the responses of GPT to BK, since the removal of the epithelium did not significantly alter PGI_2 release. This is in agreement with observations by Hay and co-workers (Hay *et al.*, 1988).

The intrinsic tone of the GPT is thought to be an important modulating factor in influencing responses to agonists (Braunstein *et al.*, 1988). Indomethacin causes a considerable reduction of the resting tone in the intact GPT, but after removal of the epithelium GPT does not gain tone. These observations suggest that a cyclo-oxygenase product mediates the tone which modulates agonist responses, but it is unlikely to be PGE_2 since the basal level of this prostaglandin was not significantly reduced by indomethacin. Another contractile cyclo-oxygenase metabolite may be involved, such as $PGF_{2\alpha}$ or PGD_2. However, it is not known whether at higher doses indomethacin could significantly reduce the basal release of PGE_2 and this needs further investigation.

The results of the experiments with ACh-induced tone on the rubbed GPT, where the contractile response is altered to a relaxation, illustrate that the induction of tone other than via a cyclo-oxygenase-dependent process still influences responses to BK. Therefore, it is not clear whether specific cyclo-oxygenase-mediated tone is necessary to alter the BK responses. The results of experiments employing salbutamol to reduce the tone of the intact GPT demonstrate that a loss of tone alone is not sufficient to alter the BK response. In fact, a reduction followed by a complete inhibition of the BK-induced relaxation of the intact GPT were achieved with salbutamol. This is an interesting observation since it seems that inhibition of cyclo-oxygenase is required and not only a reduction in tone, before a change in the BK response is obtained.

Recently, it has been suggested that removal of the epithelium or addition of indomethacin suppresses a contractile cyclo-oxygenase input, thus causing a reduction in tone of the GPT (Braunstein *et al.*, 1988). Our results suggest that PGE_2 is probably important in the BK-induced relaxations of GPT, as was described by Folkerts for the AA-induced relaxations of intact GPT (Folkerts *et al.*, 1989). These observations are also supported by the finding that bolus administration of PGE_2 induced relaxations of the intact GPT.

In addition, it appears that a cyclo-oxygenase product is involved in the maintenance of background tone of GPT, which also influences agonist-induced responses of the trachea.

It is possible that there is a cyclo-oxygenase system in both the epithelium and the smooth muscle of the GPT, which would account for the effects observed with indomethacin in both preparations. Peptidases in the epithelium might also affect the responses of GPT to BK, and their reduction when the epithelium is removed could contribute to changes observed in BK responses in the absence of the epithelium.

However, the peptidase inhibitor captopril had no significant effect on responses of either intact or rubbed GPT to BK (unpublished observation). There is also the possibility that more than one mechanism is involved in the modulation of responses to BK and PGE_2.

As damage to or loss of the epithelial cells leads to a diminished PGE_2 generation, with an associated decrease in phospholipase A_2 activity, it has been suggested that the level of phospholipase A_2 activity could be important in modulating the generation of PGE_2 from the epithelium (Folkerts *et al.*, 1989).

In conclusion, our results show that responses of GPT to BK are influenced by tone and an epithelium-dependent cyclo-oxygenase mechanism, with PGE_2 as the primary mediator. These observations may help explain the mechanism of changed responses of airways to agonists and non-specific stimuli in respiratory diseases where epithelial damage occurs.

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Pharmacological analysis of [^{3}H]-senktide binding to NK_3 tachykinin receptors in guinea-pig ileum longitudinal muscle-myenteric plexus and cerebral cortex membranes

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1 The binding properties and pharmacological specificity of the selective NK_3 tachykinin receptor agonist [^{3}H]-senktide ([^{3}H]-succinyl[Asp^6,MePhe^8] substance P (6–11)) have been examined in homogenates of guinea-pig ileum longitudinal muscle-myenteric plexus (LM/MP) and cerebral cortex.

2 Scatchard analysis of saturation binding studies in guinea-pig ileum LM/MP and cerebral cortex membranes indicated that [^{3}H]-senktide bound to a single site with apparent high affinity, $K_D = 2.21 \pm 0.65$ nM; $B_{max} = 13.49 \pm 0.04$ fmol mg^{-1} protein in ileum and $K_D = 8.52 \pm 0.45$ nM; $B_{max} = 76.3 \pm 1.6$ fmol mg^{-1} protein in cortex (values are means ± ranges; $n = 2$).

3 The pharmacological profile for tachykinins and analogues in displacing [^{3}H]-senktide from ileum membranes was: [MePhe^7] neurokinin B > neurokinin B (NKB) ≃ senktide > eledoisin > substance P (SP) > neurokinin A(NKA) > physalaemin > [Sar^9,Met(O$_2$)^{11}]SP > [Nle^{10}]NKA(4–10) = [Glp^6,L-Pro^9]-SP(6–11) > substance P methyl ester, consistent with [^{3}H]-senktide binding to an NK_3 subtype of tachykinin receptor. A similar rank order of affinity was obtained for these peptides in displacing [^{3}H]-senktide from cortex membranes

4 Several tachykinin receptor agonists were tested for their ability to displace [^{3}H]-senktide from ileal and cortical NK_3 binding sites and were found to be either weak displacers ($pIC_{50} < 5.00$) or inactive.

5 The binding of [^{3}H]-senktide to cortex membranes was inhibited by GTP ($pIC_{50} = 6.49$)and GTP-γ-S ($pIC_{50} = 6.67$) with ATP being at least three orders of magnitude less potent ($pIC_{50} = 3.55$).

6 These results indicate that both central and peripheral NK_3 receptors share a similar pharmacological specificity and that they may be labelled selectively with the NK_3 receptor agonist [^{3}H]-senktide.

Introduction

Substance P (SP) and the recently discovered mammalian neuropeptides, neurokinin A (NKA) and neurokinin B (NKB), belong to a family of peptides known as tachykinins, the biological actions of which are mediated by multiple receptors (for reviews see: Watson, 1984; 1987; Regoli *et al.*, 1987a; Quirion & Dam, 1988). The current classification of tachykinin receptors, based primarily on relative potencies of agonists in various bioassays and radioligand binding assays, recognizes three subtypes: NK_1, NK_2 and NK_3 (Henry *et al.*, 1987) where SP, NKA and NKB appear to be the preferred agonists respectively, although each peptide can activate all three receptor types. Alternative tachykinin receptor nomenclature and classification has been discussed elsewhere (see Mantyh *et al.*, 1989).

NK_3 receptors were identified as the tachykinin receptor type which mediates the release of acetylcholine from myenteric plexus neurones of the guinea-pig ileum (Laufer *et al.*, 1985). NKB is approximately two orders of magnitude more potent than NKA and SP in activating this neuronal tachykinin receptor in the guinea-pig ileum (Wormser *et al.*, 1986). A similar rank order of potency, indicative of an NK_3 receptor, is observed for tachykinin-induced contraction of the rat portal vein (Mastrangelo *et al.*, 1986; McKnight & Maguire, 1987).

The presence of NK_3 tachykinin binding sites in the mammalian brain was first demonstrated with [^{125}I]-Bolton-Hunter conjugated eledoisin ([^{125}I]-BHE; Cascieri & Liang, 1984; Beaujouan *et al.*, 1984). In these studies [^{125}I]-BHE labelled a tachykinin binding site which was distinct from that labelled with [^{125}I]-BH-substance P ([^{125}I]-BHSP), or [^{3}H]-SP, both in terms of pharmacology and distribution. Subsequent radioligand binding studies showed that [^{125}I]-BHE and [^{3}H]-NKB labelled the same site in rat brain and exhibited identical pharmacological profiles and distribution (Bergström *et al.*, 1987).

The presence of NK_3 receptors has been further confirmed by the development of a highly selective agonist, senktide (succinyl-[Asp^6,MePhe^8]SP(6–11)). This analogue of substance P has been shown to be extremely selective for NK_3 receptors while having little, if any, activity at NK_1 or NK_2 receptors (Wormser *et al.*, 1986). The Bolton Hunter-conjugated analogue of senktide ([^{125}I]-BH-[Asp^{5,6}, MePhe^8]-SP(6–11)) has been synthesized and shown to label a tachykinin binding site in rat cerebral cortex indicative of an NK_3 receptor (Laufer *et al.*, 1986).

Although the existence of NK_3 receptors is now widely accepted, previous authors have been unable to detect NK_3 binding sites in peripheral tissues using a range of radioligands including [^{125}I]-BHE, [^{125}I]-BH-NKB, [^{3}H]-NKB, 2-[^{125}I]-iodohistidyl-NKB, or [^{125}I]-BH-senktide (Burcher *et al.*, 1986; Mantyh *et al.*, 1988; 1989). This may not be surprising, given the low selectivity of many of these ligands for NK_3 receptors in addition to the possibility that the densities of NK_3 sites in peripheral tissues may be quite low. In this study, therefore, we have used [^{3}H]-senktide as a selective radioligand for the NK_3 receptor in an attempt to make a direct comparison of both peripheral (myenteric plexus) and central (cerebral cortex) NK_3 tachykinin receptors. The pharmacological specificity of these sites has been determined with a range of naturally occurring tachykinins and several tachykinin receptor agonists and antagonists shown to be selective

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for the NK_1, NK_2 or NK_3 subtype of receptor. In addition, the affinities of these analogues and naturally occurring tachykinins for [^{3}H]-senktide binding sites have been compared with their biological potencies in the rat portal vein bioassay (Drapeau *et al.*, 1987; McKnight & Maguire, 1987). A preliminary account of this work has been presented to the British Pharmacological Society (Guard *et al.*, 1989).

Methods

Preparation of ileal longitudinal muscle-myenteric plexus membranes

Ileal longitudinal muscle-myenteric plexus membranes (LM/MP) were prepared from Hartley strain guinea-pigs of either sex (300–500 g), essentially as previously described (Watson & Iversen, 1984) with minor modifications. Tissue from several animals was pooled and homogenised in 10 vol of ice-cold 20 mM HEPES buffer (pH 7.4) by polytron at position 7 for 10–15 s. The homogenate was centrifuged at 48,000 *g* for 30 min at 4°C. The pellet was resuspended in 20 mM HEPES buffer (pH 7.4) by polytron at position 7 for 5 s and incubated on ice in the presence of 300 mM KCl and 10 mM EDTA for 30 min with intermittent mixing. The homogenate was then filtered through nylon mesh (250 × 250 μm) and the filtrate centriged at 48,000 *g* for 30 min. The final pellet was resuspended in Krebs-HEPES buffer (pH 7.4), composition (mM): NaCl 120, KCl 4.8, $CaCl_2$ 1, $MgSO_4$ 1, HEPES 20, to a protein concentration of 2–3 mg ml^{-1}. Membranes were used immediately or stored in aliquots at −20°C. Protein concentration was determined by the method of Bradford (1976) with bovine serum albumin (BSA) as a standard.

Preparation of cerebral cortex membranes

Cerebral cortex membranes were prepared from Hartley strain guinea-pigs of either sex (300–500 g) essentially as described by Laufer *et al.* (1986). Brains were dissected on ice and cerebral cortices placed in ice-cold 10% sucrose (w/v). Pooled tissues were homogenised in 10 vol of 10% sucrose using a teflon glass homogeniser (10–12 strokes, 800 r.p.m.). The homogenate was centrifuged at 1,600 *g* for 10 min and the resulting supernatant was centrifuged at 20,000 *g* for 10 min. The crude synaptosomal pellet was lysed by resuspension (polytron, position 7 for 5 s) in 10 vol of 5 mM HEPES, pH 7.5. This suspension was centrifuged at 30,000 *g* for 30 min. The pellet was resuspended in the same buffer and centrifuged at 30,000 *g* for a further 30 min. The final pellet was resuspended in Krebs-20 mM HEPES buffer, pH 7.4 (composition as described above) to give a protein concentration of 2–3 mg ml^{-1}.

Radioligand binding assays

Binding assays were performed in polypropylene tubes containing, in a final volume of 0.2 ml, assay buffer (Krebs-20 mM HEPES plus 0.1% BSA, pH 7.4) 2 mM $MnCl_2$, 2 μg ml^{-1} chymostatin, 4 μg ml^{-1} leupeptin, 40 μg ml^{-1} bacitracin, [^{3}H]-senktide (1.3–1.5 nM), membranes (~300 μg protein) and various test substances. After incubation at 20°C for 60 min, the reaction was terminated by the addition of 2 ml ice-cold assay buffer containing 2 mM $MnCl_2$, 0.05% BSA and 0.01% sodium dodecyl sulphate (SDS). Membranes were then filtered, using a Brandel Cell Harvester, through Whatman GF/C filters (presoaked in a solution of 0.3% polyethylenimine/0.5% Triton X-100 for 1–2 h) and washed 3 times with 3 ml of ice-cold assay buffer containing 2 mM $MnCl_2$, 0.05% BSA and 0.01% SDS. Non-specific binding was estimated from determinations in the presence of 1 μM non-radioactive senktide. Bound radioactivity was measured by liquid scintillation spectrometry.

Data analysis

The ability of natural and synthetic tachykinins to inhibit the binding of [^{3}H]-senktide was determined using 6–9 test concentrations (0.1 nM–100 μM) assayed in duplicate or triplicate. Data were analysed by nonlinear regression using an iterative curve fitting programme (GraphPAD, ISI Software).

Saturation analyses of [^{3}H]-senktide binding to ileum LM/MP and cerebral cortex membranes were carried out over a concentration range of 0.10–100 nM. At concentrations greater than 2.0 nM, [^{3}H]-senktide was supplemented with non-radioactive peptide. Equilibrium dissociation constants (K_D) and maximum number of binding sites (B_{max}) were determined by non-linear regression by the above curve fitting programme.

Degradation of [^{3}H]-senktide

[^{3}H]-senktide (1.5 nM) was incubated with ileal or cortical membranes (~300 μg tissue per tube) in assay buffer with or without the peptidase inhibitors bacitracin (40 μg ml^{-1}), leupeptin (4 μg ml^{-1}) and chymostatin (2 μg ml^{-1}) for 60 min at room temperature. Incubations were terminated by the addition of 1 ml 5% acetonitrile-0.1% trifluoroacetic acid followed by centrifugation at 10,000 *g* for 5 min. Approximately 1 nmol of non-radioactive carrier senktide was added to the supernatant and a sample then examined by h.p.l.c. The stability of [^{3}H]-senktide was determined by reverse phase h.p.l.c. on a μBondapak C_{18} column (3.9 × 300 mm). Parallel incubations were performed with [^{125}I]-BHSP, instead of [^{3}H]-senktide, in the presence or absence of peptidase inhibitors as a control for enzyme activity. The peptides were eluted with a linear gradient: 25–41% B over 16 min at 1 ml min^{-1}. In this system, solvent A was 0.01 M trifluoroacetic acid/water and solvent B was 0.01 M trifluoroacetic acid/acetonitrile. Thirty second fractions were collected and radioactivity counted.

Materials

Substance P, eledoisin, neurokinin B and physalaemin were purchased from Cambridge Research Biochemicals, U.K. Neurokinin A and neuropeptide K were obtained from Peninsula Laboratories, U.K. Substance P methyl ester and [D-Pro^4,D-Trp^{7,9,10}]SP(4–11) were obtained from Bachem Biochemicals, Basel, Switzerland. [Sar^9,Met(O$_2$)^{11}]SP, [Nle^{10}]NKA(4–10) and [MePhe^7]NKB were purchased from IAF Biochem International, Québec, Canada. Senktide (succinyl-[Asp^6,MePhe^8]SP(6–11)), cyclo(Gln,Trp,Phe,Gly, Leu,Met) (L-659,877), Ac-Leu,Met,Gln,Trp, Phe.NH_2(L-659, 874) and [Glp^6,L-Pro^9]SP(6–11), were synthesized and characterized by Dr B.J. Williams at Merck Sharp and Dohme Research Laboratories, Harlow, U.K. [Gly^6]NKB(3–10) and [Arg^3,D-Ala^6]NKB(3–10) were a kind gift from Prof. Hashimoto, Kanazawa, Japan. Guanine and adenine nucleotides were obtained from Sigma, Poole, U.K. [^{3}H]-senktide (specific activity 50 Ci mmol^{-1} labelled at the N-methyl group, supplied in ethanol containing 5 mM β-mercaptoethanol) was a kind gift from New England Nuclear, Boston, U.S.A. [^{125}I]-BH-substance P (2,200 Ci mmol^{-1}) was obtained from Amersham International.

Results

Degradation of [^{3}H]-senktide by ileal and cortical membranes

Chromatographic analysis of [^{3}H]-senktide incubated at 20°C with either ileal or cortical membranes, in the absence of peptidase inhibitors, indicated that approximately 92% of the radioactivity eluted in the same position as unlabelled senktide. In the presence of peptidase inhibitors, degradation of [^{3}H]-senktide was almost completely prevented in both ileum and cortex with >98% of the radioactivity co-eluting with standard senktide. This same cocktail of peptidase inhibitors

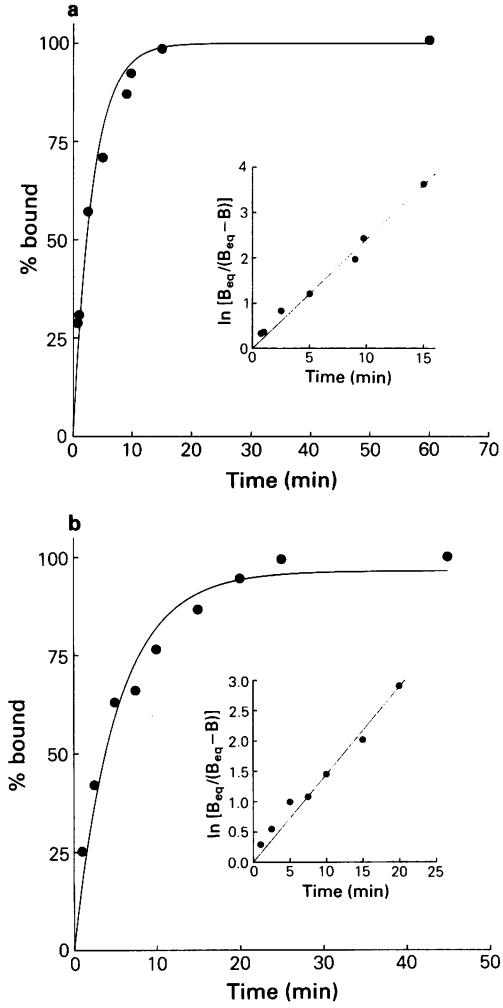


Figure 1 Association time course of [^{3}H]-senktide specific binding to (a) guinea-pig ileum longitudinal muscle-myenteric plexus (LM/MP) and (b) cerebral cortex membranes. Membranes (~300 μg protein) were incubated with radioligand (2.5 nM) and incubations terminated at various times by filtration. Non-specific binding (in the presence of 1 μM non-radioactive senktide) was determined simultaneously for all the time points. Insets: plots of ln $(B_{eq}/B_{eq} - B_t)$ versus time, where B_{eq} = amount of [^{3}H]-senktide specifically bound at equilibrium and B_t = amount specifically bound at time t.

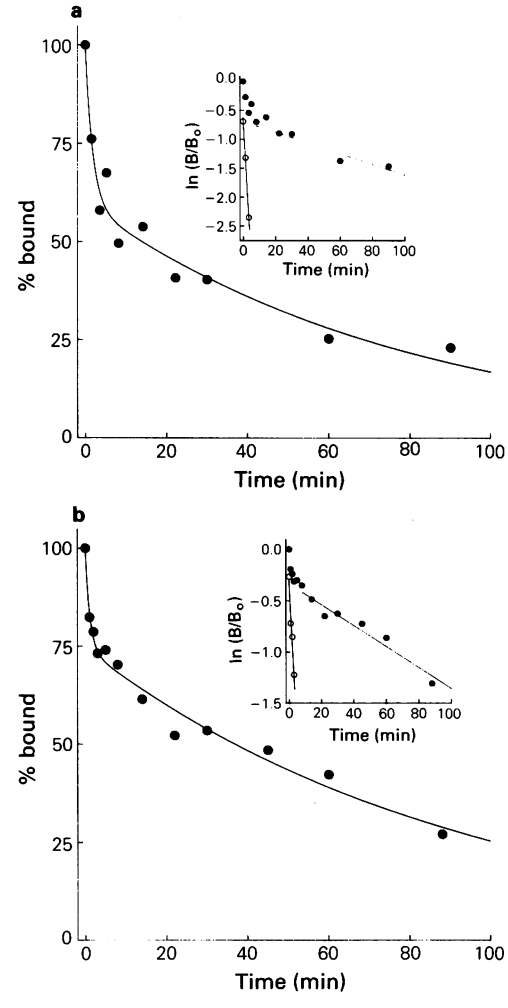


Figure 2 Dissociation time course of [^{3}H]-senktide specific binding to (a) guinea-pig ileum LM/MP and (b) cerebral cortex membranes. Membranes were incubated with radioligand for 45 min and senktide was then added to a final concentration of 1 μM (at zero time). The reaction mixture was diluted and filtered at various times thereafter. Each time point represents the mean of triplicate determinations (with s.e.mean < 10%) from a single representative experiment which was repeated one further time with similar results. Insets: data plotted as a first order reaction according to the formula ln $(B_t/B_0) = -K_{-1}t$, where B_0 represents the amount of radioligand specifically bound before dissociation and B_t is the amount bound at various times t. The fast phase of dissociation (○) was obtained following subtraction of the contribution of the slow phase (●).

also effectively inhibited the metabolism of [^{125}I]-BHSP (not shown).

Kinetic characteristics of [^{3}H]-*senktide binding*

The association time courses for the binding of [^{3}H]-senktide to guinea-pig ileal LM/MP and cerebral cortex membranes are shown in Figure 1a and b respectively. The specific binding of [^{3}H]-senktide increased with time, reaching equilibrium after 30 min of incubation at 20°C and remained stable for 60 min. As the total binding did not exceed 10% of the amount of [^{3}H]-senktide added, and there was no degradation of the radioligand detectable by h.p.l.c., the data were linearised according to a pseudo first order rate equation (Weiland & Molinoff, 1981). Plots of ln $(B_{eq}/B_{eq} - B_t)$ versus time are shown (insets to Figure 1a and b) where B_{eq} is the amount of [^{3}H]-senktide specifically bound at equilibrium and B_t is the amount specifically bound at time t. The apparent association rate constants for ileum and cortex sites were calculated to be 0.24 min^{-1} and 0.15 min^{-1}, respectively. [^{3}H]-senktide specific binding was reversible as shown by the dissociation of the radioligand following addition of 1 μM non-radioactive senktide (Figure 2a and b). The dissociation curves were curvilinear and were best fitted to a double exponential decay equation using the curve fitting programme GraphPAD. The insets to Figure 2a and b show the data plotted as a first order reaction according to the formula ln $(B_t/B_0) = -k_{-1}t$ where B_0 represents the amount of radioligand specifically bound before dissociation and B_t is the amount bound at various times t. The receptor-ligand dissociation comprised two distinct phases. The initial fast phase of dissociation was calculated after subtraction of the contribution of the slow phase and had a dissociation rate (k_{-1}) of 0.48 min^{-1} ($t_{1/2}$ = 1.26 min) and 0.30 min^{-1} ($t_{1/2}$ = 0.78 min) in ileum and cortex, respectively. The second phase was much slower: k'_{-1} = 0.004 min^{-1} ($t_{1/2}$ = 54.7 min) and k'_{-1} = 0.010 min^{-1} ($t_{1/2}$ = 64.2 min) in ileum and cortex, respectively. Similar biphasic dissociations have been described for the binding of [^{125}I]-BHE and [^{3}H]-NKB to NK_3 receptors in rat cerebral cortex membranes (Beaujouan *et al.*, 1984; Bergström *et al.*, 1987). Since the dissociation time courses were biphasic, estimates of the kinetic K_D values have not been calculated.

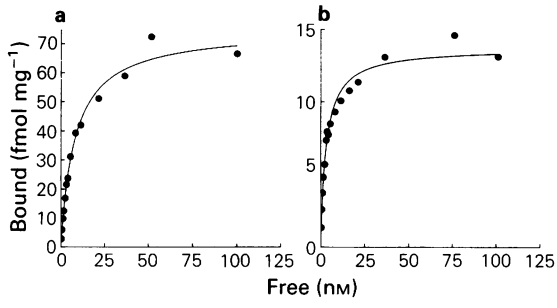


Figure 3 Saturation analysis of [^{3}H]-senktide specific binding to (a) guinea-pig cerebral cortex membranes and (b) ileum LM/MP membranes. Membranes (~300 μg protein) were incubated with [^{3}H]-senktide over a concentration range of 0.10–100 nM. At concentrations greater than 2.0 nM [^{3}H]-senktide was supplemented with non-radioactive peptide. Each time point represents triplicate determinations from a single representative experiment which was repeated one further time with similar results.

Equilibrium binding studies

[^{3}H]-senktide specific binding represented 70–75% of total binding in ileum LM/MP and 80–85% of total binding in cortex. Non-specific binding to filters amounted to approximately 10% of the total binding and 0.2% of total radioactivity added. Equilibrium binding assays were performed using ~300 μg protein at room temperature (20 ± 2°C) for 45 min.

Saturation analysis of [^{3}H]-senktide binding (0.10–100 nM) in ileum LM/MP and cerebral cortex membranes showed that specific binding was saturable (Figure 3a and b). Scatchard analysis of saturation experiments, performed in parallel to ileum LM/MP and cerebral cortex membranes, showed that the binding of [^{3}H]-senktide in ileum was to a single site of high affinity, $K_D = 2.21 \pm 0.65$ nM and $B_{max} = 13.49 \pm 0.04$ fmol mg^{-1} protein, whereas in cerebral cortex [^{3}H]-senktide bound to a single site of $K_D = 8.52 \pm 0.45$ nM and $B_{max} = 76.3 \pm 1.6$ fmol mg^{-1} protein (means ± ranges of 2 separate experiments; Figure 4).

Effect of nucleotides

The effect of guanine and adenine nucleotides (10 nM–1 mM) on [^{3}H]-senktide specific binding to cerebral cortex membranes was examined using guanosine triphosphate (GTP), the non-hydrolysable analogue guanosine 5'-O-(3-thiotriphosphate) (GTP-γ-S), and adenosine triphosphate (ATP). GTP and GTP-γ-S produced a concentration-dependent inhibition of [^{3}H]-senktide specific binding with similar pIC_{50} values, whereas ATP was approximately three orders of magnitude less potent (Table 1).

Table 1 Effect of GTP, GTP-γ-S, and ATP (30 nM–1 mM) on specific binding of [^{3}H]-senktide (1.5 nM) to guinea-pig cerebral cortex membranes

Nucleotide	*% maximal inhibition*	*pIC_{50}*
GTP	82.8 ± 5.8	6.49 ± 0.19
GTP-γ-S	83.1 ± 5.7	6.67 ± 0.09
ATP	57.6 ± 14.9	3.55 ± 0.12

Data are expressed as the percentage maximal inhibition of specific binding (defined by 1 μM senktide) in the presence of 1 mM nucleotide, and as pIC_{50} values (the negative logarithm of the concentration required to inhibit 50% of the maximal displaceable binding). Values represent the mean ± s.e.mean of 4 separate determinations.

Pharmacological specificity of [^{3}H]-senktide binding

The naturally occurring mammalian tachykinins NKB, NKA and SP all produced a concentration-dependent inhibition of [^{3}H]-senktide binding in guinea-pig cerebral cortex (Figure 5b) with a rank order of affinity of NKB > NKA ≃ SP. A similar rank order was observed in ileum (Figure 5a), NKB having between 25 and 80 times greater affinity than SP and NKA. Neuropeptide K displayed approximately one order of magnitude lower affinity than SP and NKA. The non-mammalian tachykinins, physalaemin and eledoisin also displaced [^{3}H]-senktide binding with eledoisin having approximately 100 times greater affinity than physalaemin. The affinities of these peptides and synthetic analogues for [^{3}H]-senktide binding sites in cortex and ileum are shown in Table 2. A strong correlation was observed between the affinities of tachykinins and synthetic analogues for myenteric plexus and cortical [^{3}H]-senktide binding sites ($r = 0.98$, Figure 6). A good correlation was also obtained between biological potencies of these peptides in the rat portal vein bioassay (data from McKnight & Maguire, 1987; Drapeau *et al.*, 1987) and their affinities for cortical ($r = 0.95$) and ileal sites ($r = 0.92$).

Of the synthetic analogues tested which exhibit receptor selectivity, a recently described NK_3-selective agonist, [MePhe^7]NKB (Drapeau *et al.*, 1987), had approximately 4–5 times greater affinity than senktide itself in both cortex and ileum. The NK_2 receptor-selective analogue [Nle^{10}]NKA(4–10) (Drapeau *et al.*, 1987) was virtually inactive, as were the

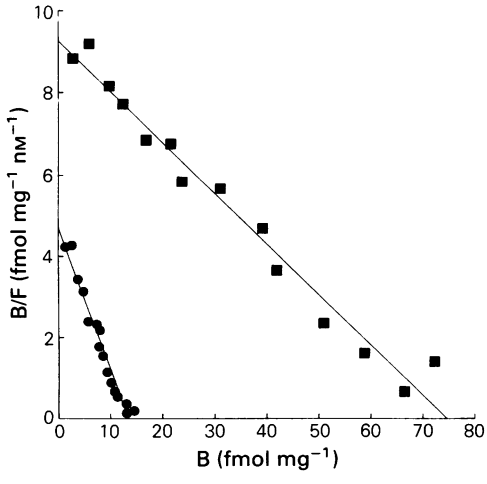


Figure 4 Scatchard analysis of the data shown in Figure 3; cortex (■) and ileum (●); see text for mean K_D and B_{max} values.

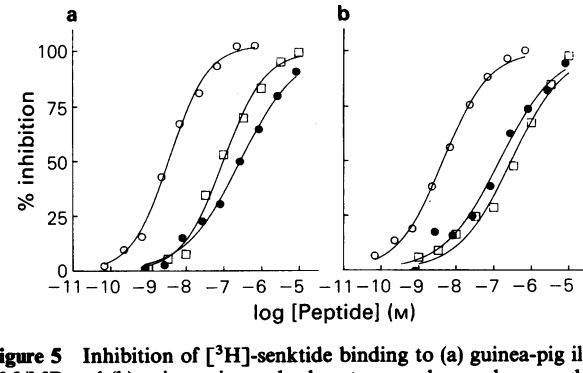


Figure 5 Inhibition of [^{3}H]-senktide binding to (a) guinea-pig ileum LM/MP and (b) guinea-pig cerebral cortex membranes by neurokinin B (○), neurokinin A (●) and substance P (□). Curves were fitted using an iterative curve-fitting procedure described in the text and the values are the mean percentage inhibition of specific binding from 3–5 separate experiments.

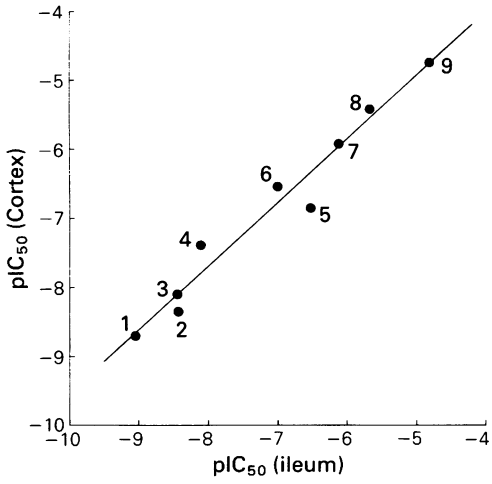


Figure 6 Correlation between pIC_{50} values for inhibition of [^{3}H]-senktide binding to guinea-pig cerebral cortex and ileum LM/MP membranes by tachykinins and analogues. pIC_{50} values were taken from Table 2. The line was fitted by linear regression and had a slope of 0.92. The correlation coefficient was 0.98. (1) [MePhe^7]NKB, (2) neurokinin B, (3) senktide, (4) eledoisin, (5) neurokinin A, (6) substance P, (7) physalaemin, (8) [Sar^9,Met(O$_2$)^{11}]SP, (9) [Gly^6]NKB(3–10).

selective NK_1 agonists substance P methyl ester (Watson *et al.*, 1983), [Glp^6,L-Pro^9]SP(6–11) (Wormser *et al.*, 1986) and [Sar^9,Met(O$_2$)^{11}]SP (Drapeau *et al.*, 1987).

The affinities of several tachykinin receptor antagonists for NK_3 sites were also determined (Table 2). The non-selective NK_1/NK_2 antagonist [D-Pro^4,D-Trp^{7,9,10}]SP(4–11) (Featherstone *et al.*, 1986) and the recently described selective NK_2 receptor antagonists cyclo-(Gln,Trp,Phe,Gly,Leu,Met) (L-659,877) and Ac-Leu,Met,Gln,Trp,Phe.NH$_2$ (L-659,874) (McKnight *et al.*, 1988) failed to produce any significant displacement of [^{3}H]-senktide specific binding at concentrations up to 10 μM. The putative NK_3 antagonists [Gly^6]NKB(3–10) and [Arg^3, D-Ala^6]-NKB(3–10) (Hashimoto *et al.*, 1987) were similarly weakly active or inactive respectively.

Discussion

Until recently, some relatively non-selective radioligands have been used to label tachykinin binding sites. For example, [^{125}I]-BHE not only labels NK_3 sites, but also NK_2 (Lee *et al.*, 1986) and NK_1 sites (Lee *et al.*, 1986; Guard *et al.*, 1988a). Similarly, [^{125}I]-BHNKA labels not only NK_2 sites (Buck *et al.*, 1984; Burcher & Buck, 1986), but also NK_3 (Foster & Tridgett, 1988) and NK_1 sites (Souquet *et al.*, 1987). Furthermore, recent attempts at labelling peripheral NK_3 sites with non-selective ligands such as [^{3}H]-NKB and 2-[^{125}I]-iodohistidyl-NKB (Mantyh *et al.*, 1988; 1989) have proved unsuccessful. In this study we have used the selective NK_3 receptor agonist [^{3}H]-senktide to investigate NK_3 receptors in longitudinal muscle-myenteric plexus membranes rather than whole ileum, thereby effectively increasing the density of neuronal NK_3 sites. Thus, we have been able to characterize pharmacologically both peripheral and central NK_3 tachykinin binding sites.

Under the conditions used, [^{3}H]-senktide bound with high affinity (nM) to both ileum LM/MP and cerebral cortex membranes as determined by Scatchard analysis of saturation data. In competition experiments, the naturally occurring tachykinins displaced [^{3}H]-senktide binding from ileal and cortical high affinity sites with the following rank order of affinities: NKB > eledoisin > NKA $\geqslant$ SP > physalaemin $\geqslant$ NPK. This is in good agreement with the rank order of potencies observed for these peptides in the rat portal vein (Mastrangelo *et al.*, 1986; McKnight & Maguire, 1987) or the neuronal NK_3 receptor bioassay in the guinea-pig ileum (Laufer *et al.*, 1985). Indeed, the affinities of the naturally occurring tachykinins and synthetic analogues for [^{3}H]-senktide binding sites correlate well with biological data in the literature, suggesting that these are functional receptor sites.

Further evidence that [^{3}H]-senktide is labelling NK_3 receptors in these tissues comes from the use of receptor-selective agonists. The selective NK_1 agonists substance P methyl ester and [Glp^6,L-Pro^9]SP(6–11) did not displace [^{3}H]-senktide (1.3–1.5 nM) at concentrations up to 10 μM. [Sar^9, Met(O$_2$)^{11}]SP, another NK_1 selective agonist, was weakly active at [^{3}H]-senktide sites having approximately 500 times lower affinity than senktide itself. Conversely, and in agreement with these findings, we have previously shown that senktide is inactive at guinea-pig ileum longitudinal smooth muscle NK_1 sites labelled with [^{125}I]-BHSP (Guard *et al.*, 1988a). The selective NK_2 agonist [Nle^{10}]NKA(4–10) was also a very weak displacer of [^{3}H]-senktide specific binding. In contrast, the NK_3 receptor agonist [MePhe^7]NKB had approximately 5 times greater affinity than senktide, whilst NKB has similar affinity. These observations are consistent

Table 2 Affinities of natural and synthetic tachykinins for [^{3}H]-senktide binding sites in guinea-pig ileal longitudinal muscle-myenteric plexus and guinea-pig cerebral cortex membranes

	Ileum		*Cortex*	
Peptide	*pIC_{50}*	*nH*	*pIC_{50}*	*nH*
Neurokinin B	8.43 (0.05)	0.86 (0.07)	8.35 (0.03)	0.72 (0.03)
[MePhe^7]NKB	9.05 (0.05)	1.24 (0.15)	8.71 (0.10)	1.09 (0.25)
Senktide	8.44 (0.03)	1.04 (0.07)	8.06 (0.02)	1.04 (0.04)
Eledoisin	8.11 (0.02)	0.78 (0.02)	7.39 (0.06)	0.77 (0.07)
Neurokinin A	6.53 (0.07)	0.59 (0.03)	6.85 (0.07)	0.59 (0.05)
Substance P	7.01 (0.05)	0.80 (0.06)	6.54 (0.24)	0.62 (0.06)
Physalaemin	6.13 (0.05)	0.72 (0.06)	5.92 (0.02)	0.77 (0.03)
[Sar^9,Met(O$_2$)^{11}]SP	5.68 (0.05)	0.71 (0.06)	5.42 (0.03)	0.80 (0.05)
SP methyl ester	<5.00		<5.00	
Neuropeptide K	ND		5.86 (0.007)	0.89 (0.12)
[Nle^{10}]NKA(4–10)	5.38 (0.04)	0.92 (0.09)	<5.00	
[Glp^6,L-Pro^9]SP(6–11)	<5.30		<5.00	
[Gly^6]NKB(3–10)	4.82 (0.03)	0.90 (0.06)	4.74 (0.09)	0.67 (0.10)
[Arg^3,D-Ala^6]NKB(3–10)	<4.00		<4.00	
PTTT	<5.00		<5.00	
L-659,877	<5.00		<5.00	
L-659,874	<5.00		<5.00	

Data are expressed as pIC_{50} values (the negative logarithm of the molar concentration of peptide required to inhibit 50% of [^{3}H]-senktide specific binding), nH is the Hill coefficient. Results are the means (±s.e.mean) of 2–5 separate experiments performed in duplicate or triplicate; ND, not determined. PTTT: [D-Pro^4,D-Trp^{7,9,10}]SP(4–11); L-659,877 = cyclo(Gln,Trp,Phe,Gly,Leu,Met); L659,874 = AcLeu,Met,Gln,Trp,Phe.NH$_2$.

with the relative potencies obtained for these analogues in the rat portal vein bioassay (Drapeau *et al.*, 1987).

The moderately potent NK_3 antagonists [Gly^6]NKB(3–10) and [Arg^3,D-Ala^6]-NKB(3–10) described recently by Hashimoto *et al.* (1987), were poor displacers of [^{3}H]-senktide binding in both tissues. [Gly^6]NKB(3–10) has been shown to be a specific, competitive antagonist of NKB-induced contraction of the guinea-pig ileum and rat portal vein (Hashimoto *et al.*, 1987). However, the low pIC_{50} values obtained for this NKB analogue in displacing [^{3}H]-senktide from cerebral cortex and ileum membranes (<5.00) is in contrast to its reported biological activity as an NK_3 receptor antagonist. The other putative NK_3 antagonist [Arg^3,D-Ala^6]NKB(3–10) failed to inhibit [^{3}H]-senktide binding in either tissue up to 30 μM. This is in contrast to its antagonist activity against NKB in the guinea-pig ileum, but consistent with its lack of antagonist activity in the rat portal vein (Hashimoto *et al.*, 1987). The non-selective NK_1/NK_2 receptor antagonist [D-Pro^4,D-Trp^{7,9,10}]SP(4–11) and the selective NK_2 antagonists L-659,877 and L-659,874 were unable to displace [^{3}H]-senktide binding from either ileum or cortex membranes at concentrations up to 10 μM, in agreement with the lack of antagonist activity of these analogues in bioassays for NK_3 receptors (Featherstone *et al.*, 1986; McKnight *et al.*, 1988).

The neuronal NK_3 receptor in the guinea-pig ileum mediates not only acetylcholine release from myenteric plexus neurones (Laufer *et al.*, 1986), but also the release of a tachykinin-like substance (Guard & Watson, 1987). We have also recently provided evidence for the coupling of NK_3 receptors in the guinea-pig ileum to the hydrolysis of inositol phospholipids (Guard *et al.*, 1988b). Activation of phospholipase C by NK_3 receptors in this preparation most likely occurs via a GTP regulatory binding protein, since the binding of [^{3}H]-senktide is inhibited by GTP and its analogue GTP-γ-S. More recently, Hanani *et al.* (1988) have demonstrated electrophysiologically that depolarisation of myenteric neurones following application of selective NK_3 analogues is associated with an increase in input resistance which reverses at about -90 mV, indicating that this response is likely to be due to the closure of potassium channels. Senktide is 20–100 times more potent than SP in depolarizing myenteric neurones, consistent with our affinity estimates for senktide and SP at ileal NK_3 binding sites. Although the neuronal NK_3 receptor has been described as NKB-preferring (Laufer *et al.*, 1985), the origin of NKB which might act upon peripheral NK_3 receptors has not been established. Using specific radio-immunoassays we have been unable to detect NKB in extracts of whole guinea-pig ileum which contain significant concentrations of SP and NKA (Too *et al.*, 1989). In contrast, extracts of guinea-pig CNS tissues contain all three tachykinins (SP, NKA and NKB) in easily quantified amounts. Nonetheless, given that SP and NKA exhibit appreciable affinity for the NK_3 receptor and are apparently co-localised in myenteric neurones (Deacon *et al.*, 1987), it is conceivable that these peptides may be the endogenous ligands for the neuronal NK_3 receptor.

At present very little is known about the functional role of central NK_3 receptors. Stoessl *et al.* (1988a) have recently demonstrated 5-HT-mediated behaviour in mice and rats following both central and peripheral administration of senktide. In addition, and by analogy to the actions of tachykinins in the enteric nervous system, behavioural studies have revealed NK_3 receptor-mediated tachykinin-cholinergic interactions in the CNS (Stoessl *et al.*, 1988b).

In conclusion, we have compared the pharmacological specificities of peripheral and central NK_3 tachykinin binding sites using the selective NK_3 receptor agonist [^{3}H]-senktide. We have shown that there is a great deal of similarity between central and peripheral NK_3 binding sites, as revealed by the good correlation between the affinities of tachykinins and analogues (both agonists and antagonists) for [^{3}H]-senktide binding sites in cortex and ileum. Furthermore, the correlation between biological potencies and the data presented here suggest that NK_3 binding sites represent functional receptors and that both central and peripheral NK_3 receptors can be selectively labelled with the metabolically stable NK_3 receptor ligand [^{3}H]-senktide.

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Effects of neuropeptide Y and calcitonin gene-related peptide on sheep coronary artery rings under oxygenated, hypoxic and simulated myocardial ischaemic conditions

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1 The effects of calcitonin gene-related peptide (CGRP) and neuropeptide Y (NPY) were examined on sheep circumflex (2–2.5 mm o.d.) coronary artery rings, with and without endothelium, under oxygenated, hypoxic and simulated ischaemic conditions. The interaction between the vasoconstrictor effects of NPY and the thromboxane mimetic, U46619, was also studied.

2 Ischaemia was simulated by modification of the composition of the physiological salt solution by increasing potassium and H^+, including lactate and reducing glucose and Po_2.

3 Hypoxia alone and simulated ischaemia increased the maximum vasodilatation produced by CGRP. CGRP (30 nM) abolished and markedly reduced the contraction that was induced by hypoxia and simulated ischaemia respectively.

4 Hypoxia increased and simulated ischaemia reduced the contractile response to NPY in endothelium intact rings. When the endothelium was removed, NPY caused a contraction under ischaemic conditions only. The hypoxic and ischaemic-induced contractions were augmented by NPY (30 nM).

5 In the rings containing endothelium, NPY enhanced the contraction caused by U46619 during hypoxia only. In endothelium-denuded preparations, NPY increased or partially restored the contraction caused by U46619.

6 These results show that the responsiveness of coronary artery rings isolated from sheep to either CGRP or NPY is modified by hypoxia or simulated myocardial ischaemia.

Introduction

Both calcitonin gene-related peptide (CGRP) and neuropeptide Y (NPY) have been demonstrated to be present in nerve fibres around coronary blood vessels and in the myocardium (Lundberg *et al.*, 1983; 1985; Mulderry *et al.*, 1985). CGRP is a potent coronary vasodilator (Holman *et al.*, 1986; Franco-Cereceda & Lundberg, 1987) whereas NPY is a vasoconstrictor (Franco-Cereceda *et al.*, 1985) and enhances the response to various constrictor substances in isolated blood vessels (Edvinsson *et al.*, 1984; Andriantsitohaina & Stoclet, 1988). The functional significance of the nerves containing these peptides in the heart is still unknown. However, it has been shown that myocardial ischaemia can cause the release of CGRP from isolated perfused hearts (Franco-Cereceda *et al.*, 1987b) suggesting that it may be of pathophysiological importance.

The aim of this study was to investigate whether the effects of CGRP and of NPY on coronary artery rings isolated from sheep were modified by simulated myocardial ischaemia and by hypoxia, one of the important consequences of ischaemia. Moreover, the interaction between the vasoconstrictor effects of NPY and of the thromboxane mimetic, U46619, were studied under oxygenated, hypoxic and simulated ischaemic conditions. The role of the endothelium in the observed responses was also investigated.

Methods

The experiments were performed on rings of sheep left circumflex coronary arteries, o.d. = 2–2.5 mm (main circumflex trunk after its first branch). The coronary arteries were dissected free from hearts of freshly slaughtered sheep. Arteries were then cleared of fat and adhering connective tissue. Care was taken to avoid stretching and damage to the luminal surface. Lack of damage was confirmed by histological examination in a representative sample of artery rings. In some experiments, the endothelium was removed by deliberately rubbing the internal surface with a wooden stick. The removal of endothelium was also confirmed by histological examination or the absence of substance P (0.1 μM)-mediated relaxation. The arteries were cut into rings 4–5 mm long. Segments were suspended in a water-jacketed muscle chamber (10 ml) filled with Krebs-Henseleit solution (37°C) of the following composition (mM): NaCl 119, $NaHCO_3$ 25, glucose 11, KCl 4.6, $MgCl_2$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 2.5. The solution was aerated with $95\%O_2 - 5\%CO_2$ (oxygenated, $Po_2 = 620 \pm 30$ mmHg, pH = 7.51 ± 0.04, $n = 8$). Hypoxia was introduced by draining the oxygenated Krebs solution and exchanging it with pre-equilibrated Krebs solution (bubbled with $95\%N_2 - 5\%CO_2$ for 30 min), followed by bubbling continuously with $95\%N_2 - 5\%CO_2$. The pH of the hypoxic solution was 7.5 ± 0.05 and the Po_2 values (mmHg) of 21 ± 3, 16 ± 2, 10 ± 2, 9 ± 2, 8 ± 2 ($n = 8$) were obtained at 30 s, 2, 5, 15 and 25 min respectively after the addition of hypoxic Krebs solution. The major ionic and metabolic changes occurring during myocardial ischaemia were modelled through the use of a simulated ischaemic solution (Kwan *et al.*, 1989c). When simulated ischaemic conditions were introduced into the organ bath the oxygenated Krebs solution was exchanged with the pre-equilibrated (bubbled with $95\%N_2 - 5\%CO_2$ for 30 min) Krebs solution of the following composition (mM): NaCl 134.5, $NaHCO_3$ 8.5, glucose 4, KCl 7.6, $MgCl_2$ 1.2, KH_2PO_4 2.4, $CaCl_2$ 1.5 and calcium lactate 1 (ischaemic; $Po_2 = 8 \pm 2$ mmHg and pH = 6.85 ± 0.05 at 15 min after introduction of the simulated ischaemic solution, $n = 8$). Oxygen tension was measured by an oxygen electrode (Strathkelvin Instruments) immersed in the organ bath. Aliquots of the bathing medium were removed by syringe and the Pco_2 and pH were measured by using a blood gas analyzer (Radiometer Copenhagen).

The rings were suspended on a pair of stainless steel hooks, one of which was fixed to an L-shaped rod inside the chamber and the other to a Grass isometric transducer. Arterial rings were equilibrated in Krebs-Henseleit solution gassed with $95\%O_2 - 5\%CO_2$ for an hour at their optimum resting tension of 1.5 g. The isometric tension was calculated as force developed per cross sectional area. The cross sectional area (A) of the artery was calculated by using the equation:

A = blotted weight of the artery/(h × β) where h = the distance (cm) between the two stainless steel hooks with the artery ring under optimum resting tension, and β = the density of the artery ring which was taken as 1.05 g cm^{-3}. Before the start of the experimental protocol the tissues were sensitized by repeated contraction cycles with 40 mM KCl until two consecutive identical responses were observed.

Experimental protocol

Effects of CGRP and NPY on endothelium-intact and denuded coronary artery rings under oxygenated, hypoxic and ischaemic conditions Endothelium intact and denuded coronary artery rings were precontracted by 40 mM KCl. After the contraction reached steady state, CGRP was added in a cumulative manner and the relaxation was recorded. The artery ring was washed, allowed to relax for 30 min and the optimum resting tension was readjusted before the introduction of hypoxia. The oxygen tension was reduced for 45 min and the resting tension was readjusted. The artery ring was precontracted by 40 mM KCl and CGRP was then added. The relaxations caused by CGRP under oxygenated and hypoxic conditions were compared. Similar experiments were performed on the precontracted (by 40 mM KCl) artery rings under simulated ischaemic conditions. The percentage change in tension, if any, observed in the response of a parallel control tissue that did not receive CGRP was used to correct the response in the paired artery rings receiving the CGRP.

The contraction produced by KCl 40 mM was: oxygenated conditions 77.2 ± 5.6 g cm^{-2}, hypoxic conditions 62.3 ± 1.9 g cm^{-2} and simulated ischaemic conditions 67.2 ± 9.3 g cm^{-2}. Thus the contraction produced by KCl 40 mM was slightly reduced under hypoxic conditions. In a control series of experiments, the maximal relaxation produced by CGRP was determined following precontraction with KCl over a very wide range of concentrations. With KCl at its EC_{85} concentration (KCl 40 mM) the maximal relaxation was 60 ± 5%, with KCl at its EC_{40} concentration the maximum relaxation was 67 ± 2%, and with KCl at its EC_{20} concentration the maximum relaxation was 76 ± 4%. These experiments show that a small change in the size of the precontracting stimulus would have a negligible effect on the percentage relaxation in response to CGRP.

The effect of NPY on endothelium-intact or denuded coronary artery rings under resting tension was recorded. The artery ring was washed, allowed to relax for 30 min and the optimum resting tension reset before the introduction of hypoxia or simulated ischaemia. Hypoxia or simulated ischaemia was introduced as described above, the optimum resting tension reset and the contractions induced by NPY recorded and compared with those under oxygenated conditions.

Effects of CGRP and NPY on the hypoxic- and ischaemic-induced contraction These experiments were carried out in rings containing endothelium only. Hypoxia or simulated ischaemia was introduced for 30 min and the tension developed was recorded. Oxygenated conditions were re-established by washing with Krebs solution aerated with 95% O_2 − 5% CO_2. The artery was then allowed to return to baseline tension and either CGRP (30 nM) or NPY (30 nM) was added, allowed to equilibrate for 30 min and the relaxation or contraction measured. The optimal resting tension was then reset and the hypoxic or simulated ischaemic challenge repeated in the continued presence of either peptide. The relaxation or contraction was measured and compared with that in the absence of the peptide.

Effects of NPY on the contractile effect of the thromboxane mimetic, U46619, under oxygenated, hypoxic and ischaemic conditions These experiments were carried out on endothelium-intact and denuded artery rings. Under oxygenated conditions, U46619 (0.1 μM) was added to the organ bath and the tension developed was recorded. The artery ring was then washed three to four times with oxygenated Krebs solution, the artery was allowed to relax to the baseline level and resting tension was readjusted, if necessary. NPY (30 nM) was added and after 5 min U46619 (0.1 μM) was added in addition. After the contraction had plateaued the artery ring was washed with oxygenated Krebs solution. Then, hypoxia was introduced for 45 min and tension was readjusted. Under hypoxic conditions U46619 (0.1 μM) was added, the artery ring was washed with hypoxic Krebs solution and allowed to equilibrate for 30 min under hypoxic conditions and the resting tension reset. NPY (30 nM) was added, followed after 5 min by U46619 (0.1 μM). Similar experiments were performed under simulated ischaemic conditions. The U46619-mediated contractions were measured under oxygenated, hypoxic and ischaemic conditions, both in the absence and in the presence of NPY.

Drugs

U46619 (11α,9α-epoxymethono PGH_2, dissolved in absolute ethanol with further dilution in distilled water, Upjohn), neuropeptide Y (porcine sequence, anhydrous mol. wt.: 4254, dissolved in 1 mg ml^{-1} BSA in boiled, freshly distilled water), calcitonin gene-related peptide (human sequence, anhydrous mol. wt.: 3791, dissolved in 0.01 M acetic acid in boiled, freshly distilled water) (both peptides (Sigma) were stored as 100 μl (0.1 mM) aliquots in siliconized tubes at −20°C). All glass apparatus that was in contact with either peptide was siliconized. Vehicles were tested at equal volumes.

Data analysis

Results are expressed as mean ± s.e.mean. Significance tests were performed by Student's paired or unpaired t test as appropriate. P values of less than 0.05 were considered to be statistically significant. The EC_{50} values were calculated by using a least squares curve fitting programme with concentrations of drug expressed as log concentrations.

Results

Effects of calcitonin gene-related peptide on pre-contracted coronary artery rings (by 40 mM KCl) under oxygenated, hypoxic and ischaemic conditions

CGRP caused relaxation of precontracted coronary artery rings. Simulated ischaemia and hypoxia each significantly augmented the maximal inhibition (oxygenated 60 ± 5%, ischaemic 88 ± 8%, hypoxic 148 ± 20%, n = 6) without changing the IC_{50} (Figure 1). Relaxation produced by CGRP (30 nM, the IC_{50} concentration) was greater in endothelium-denuded

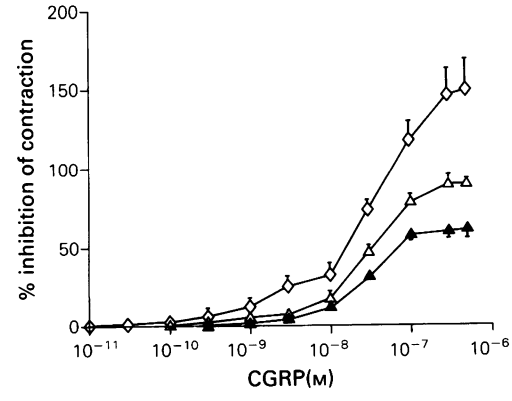


Figure 1 Cumulative concentration-response curves to calcitonin gene-related peptide (CGRP) in KCl-precontracted rings of sheep coronary artery with intact endothelium under oxygenated (▲), hypoxic (◇) and ischaemic conditions (△). Each point represents the mean and vertical lines show s.e.mean; n = 6.

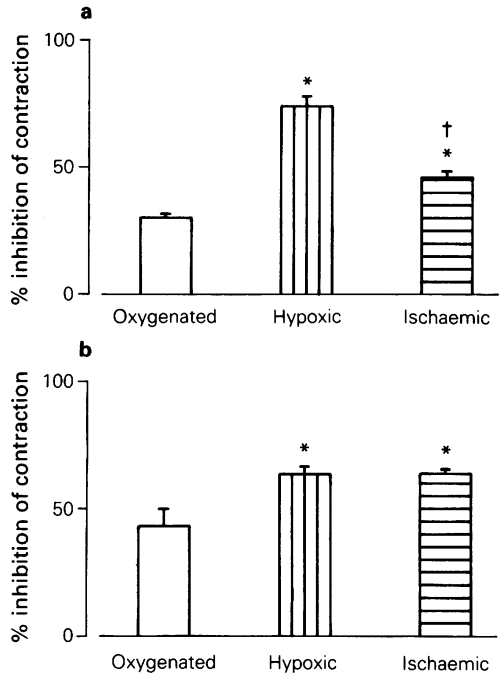


Figure 2 The calcitonin gene-related peptide (CGRP, 30 nM)-induced relaxation of KCl precontracted coronary artery rings under oxygenated, hypoxic and simulated ischaemic conditions: endothelium intact (a) and denuded (b). Each column represents the mean and bars show s.e.mean; $n = 6$. $^*P < 0.05$, significantly different from inhibition under oxygenated conditions. $\dagger P < 0.05$, significantly different from inhibition under hypoxic conditions.

(46 ± 3%) compared with endothelium-intact preparations (30 ± 1%). Hypoxia and ischaemia each potentiated CGRP-induced relaxation whether the endothelium was intact or denuded (Figure 2).

Effects of CGRP (30 nM) on the hypoxic and ischaemic-induced contraction

In coronary artery rings containing endothelium, introduction of hypoxia caused a contraction and introduction of simulated ischaemia caused a relaxation followed by contraction as previously described. Baseline force was regained after the tissues had been washed with oxygenated Krebs and the responses could be replicated several times (Kwan *et al.*, 1989a,c). CGRP abolished the hypoxic contraction and a relaxation was

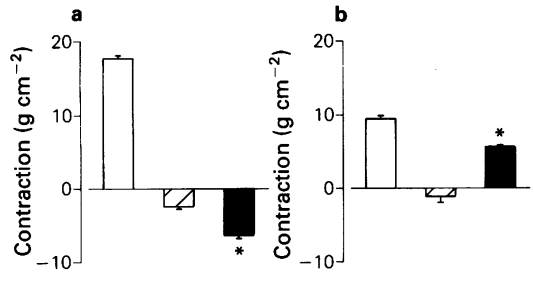


Figure 3 Effect of calcitonin gene-related peptide (CGRP, 30 nM) on the hypoxic (a)- and ischaemic (b)-induced contraction in sheep coronary artery rings. Open columns: hypoxic (a)- or ischaemic (b)-induced contraction (control); solid columns: hypoxic (a)- or ischaemic (b)-induced contraction in the presence of CGRP (30 nM). The relaxation caused by CGRP (30 nM) alone on basal tension (i.e. not precontracted) is also shown (hatched columns). Each column represents the mean and bars show s.e.mean; $n = 6$. $^*P < 0.05$ denotes a significant difference from the control hypoxic or ischaemic contraction.

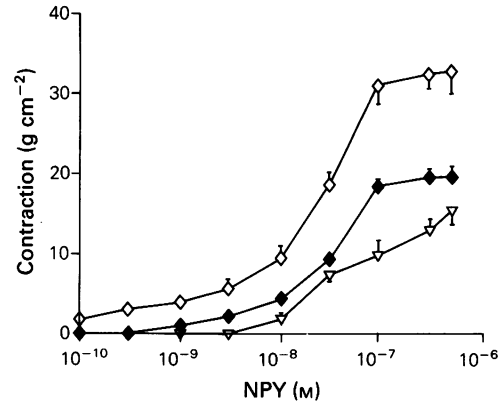


Figure 4 Cumulative concentration-response curves to neuropeptide Y (NPY) in sheep coronary artery rings with intact endothelium under oxygenated (◆), hypoxic (◇) and ischaemic (▽) conditions. Each point represents the mean and vertical lines show s.e.mean; $n = 8$.

observed (Figure 3a). In contrast, the ischaemic-induced contraction was reduced but not reversed by CGRP (Figure 3b). The initial transient relaxation phase produced by simulated ischaemia was not affected by CGRP. Under oxygenated conditions, this concentration of CGRP caused a slight relaxation below baseline tension as shown in Figure 3.

Effect of NPY (30 nM) on coronary artery rings under oxygenated, hypoxic or ischaemic conditions

NPY contracted sheep coronary artery rings. Under hypoxic conditions, there was a significant increase in the maximal contraction (19 ± 1 to 33 ± 3 g cm^{-2}, $n = 8$) and decrease in the EC_{50} (29 ± 1 to 21 ± 5 nM, $n = 8$). In contrast, simulated ischaemia reduced the maximal contraction (to 15 ± 2 g cm^{-2}, $n = 8$) and increased the EC_{50} (to 32 ± 7 nM, $n = 8$) (Figure 4). Contractions produced by NPY (30 nM, the EC_{50} concentration in oxygenated condition) in endothelium-intact and endothelium-denuded rings are shown in Figure 5. When the endothelium was absent, NPY did not cause a contraction under oxygenated or hypoxic conditions but did so during simulated ischaemia. This contraction during ischaemia was observed in all 6 preparations tested.

Effect of NPY (30 nM) on the hypoxic- and ischaemic-induced contraction

Both the hypoxic- and the ischaemic-induced contractions were augmented by NPY. In the presence of NPY the hypoxic contraction was 22.7 ± 1.6 g cm^{-2} compared with the control value of 17.2 ± 1.0 g cm^{-2} ($n = 6$, $P < 0.05$) and the ischaemic contraction was 22.3 ± 1.2 g cm^{-2} compared with 10.8 ± 0.6 g cm^{-1} ($n = 6$, $P < 0.05$).

Effect of NPY (30 nM) on the contractile effect of U46619 (0.1 μM) under oxygenated, hypoxic and ischaemic conditions

The contractile effects of the thromboxane mimetic U46619 (0.1 μM) alone and in combination with NPY (30 nM) on endothelium-intact and denuded coronary artery rings during normal oxygenation, hypoxia and simulated ischaemia are shown in Figure 6. In endothelium-intact rings the contraction caused by U46619 was enhanced by NPY during hypoxia but not in oxygenated or simulated ischaemic conditions. Removal of the endothelium potentiated the U46619-induced contraction under oxygenated conditions but abolished it in the presence of hypoxia or ischaemia. NPY increased the contraction caused by U46619 under oxygenated conditions and partially restored that during hypoxia and ischaemia.

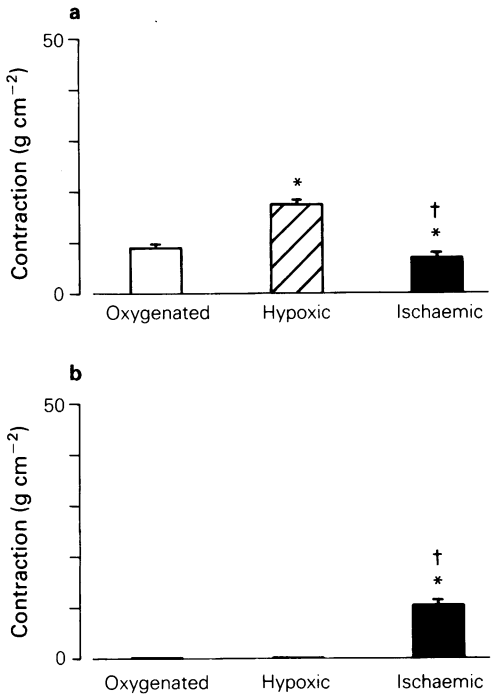


Figure 5 The contraction induced by neuropeptide Y (NPY, 30 nM) under oxygenated, hypoxic and ischaemic conditions in endothelium-intact (a) and denuded (b) sheep coronary artery rings. Each column represents the mean and bars show s.e.mean; $n = 6$. $*P < 0.05$, significantly different from contraction obtained under oxygenated conditions. $\dagger P < 0.05$, signifiantly different from contraction-obtained under hypoxic conditions.

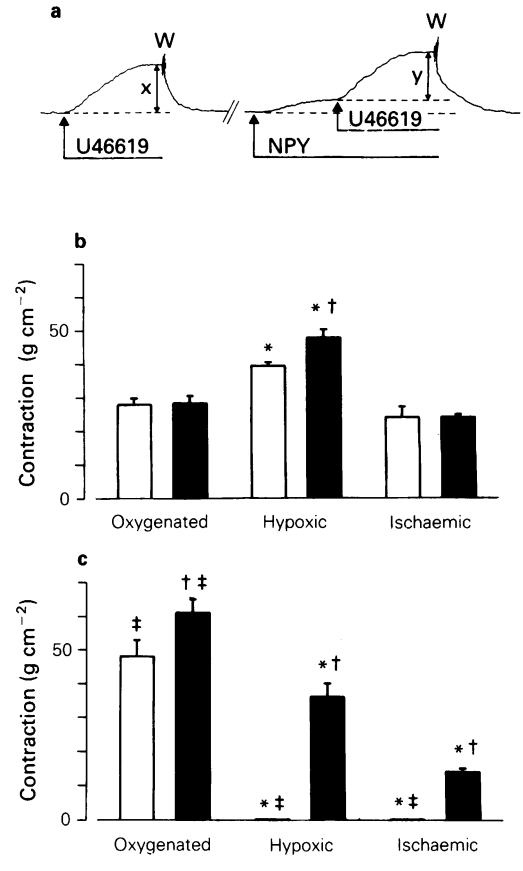


Figure 6 The effect of U46619 (0.1 μM) alone and in combination with neuropeptide Y (NPY, 30 nM) under oxygenated, hypoxic and ischaemic conditions in sheep coronary artery rings. (a) A trace to show the method of measuring U46619 contraction alone and in the presence of NPY. The data are presented in (b) for endothelium-intact and in (c) for denuded preparations. Open columns: effects of U46619 (0.1 μM) alone; solid columns: effects of U46619 (0.1 μM) + NPY (30 nM). Each column represents the mean and bars show s.e.mean; $n = 6$. $\dagger P < 0.05$ significantly different from the contraction obtained with U46619 alone. $*P < 0.05$ significantly different from contraction obtained under oxygenated conditions. $\ddagger P < 0.05$ significantly different from contraction in endothelium-intact rings.

Discussion

This study has shown that relaxation produced by CGRP, contraction produced by NPY, and the interaction between U46619 and NPY are profoundly modified by hypoxia and simulated ischaemia. Many of these effects may be explained by modulation of the release of vasoactive factors from the vessel wall.

Calcitonin gene-related peptide

In the sheep coronary artery, CGRP was found to be an endothelium-independent vasodilator, as it is in cattle and pig coronary arteries (Greenberg *et al.*, 1987; Franco-Cereceda *et al.*, 1987a). In the absence of the endothelium, CGRP-induced relaxation was augmented by hypoxia and by simulated ischaemia. Since the actions of CGRP are mediated via adenosine 3′:5′-cyclic monophosphate (cyclic AMP) (Kubota *et al.*, 1985), it is of interest that hypoxia and ischaemia potentiate other vasodilators acting through the cyclic AMP mechanism, such as adenosine and iloprost (Kwan *et al.*, 1989b,c). However, in the presence of the endothelium, although hypoxia strongly potentiated CGRP, simulated ischaemia caused less potentiation. This suggests that one of the mediators released during ischaemic conditions opposes cyclic AMP-dependent vasodilatation.

Hypoxia and simulated ischaemia each produce contraction (preceded, in the case of simulated ischaemia, by relaxation) of isolated rings of sheep coronary artery. The contractile phase is caused by the release, from the endothelium, of a vasoconstrictor combined with a reduction in the basal output of a vasodilator (Amatya *et al.*, 1988; Kwan *et al.*, 1989c). The present results suggest that CGRP antagonizes either the action or the release of these mediators, since it reduces the simulated ischaemic- and abolishes the hypoxic-induced contraction. The smaller effect of CGRP during ischaemia compared with hypoxia, supports the suggestion made above that one of the mediators released during simulated ischaemia opposes the CGRP coupling mechanisms.

Neuropeptide Y

NPY induced a contraction in endothelium-intact but not in endothelium-denuded rings of sheep coronary artery. The NPY-induced contraction is also endothelium-dependent in the rabbit ear artery (Daly & Hieble, 1987). This suggests that the NPY-induced contraction is caused either by the release of a vasoconstrictor substance from the endothelium, or by inhibition of the basal release of a vasodilator from the endothelium. The latter explanation is particularly attractive since there is a high basal output of vasodilators from the sheep coronary artery endothelium, and contraction is produced either by inhibition of prostacyclin release (Amatya *et al.*, 1988) or of endothelium-derived relaxing factor (EDRF) (Kwan *et al.*, 1990). Augmentation of the NPY-induced contraction during hypoxia (Figure 3) is to be expected since hypoxia itself inhibits vasodilator output from the endothelium (Amatya *et al.*, 1988). However, simulated ischaemia reduced the NPY contraction, probably because of the presence, in the simulated ischaemic solution, of substances that inhibit smooth muscle contraction, such as lactate (Hester *et al.*, 1980) and H^+ (Rooke & Sparks, 1981).

In endothelium-denuded sheep coronary artery rings, simulated ischaemia causes a relaxation (Kwan *et al.*, 1989c). This relaxation is probably caused by release of a vasodilator (such as adenosine or noradrenaline) from cells in the medial or adventitial layer. In the absence of endothelium, NPY caused a contraction during simulated ischaemia, but not during

hypoxia or oxygenation. This suggests that NPY inhibits the release of the vasodilator, released by cells in the medial or adventital layer, during simulated ischaemic conditions.

NPY augmented the contractions produced by hypoxia and by simulated ischaemia in endothelium-containing rings of sheep coronary artery. If, as hypothesized, NPY is acting by inhibiting vasodilator release, then this may be additive with hypoxia and ischaemia, both of which, as stated previously, inhibit release of a vasodilator.

Interaction of neuropeptide Y with U46619

NPY has been found to augment the action of a variety of vasoconstrictors in several isolated artery preparations (Neild, 1987; Andriantsitohaina *et al.*, 1988). However, in sheep coronary artery rings with endothelium intact under oxygenated conditions, contraction caused by the thromboxane mimetic U46619 was not affected by pretreatment with NPY. This may be because the sheep coronary artery has a high basal output of vasodilator substances from the endothelium (Amatya *et al.*, 1988; Kwan *et al.*, 1989c). In support of this view, removal of the endothelium both augmented the U46619-induced contraction and permitted NPY to potentiate U46619 (Figure 4). Similar effects were observed in endothelium-intact sheep coronary artery rings under conditions of hypoxia, which inhibits prostacyclin release from the endothelium (Amatya *et al.*, 1988). In endothelium-intact rings under conditions of simulated ischaemia, NPY had no augmenting effect, probably because the solution simulating ischaemia contains vasodilator substances (lactate, H^+). Following removal of the endothelium, U46619 is unable to generate a contraction if the conditions are hypoxic or simulated ischaemic, as previously described (Kwan *et al.*, 1989b). NPY may inhibit the vasodilator release thought to occur from cells in the medial or adventitial layer during hypoxia or simulated ischaemia, thus permitting a contraction to occur when the combination of U46619 and NPY is given together.

The results of this study may be of importance to the pathophysiological events of myocardial ischaemia. CGRP and NPY are neuropeptides present in the myocardium and coronary arteries. CGRP is co-localized with substance P in sensory neurones (Lundberg *et al.*, 1983; Mulderry *et al.*, 1985). A short period of ischaemia has been shown to release CGRP from the guinea-pig perfused heart (Franco-Cereceda *et al.*, 1987b). Our results show that the vasodilator action of CGRP is enhanced by simulated ischaemia, which would make CGRP a good mediator of reactive hyperaemia. NPY is co-localized with noradrenaline in sympathetic neurones and is probably released in the heart when these nerves are activated. High frequencies of neuronal stimulation release NPY preferentially (Pernow, 1988). Although we found NPY to be endothelium-dependent under oxygenated conditions, during simulated ischaemia, NPY caused contraction whether or not the endothelium was present. Thus a combination of sympathetic stimulation, myocardial ischaemia and damage to the endothelium (e.g. atherosclerosis) could lead to a NPY-mediated coronary vasoconstriction.

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Potassium channel blockers and the effects of cromakalim on the smooth muscle of the guinea-pig bladder

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1 The K^+ channel blocking drugs tetraethylammonium Cl (TEA), procaine, 4-aminopyridine (4AP) and quinidine all produced concentration-dependent contractions of strips of smooth muscle from the guinea-pig urinary bladder. Apamin and glibenclamide caused little increase in the mechanical activity, and tolbutamide inhibited it.

2 TEA, procaine, 4AP, quinidine and apamin all increased the frequencies of spontaneous action potentials recorded with microelectrodes. TEA, quinidine and procaine all caused prolongation of the falling phase of the spike, and procaine and apamin completely abolished the after-hyperpolarization.

3 TEA and procaine increased K^+ efflux from the tissue, an effect blocked by nifedipine. TEA and apamin increased, whereas quinidine, procaine and 4AP decreased K^+ uptake.

4 Cromakalim caused a concentration-dependent hyperpolarization of the membrane, abolished spike activity, increased K^+ fluxes and relaxed the smooth muscle. The relaxant effect of cromakalim was unaffected by apamin, and in its presence the effects of cromakalim on membrane potential and K^+ fluxes were unchanged. Procaine abolished all the effects of cromakalim, and TEA at high concentrations reduced but did not abolish these effects. Quinidine reduced the effects of cromakalim on tension and membrane potential, but its actions were surmounted by higher concentrations of cromakalim. The effects of 4AP on tension and membrane properties were transitory, but it had some effects on the actions of cromakalim. Glibenclamide and tolbutamide reversed the relaxant effects of submaximal cromakalim concentrations, tolbutamide only transiently.

5 It is concluded that the channels opened by cromakalim are not those involved in generating the spike after-hyperpolarization. They have properties similar to the delayed rectifier K^+ channels responsible for spike repolarization, and also are similar to the ATP-dependent K channels in vascular smooth muscle.

Introduction

The excitable properties and spike shapes of smooth muscles vary widely from tissue to tissue, and it is likely that each smooth muscle possesses a unique set of K^+ channels, whether in channel type, or in the proportions of the various types present. One common feature of all smooth muscles so far studied, is the ability of cromakalim, a drug classified as a K^+ channel activator, to induce relaxation (Weston & Abbott, 1987; Cook, 1988). To investigate the types of K^+ channel present in smooth muscles, potassium channel inhibitors have been used as tools (Benham *et al.*, 1985; Inoue *et al.*, 1985; McCann & Welsh, 1987; Okabe *et al.*, 1987), although there is considerable overlap in the channels each inhibitor can block and there are few, if any, compounds which selectively block a single class of K^+ channel. At the time of writing, there have been two proposals as to the channels that can be activated by cromakalim in smooth muscle, the delayed rectifier channel (Beech & Bolton, 1987; Beech, 1989), and an ATP-dependent K^+ channel (Standen *et al.*, 1989).

There is at present little detailed information about the electrical properties of bladder smooth muscle, and less about the K^+ channels present. The best studied bladder is that of the guinea-pig, on which microelectrode techniques have been applied by Creed and her colleagues (Creed, 1971a,b; Callahan & Creed, 1981; 1986; Kurihara & Creed, 1972), Mostwin (1986) and Fujii (1988). Membrane currents have been recorded by Klöckner & Isenberg (1985) using a whole cell patch clamp. We have previously shown that cromakalim increases potassium permeability in the smooth muscle of the guinea-pig urinary bladder. This results in membrane hyperpolarization, cessation of spike activity and relaxation of the tissue. The channels opened by the drug in the guinea-pig bladder seem to be selectively more permeable to K^+ than to Rb^+ ions, and have similarities with channels involved in spike repolarization (Foster *et al.*, 1989).

In this paper we have investigated the effects of K^+ channel inhibitors on the mechanical and electrical activity in guinea-pig bladder and on transmembrane K^+ fluxes, and have examined the interaction of these inhibitors with the effects of cromakalim.

Methods

Details of the methods used are described in Foster *et al.* (1989), and are given in outline below.

Contractile studies

Isometric tension was recorded from strips of detrusor mounted in 0.2 ml chambers, continuously superfused at 1 ml min^{-1} with solutions warmed to 35–37°C, and equilibrated with 97% O_2, 3% CO_2. On mounting, tissues were stretched to an initial tension of 0.5 g wt. Drugs were administered in the desired concentrations in the superfusing solution.

Electrophysiology

Membrane activity was recorded from strips of detrusor mounted in a small organ bath and superfused at a rate of 2–3 ml min^{-1} with warmed (36°C), gassed solutions made hypertonic by addition of 12 g sucrose to 100 ml Krebs solution to reduce tissue movement. Microelectrodes had a tip resistance of 40–70 MΩ when filled with 3 M KCl. Potentials were displayed on a penwriting recorder (Gould Brush 220).

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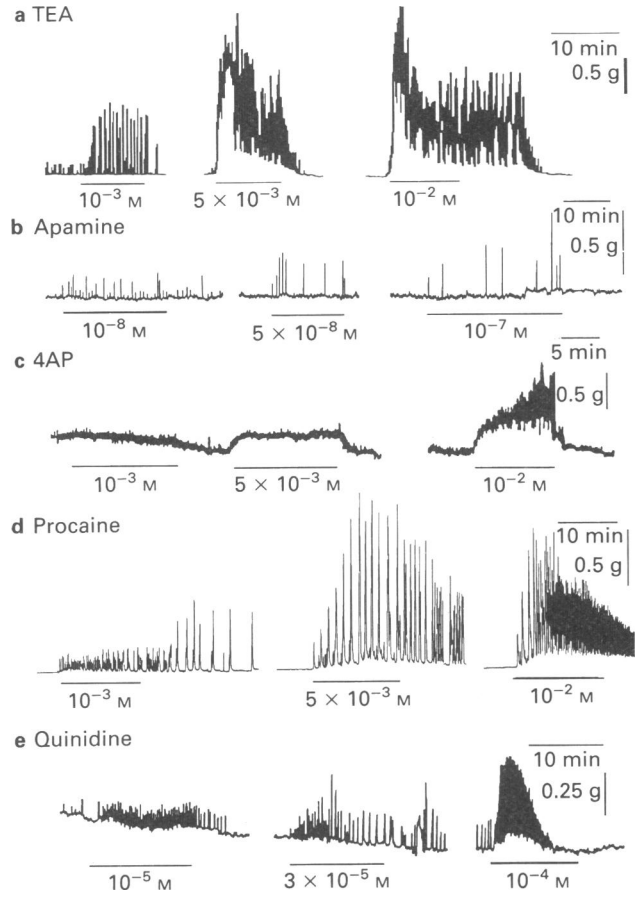


Figure 1 The effect of K^+ channel blockers on contractile activity of strips of detrusor muscle from the guinea-pig bladder. Each row of recordings is taken from a different preparation. TEA, tetraethylammonium chloride; 4AP, 4-aminopyridine.

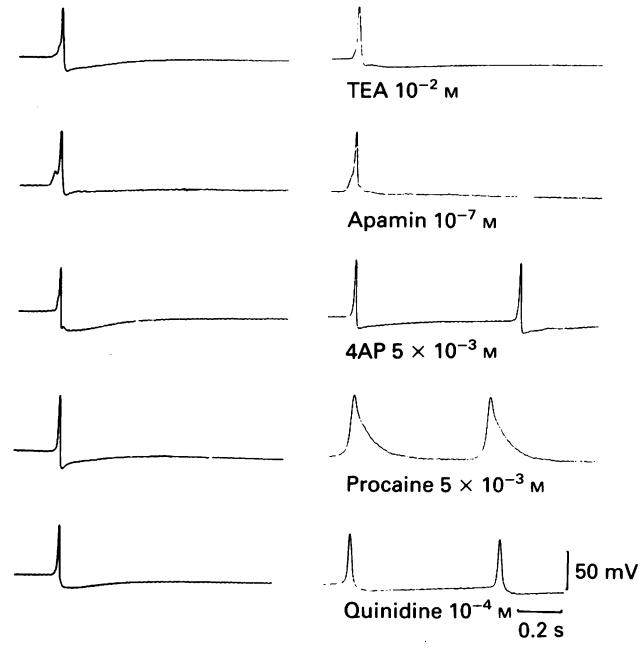


Figure 2 Detrusor muscle of guinea-pig urinary bladder: the effect of K^+ channel blockers on the action potential shape (membrane potential recorded with microelectrodes). In each row the left hand record shows a control action potential recorded in normal solution, and the right hand record shows an action potential after the maximum effect of the blocker on spike frequency had occurred during the same recording. Each row of traces is from a different tissue. Note the variability of shapes recorded in the control spikes, but the common fast repolarization and after-hyperpolarization. At the concentrations used, tetraethylammonium Cl (TEA) slows the repolarization and reduces the after-hyperpolarization. Apamin and 4-aminopyridine (4AP) have no effect on repolarization, but apamin completely abolishes and 4AP slightly reduces the after-hyperpolarization. Procaine greatly slows repolarization and abolishes the after-hyperpolarization. Quinidine slows repolarization and reduces the after-hyperpolarization.

$^{43}K^+$ fluxes

Strips of detrusor were mounted on stainless-steel holders. For uptake experiments tissues were exposed to isotope-containing solution for 10 min and then washed in ice cold Krebs solution for 5 min to remove extracellular isotope. The solution used to study tissue uptake of isotope contained 2–5 μCi ml^{-1} $^{43}K^+$. When K^+ channel inhibitors were studied, tissues were preincubated with the appropriate inhibitor for 10 min before exposure to isotope-containing solution also containing the inhibitor. In experiments where the effects of cromakalim (10^{-5} M) were studied, this agent was added to the isotope-containing solution. For efflux experiments, tissues were loaded in Krebs solution containing 1–3 μCi ml^{-1} $^{43}K^+$ for 4.5 h, and washed out in a superfusion apparatus with Krebs solution (35–38°C) at a flow rate of 1 ml min^{-1}. Two min samples were collected from the 10th min of the washout, and 30 min later cromakalim was applied for 10 min. K^+-channel inhibitors were applied 10 min before cromakalim and were present until the end of the washout. When nifedipine was used, this was present in all the washout solutions.

Solutions and drugs

The Krebs solution contained (mM): NaCl 120, KCl 5.9, $NaHCO_3$ 15.4, $MgCl_2$ 1.2, NaH_2PO_4 1, $CaCl_2$ 2.5, glucose 11 and was equilibrated with 97% O_2, 3% CO_2. For electrophysiological recordings, the osmolarity was increased by the addition of 12 g sucrose to 100 ml Krebs, to reduce tissue movement. Drugs used were: cromakalim (BRL 34915, (±)-6-cyano-3,4-dihydro-2,2-dimethyl-*trans*-4-(2-oxo-1-pyrrolidyl)-2H-benzo[b]pyran-3-ol), was a gift from Beecham Pharmaceuticals U.K. It was made up as a stock solution (10^{-2} M) with 70% ethanol, and diluted with the experimental Krebs solution to the desired concentration. Apamin, 4-aminopyridine (4AP), procaine and tetraethylammonium Cl (TEA) were obtained from Sigma, and made up as concentrated stock solutions in distilled water. Apamin was kept frozen and prepared freshly no more than an hour before it was required. Nifedipine was obtained from Bayer and PN200-110 (isradipine (isopropyl methyl-4-(4-benzofurazanyl)-1,4-dihydro-7,6-dimethyl-3,5-pyridinedicarboxylate) from Sandoz. Stocks of these two drugs and quinidine (Sigma), were prepared in 70% ethanol and the solutions containing nifedipine were shielded from light during use.

Statistics

Experimental results are given as means and standard errors of the mean, and significance was tested by Student's unpaired *t* test. *P* values of less than 0.05 are considered significant.

Results

Effect of K^+-channel inhibitors on mechanical and electrical activity of normal tissues

Figure 1 shows the effects of the five potassium channel blocking drugs on the contractile activity of strips of detrusor. With the exception of apamin, the tissue responded to the drugs with a concentration-dependent increase in contractile activity. TEA and procaine elicited vigorous contractile activity, and there were significant effects at concentrations of 10^{-3} M

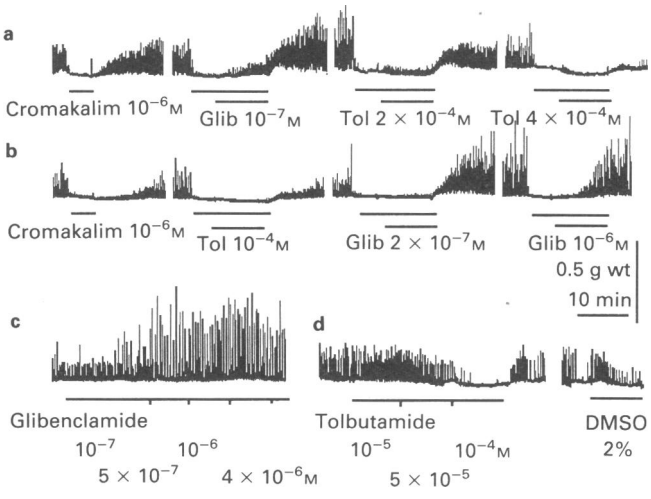


Figure 3 The effects of tolbutamide (Tol) and glibenclamide (Glib) on contractile activity of guinea-pig bladder strips, and their ability to reverse the actions of cromakalim (10^{-6} M). Traces (a) and (c) are from one tissue, traces (b) and (d) from two others. The effects of cromakalim were more easily reversed in tissue (a), where 10^{-7} M glibenclamide caused more recovery than did 2×10^{-7} M in tissue (b). Glibenclamide 10^{-6} M caused complete recovery of the spontaneous activity in all tissues tested. Tolbutamide caused a small recovery at 2×10^{-4} M, and a transient recovery at 4×10^{-4} M. Note the reduced spontaneous activity of the tissues after tolbutamide had been washed off, and the increased activity after washing off glibenclamide. In trace (c) the tissue had not completely recovered from previous exposure to tolbutamide by the beginning of the trace. The 10^{-4} M tolbutamide solution contained 1% dimethylsulphoxide (DMSO). The inhibitory effect of 2% DMSO shown at the end of trace (d) is clearly less than that produced by 10^{-4} M tolbutamide.

TEA and 10^{-3} M procaine. 4AP had a less dramatic effect, but in most tissues caused a contractile response at 10^{-3} M. Quinidine was more potent, eliciting contractile activity at 10^{-5} M, but even at 10^{-4} M the response was transient and not as striking as with TEA or procaine. Apamin had little effect. In 2 out of 8 strips it caused a small increase in contractile activity at 10^{-8} M, but in the rest it caused a slight inhibition. Since it has recently been reported that ATP-dependent K^+ channels may be involved in the relaxant effects of cromakalim on vascular smooth muscle (Quast & Cook, 1988; Cavero *et al.*, 1988; Standen *et al.*, 1989), the effects of the sulphonylureas tolbutamide and glibenclamide on contractile activity were also studied, as these drugs block such channels in the pancreatic beta cells (Ashcroft, 1988), although at much lower concentrations than needed to inhibited the cromakalim-activated channels (Standen *et al.*, 1989; Buckingham *et al.*, 1989). Glibenclamide at 10^{-7} M caused a small increase in the amplitude of the spontaneous activity, but increasing the concentration to 4×10^{-6} M had little further effect. Tolbutamide had no effect at 10^{-5} M, but suppressed the spontaneous activity at 10^{-4} M.

An investigation of the effect of the first five of the K^+-channel inhibitors on the electrical activity showed that they all increased the spike frequency with no detectable depolarization of the cell membrane, apart from procaine (5×10^{-3} M) which caused a small depolarization. Because of the variability of the spike shape even in the absence of drugs (see Figure 2), it is difficult to be precise about the effects of the K^+-channel inhibitors on it, but apamin (10^{-7} M) and 4AP (5×10^{-3} M) had no measureable effect on the rate of repolarization of the spikes, TEA (10^{-2} M) and quinidine (10^{-4} M) caused some prolongation of the falling phase, (the time for half repolarization increasing to 220% and 340% of the

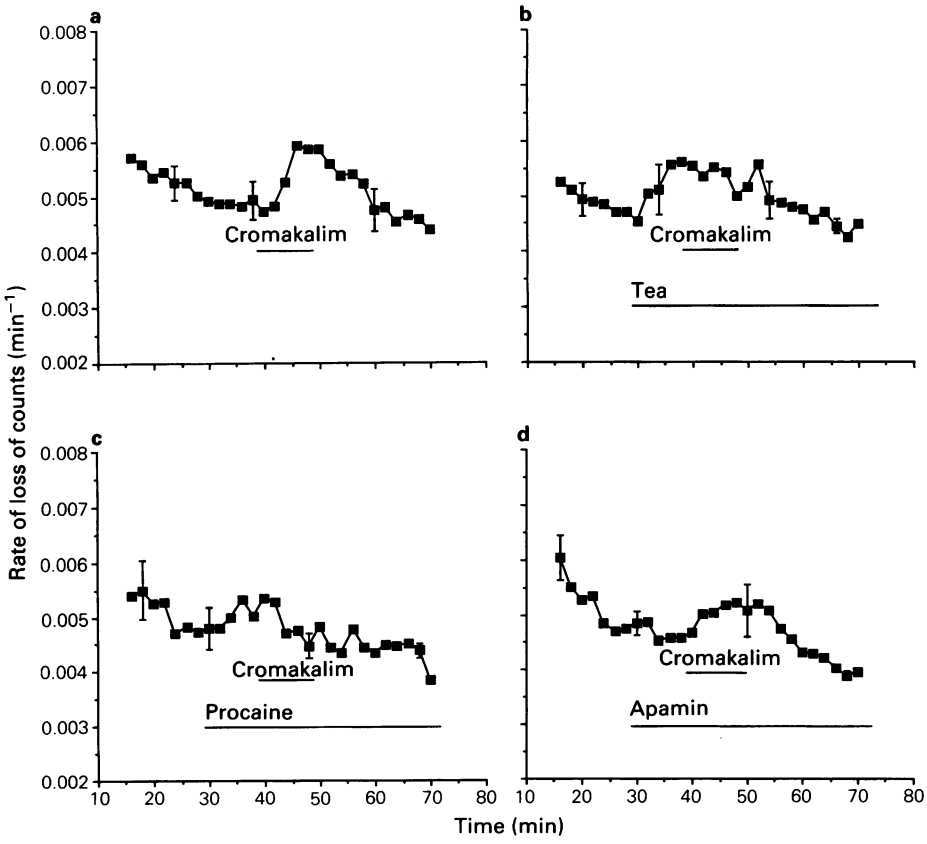


Figure 4 The effect of cromakalim (10^{-5} M) on the rate of loss of $^{43}K^+$ from strips of guinea-pig bladder smooth muscle, on its own, and in the presence of tetraethylammonium Cl (TEA, 5×10^{-3} M), procaine (5×10^{-3} M) or apamin (10^{-7} M). Procaine and TEA cause an increase in the efflux rate which obscures the effects of cromakalim. Values are means with s.e.mean shown by vertical bars, $n = 3$ tissues (from 2 animals).

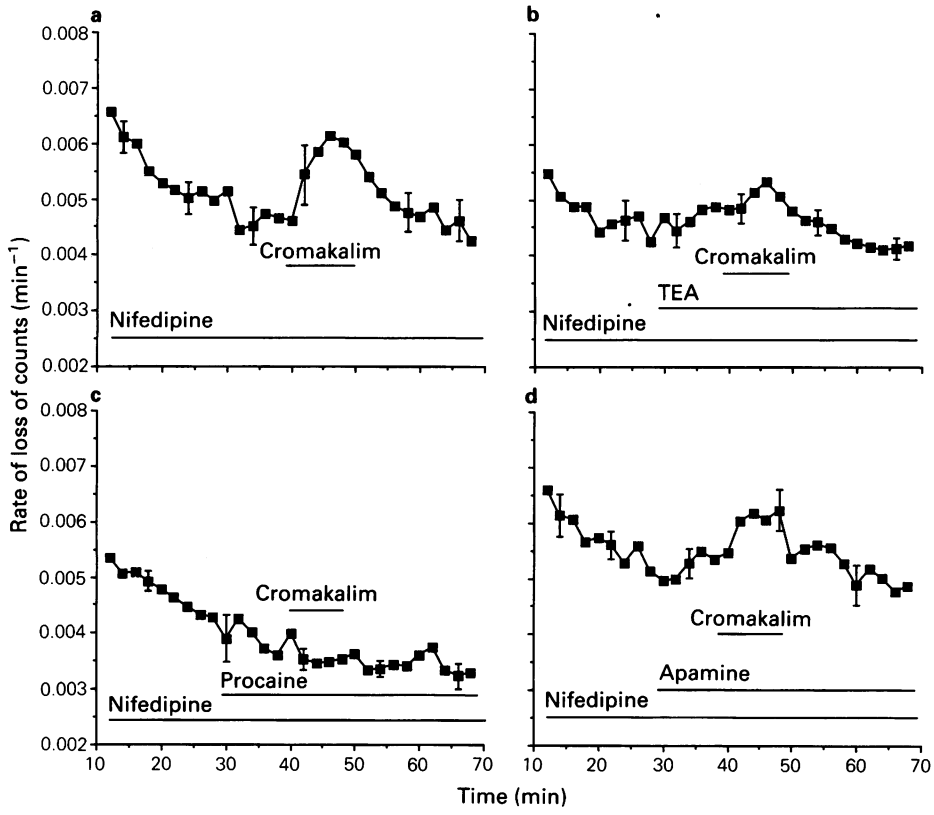


Figure 5 The effect of cromakalim (10^{-5} M) on the rate of loss of $^{43}K^+$ from strips of guinea-pig bladder smooth muscle in the presence of nifedipine (5×10^{-7} M). Nifedipine abolishes the increase in rate of loss of $^{43}K^+$ induced by tetraethylammonium Cl (TEA, 5×10^{-3} M) and procaine (5×10^{-3} M), and it is apparent that TEA reduces, and procaine abolishes the stimulation of the efflux produced by cromakalim. Apamin (10^{-7} M) has no significant effect. Values are means with s.e.mean shown by vertical bars, $n = 3$ tissues (from 2 animals).

control in the examples shown). Procaine (5×10^{-3} M) caused a marked prolongation of the falling phase, which took more than 10 times the control for half repolarization. The drugs also had variable effects on the pronounced after-hyperpolarization seen in normal tissues. Procaine and apamin completely eliminated this phase of the spike, 4AP and TEA had little effect, and quinidine reduced the after-hyperpolarization. Figure 2 shows a typical control spike, and spikes in the presence of the five K^+-channel blockers. An unexpected finding was that the effect of 4AP on spike frequency was transient. The frequency, after an initial large rise, fell to control levels after about 10 min in the continuous presence of the drug, and remained low even on further application of 4AP from a freshly made-up solution. A similar phenomenon was also seen in its effects on contractile activity.

Effect of K^+-channel inhibitors on the response of the detrusor to cromakalim

In the guinea-pig detrusor, it proved impossible to establish a dose-response relationship for the relaxant effects of cromakalim on smooth muscle strips, since the spontaneous activity consisted of contractions which rose from and returned to zero basal tension. Cromakalim at concentrations above 5×10^{-7} M simply abolished this activity. As it was not possible to study the effect of the K^+-channel inhibitors on the dose-response curve, their ability to block the effect of 10^{-6} M, and in some cases 10^{-5} M cromakalim was studied. Cromakalim 10^{-6} M is a sub-maximal concentration for both the hyperpolarization and increase in K^+ flux (Foster *et al.*, 1989). TEA was relatively ineffective at blocking the relaxant effect of cromakalim, and even at 10^{-2} M only partially inhibited the effect of 10^{-6} M cromakalim. Apamine did not antagonize the effect of 10^{-6} M cromakalim at any concentration applied (up to 10^{-7} M). 4AP 10^{-3} M was ineffective, cromakalim still causing a relaxation in its presence, but at higher concentrations of 4AP, although cromakalim (10^{-6} M) slowed the frequency of the contractions, it increased their amplitude. Procaine (10^{-4} M) completely blocked the relaxant effect of 10^{-6} and 10^{-5} M cromakalim. Quinidine blocked the effect of 10^{-6} M cromakalim partially at 10^{-5} M, and completely at 5×10^{-5} M, but was less effective against higher concentrations. This series of K channel blockers all clearly enhanced the spontaneous activity on their own. Glibenclamide had little effect on its own, but was able to block the relaxant effect of 10^{-6} M cromakalim partially at a concentration of 2×10^{-7} M, and completely at 10^{-6} M, although these concentrations did not prevent the action of 10^{-5} M cromakalim. Tolbutamide had a supressant effect on the spontaneous activity on its own at 10^{-4} M, but showed some evidence of partially blocking the effects of 10^{-6} M cromakalim, and more clearly, but transiently antagonized the effect at 4×10^{-4} M. The effects of glibenclamide and tolbutamide are shown in Figure 3.

Cromakalim (10^{-5} M) caused an increased rate of loss of $^{43}K^+$ from the tissue. The effect of the potassium channel inhibitors apamin, procaine and TEA on this action of cromakalim is shown in Figure 4. Apamine itself did not increase the $^{43}K^+$ efflux rate, and in the presence of apamin (10^{-5} M) cromakalim retained its ability to promote $^{43}K^+$ efflux. Both procaine (5×10^{-3} M) and TEA (10^{-2} M) themselves caused an increase in efflux of $^{43}K^+$, and cromakalim caused no further increase. Since the stimulation of the efflux observed on addition of TEA and procaine is probably a result of the increased contractile activity (for instance, an increase in intracellular Ca ions might open Ca^{2+}-dependent K^+ channels), the experiments were repeated in the presence of the Ca^{2+}-channel blocker nifedipine (5×10^{-7} M). In the presence of nifedipine, TEA and procaine no longer caused an increase in

the $^{43}K^+$ efflux, and it could then be seen that TEA partially antagonized and procaine completely abolished the effects of cromakalim. In the presence of both nifedipine and apamin (10^{-7} M), cromakalim retained its ability to promote $^{43}K^+$ efflux (Figure 5).

The effects of the first five inhibitors on the uptake of $^{43}K^+$ and the stimulatory effect of cromakalim on this uptake are illustrated in Figure 6. The results in this figure came from 6 experiments in which a clear stimulation of the $^{43}K^+$ uptake was seen by cromakalim on control tissues. In another 12 experiments analysis of the results showed no effect of cromakalim on the $^{43}K^+$ uptake and we did not include the results of the K^+ channel inhibitors from these experiments. Figure 6 shows that apamin and TEA significantly increased the uptake of $^{43}K^+$ on their own (to 114.8 ± 3.8 and 119.0 ± 10.8% of the control), quinidine and procaine cause a small but significant reduction (to 85.8 ± 7 and 83.8 ± 8%), and 4AP a large reduction (to 62.4 ± 1.7% of the control). With the exception of apamin, all the K^+-channel inhibitors reduced the stimulatory effect of cromakalim on the uptake.

In case the variability of the response to cromakalim of the normal tissues was due to differences in the spontaneous activity of the tissues, the experiments were also conducted in the presence of the Ca-channel antagonist PN 200-110. This treatment did not increase the proportion of tissues in which cromakalim induced significant $^{43}K^+$ uptake. Out of a total of 12 experiments carried out in the presence of PN 200-110, in only 4 did cromakalim cause a significant increase of $^{43}K^+$ uptake. PN200-110 abolished the spontaneous spikes of the tissue, but did not affect the response of the tissue to 30 mM K^+ or to cromakalim measured with microelectrodes, as shown in Figure 7. The membrane was depolarized in 30 mM K^+ and cromakalim 10^{-5} M still caused a hyperpolarization of the membrane by about 20 mV.

The effects of the K^+-channel inhibitors on the electrical response of the tissues to cromakalim is shown in Figure 8. In this figure the effects of 5×10^{-6} M cromakalim are shown. As can be seen, this concentration of cromakalim produced membrane hyperpolarization and loss of spike activity even in the presence of 10^{-2} M TEA, 10^{-7} M apamin and 5×10^{-3} M 4AP, whereas it had no effect at all in the presence of 5×10^{-3} M procaine and only caused a slowing of the spike frequency in the presence of 10^{-4} M quinidine. Procaine (5×10^{-3} M) was

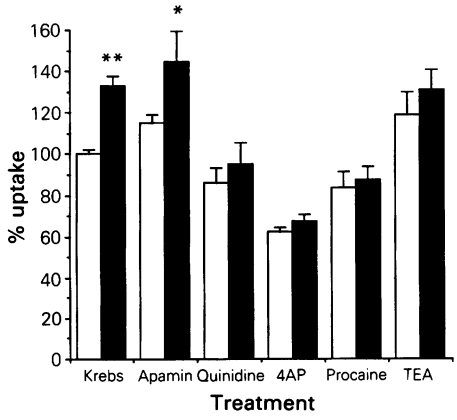


Figure 6 The effect of cromakalim (10^{-5} M) and K^+ channel blockers on the 10 min uptake of $^{43}K^+$ by strips of smooth muscle from guinea-pig bladder. Open columns show the uptake (as a percentage of the counts present in the control tissues) in the absence of cromakalim, and the solid columns in its presence. The concentrations of the K^+ channel blockers were: apamin, 10^{-7} M; quinidine, 3×10^{-5} M; 4-aminopyridine (4AP) 5×10^{-3} M; procaine, 5×10^{-3} M; tetraethylammonium Cl (TEA) 5×10^{-3} M. For each blocker $n = 6$ strips from 3 different guinea-pigs. For the control group, $n = 32$ strips from 7 different guinea-pigs. The uptakes in the presence of the blockers are all significantly different from the control uptake, but cromakalim significantly increases the uptake only in normal Krebs solution or in the presence of apamin.

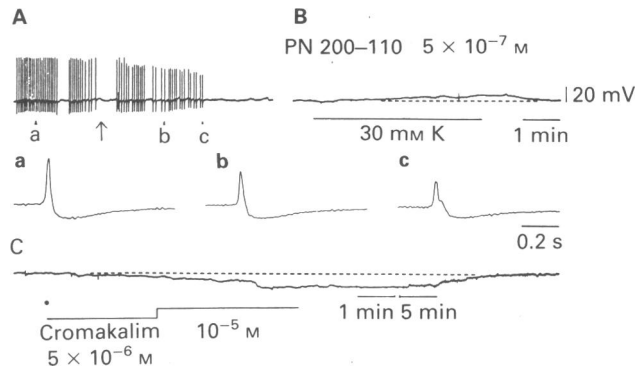


Figure 7 Detrusor muscle from guinea-pig urinary bladder: microelectrode recording of membrane potential activity. (A) The effect of the Ca-antagonist PN 200-110 (10^{-7} M) added at the arrow. The second line shows action potentials on an expanded time-scale. (B) and (C) are in the presence of 5×10^{-7} M PN 200-110. (B) Depolarization still occurs when the K^+ is elevated to 30 mM. (C) Cromakalim still causes hyperpolarization of the membrane. Note reduction in chart speed during latter portion of trace (C).

capable of blocking the effects of higher concentrations of cromakalim. In the presence of 10^{4} M quinidine, however, although the effect of 5×10^{-6} M cromakalim was virtually abolished, if the concentration of cromakalim was increased, the effects of quinidine were progressively antagonized. At 10^{-5} M cromakalim, the spike frequency was reduced, and the

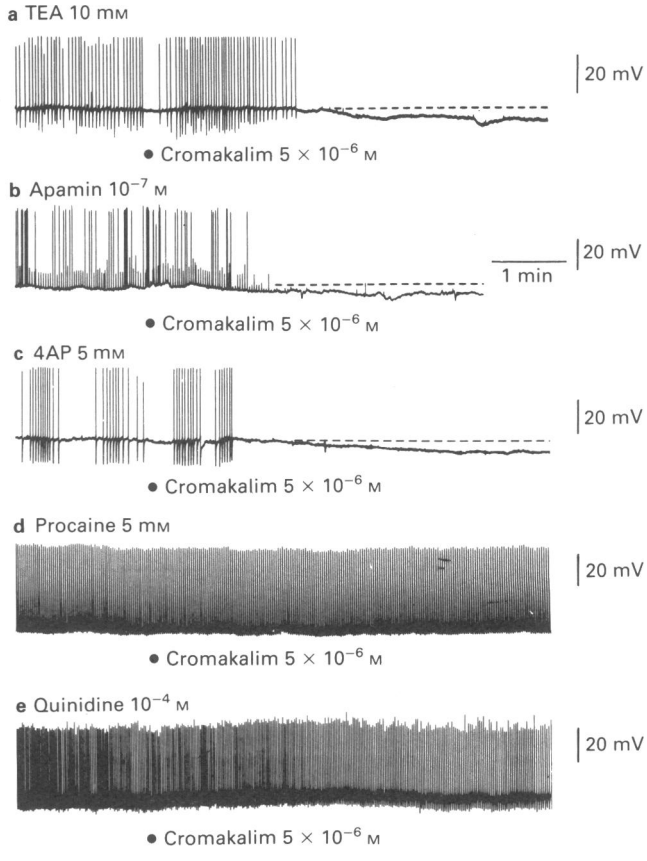


Figure 8 Detrusor muscle from guinea-pig urinary bladder: microelectrode recordings of the effects of cromakalim (5×10^{-6} M) in the presence of K^+ channel blocking drugs. Each tracing comes from a different tissue. Note the lack of an after-hyperpolarization to the spikes in the presence of apamin and procaine. At the concentrations used only procaine (d) and quinidine (e) abolish the action of cromakalim.

prolongation of the falling phase of the action potential (often leading to double spikes) and the reduction in the size of the after-hyperpolarization were both partially reversed, as shown in Figure 9. At 5×10^{-5} M cromakalim, the spike activity was abolished.

Discussion

The results in this paper suggest that there are at least two classes of K^+ channels present in the membranes of the smooth muscle of the guinea-pig urinary bladder and involved in action potential production: one that is responsible for the after-hyperpolarization and is blocked by apamin and procaine and partially blocked by quinidine, and another responsible for the falling phase of the spike which is resistant to apamin and 4AP, but blocked by procaine, TEA and quinidine. The channels that are responsible for the after-hyperpolarization do not appear to be involved in the response of the tissue to cromakalim, since apamin, at a concentration which abolishes the after-hyperpolarization and increases contractile activity, has no effect on the ability of cromakalim to hyperpolarize the cell membranes or relax the tissue. Apamine also fails to prevent the increase in efflux or uptake of $^{43}K^+$ induced by 10^{-5} M cromakalim. Several pieces of evidence suggest that the channels responsible for the repolarization of the spike may have similar properties to the channels opened by cromakalim. Procaine, at a concentration which causes the greatest prolongation of the spike repolarization, is also the most effective at blocking the effects of cromakalim on the tension, membrane potential and the K^+ fluxes. Potassium channel inhibitors which do not, or at concentrations which do not prolong the falling phase of the spike, are not effective in antagonizing the effects of cromakalim on tension, spike activity and membrane hyperpolarization. In an earlier paper (Foster *et al.*, 1989) we also showed that replacing the intracellular K^+ with Rb^+ blocked the effects of cromakalim and caused a significant prolongation of the falling phase of the spike. This suggests that the channels opened by cromakalim have a similar ionic selectivity to the channels responsible for spike repolarization.

In vascular smooth muscle (Standen *et al.*, 1989), ATP-dependent K^+ channels have been demonstrated which are activated by cromakalim and blocked by glibenclamide and tolbutamide, and glibenclamide shifts the concentration-relaxation curves for cromakalim to the right (Buckingham *et al.*, 1989). Using whole cell recording techniques in guinea-pig detrusor we have so far been unable to demonstrate ATP-dependent K^+ channels (R. Inoue, unpublished results). However, the fact that glibenclamide and tolbutamide are able to reverse the effects of 10^{-6} M cromakalim on the contractile activity in the same range of concentrations as they reverse its effects in vascular smooth muscle, and that TEA is relatively ineffective in both tissues, suggests that the channels are very similar. The effects of the other potassium channel blockers are much less clear cut, and it is difficult to come to any conclusions without investigating a wider range of concentrations of the inhibitors. One or two interesting points emerge. TEA and quinidine are both capable of prolonging the falling phase of the spike; however, in the presence of concentrations of the

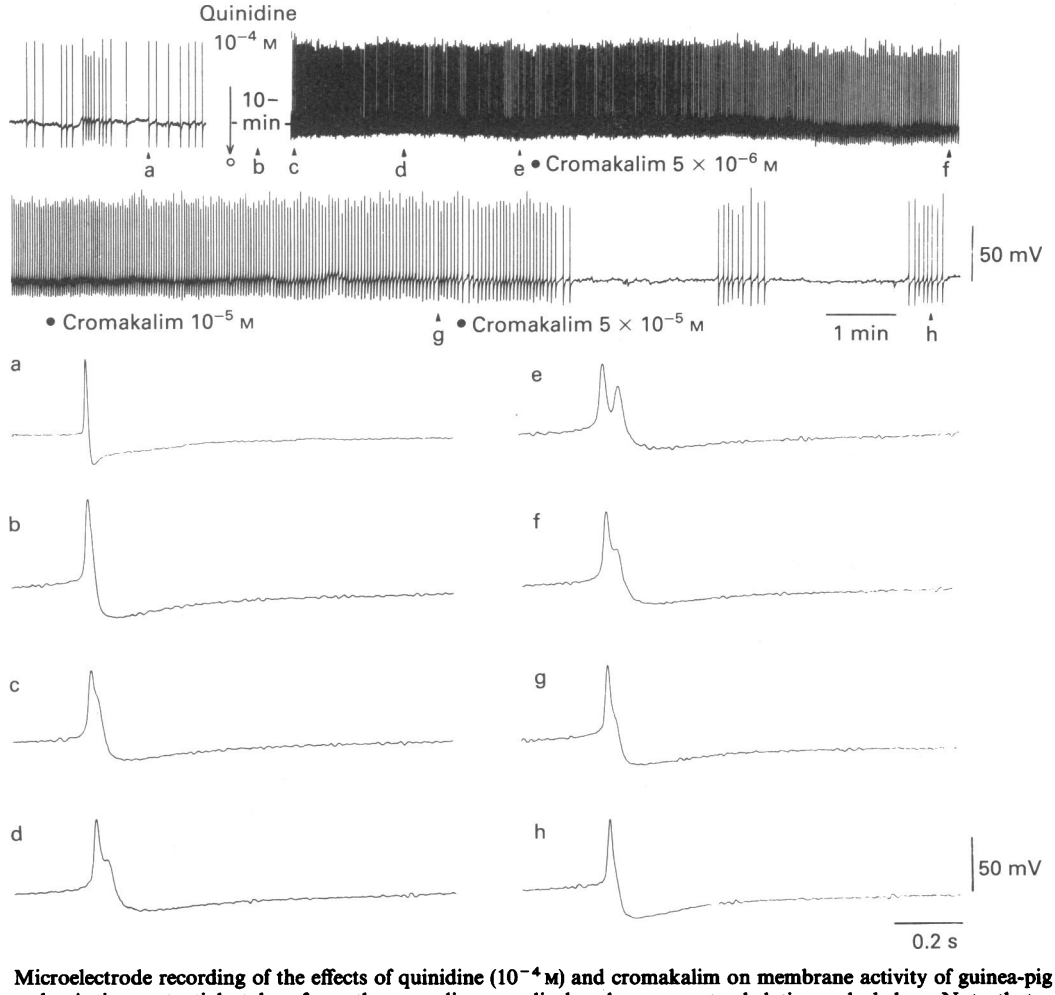


Figure 9 Microelectrode recording of the effects of quinidine (10^{-4} M) and cromakalim on membrane activity of guinea-pig bladder smooth muscle. Action potentials taken from the recording are displayed on an extended timescale below. Note that quinidine increases the spike frequency, reduces the size of the after-hyperpolarization and prolongs the repolarization phase of the action potential leading to double spikes. Increasing doses of cromakalim antagonize these effects.

inhibitors that produce similar prolongations, cromakalim (5×10^{-6} M) still caused a cessation of spike activity and hyperpolarized the membrane in the presence of TEA, but had little effect in the presence of quinidine. Cromakalim also caused an increase in efflux in the presence of TEA (although less marked than in the control tissues). In the presence of quinidine, increasing the concentration of cromakalim, without causing any apparent hyperpolarization, caused a reduction in spike frequency, shortened the spike repolarization and caused a return of the full sized after-hyperpolarization. This suggests that there might be some competition for binding between cromakalim and quinidine. The effects of 4AP are difficult to interpret because of the transience of its effect on spike frequency, and the atypical effect of cromakalim on tension in its presence. 4AP does, however, consistently and significantly reduce the tissue uptake of $^{43}K^+$ after a 10 min exposure, suggesting that it is an effective blocker of some K^+ channels at the concentrations used.

A consistent finding was that there was no clear relationship between the membrane potential of the cells and the spike frequency. Most of the K^+-channel inhibitors increased the spike frequency in the absence of any recordable depolarization of the membrane. Cromakalim on its own or in the presence of those inhibitors which did not block its effect, usually blocked spike activity at a time when there was no obvious change in membrane potential, the hyperpolarization beginning a few seconds later. The ionic mechanisms responsible for spike initiation in the bladder are unknown, but conceivably involve channels that are sensitive both to the K^+-channel inhibitors and to cromakalim.

We have again come up against the problem of lack of consistency of the effects of cromakalim on the K^+ fluxes, and this is more marked when looking at the uptake of $^{43}K^+$ than its efflux. We were only able to record an effect of 10^{-5} M cromakalim on K^+ uptake in 6 out of 15 experiments. In comparison, as we have previously reported (Foster *et al.*, 1989) we found an increased efflux in 17 out of 20 tissue strips, and have always seen cessation of mechanical and electrical activity on exposure to cromakalim at this concentration. The changes in membrane potential and spike frequency caused by cromakalim may themselves alter the pattern of opening of several types of K^+ channels, and the net effect will vary depending on the profile of channels that each strip possesses. The hyperpolarization will also reduce the driving force for K^+ efflux, but theoretically should increase the driving force for K^+ influx. The effects of cromakalim should therefore be seen more consistently on the uptake. The fact that the reverse is the case, could be due to some intrinsic properties of the channels opened by cromakalim. It has been suggested for instance, that the delayed rectifier K^+ channels in squid giant axon may show 'single file' effects, which would occur if several ions could occupy the channel simultaneously (Hodgkin & Keynes, 1955; Hille, 1984), which would alter the flux ratio in favour of efflux.

In conclusion, the results described in this paper support the suggestion put forward by Beech (1989) that the channels opened by cromakalim may be similar to the delayed rectifier K^+ channels that have been demonstrated in many smooth muscles, and whose presence in guinea-pig bladder has been suggested by Klöckner & Isenberg (1985). They also suggest that the channels are similar to the ATP-dependent K channels recently demonstrated in vascular smooth muscle (Standen *et al.*, 1989). More conclusive evidence awaits patch clamp analysis from detrusor smooth muscle cells.

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Effects of torbafylline, pentoxifylline and buflomedil on vascularisation and fibre type of rat skeletal muscles subjected to limited blood supply

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1 Blood flow (measured by radio-labelled microspheres), fibre composition and capillary/fibre ratio were estimated in rat fast twitch skeletal muscles (tibialis anterior and extensor digitorum longus) five weeks after unilateral ligation of the common iliac artery in animals treated with either saline, torbafylline, pentoxifylline or buflomedil.

2 The resting blood flow was lower in muscles with limited blood supply than in their contralateral controls; this difference became statistically insignificant after treatment. Capillary/fibre ratio was similar in all muscles with either intact or limited blood supply and did not change after administration of any of the drugs.

3 The percentage of glycolytic fibres was not changed by ligation, but it decreased significantly in animals treated with torbafylline. This may improve performance in muscles with limited blood supply.

Introduction

Limitation of blood supply to skeletal muscles, whether acute or chronic, is almost inevitably linked with impaired muscle performance. Although some collateral circulation usually develops (Imparato *et al.*, 1975), which can satisfactorily meet the oxygen demand of muscles at rest, it is not sufficient to ensure adequate supply even during moderate exercise (Gaethgens *et al.*, 1976) and the result is intermittent claudication. Reconstructive surgery which normalises muscle metabolism (Pernow *et al.*, 1975), or exercise therapy, which is very beneficial (Larsen & Lassen, 1966; Zetterquist, 1970) is not always applicable. Consequently, there is a constant search for drugs which improve muscle blood flow and performance.

Close-arterial administration of adenosine 5'-triphosphate (ATP) increases blood flow in the occluded region (Schoop, 1956; hess, 1964), but it cannot be used routinely in the clinic. Blood flow after arterial thrombosis was increased, at least temporarily, by a shortlasting infusion of noradrenaline and this effect was attributed to the increased perfusion pressure (Dahn *et al.*, 1967). Bradykinin was without effect, or even impaired blood flow (Pernow & Zetterquist, 1967) in muscles with limited blood supply. Numerous data on pentoxifylline (Ward & Clissold, 1987) indicate that this drug improves microcirculation possibly by increasing the deformability of red blood cells. It is used routinely in patients with peripheral vascular diseases, but the data on the improvement of walking distance show great variability. Buflomedil was reported to improve blood flow in patients with peripheral vascular diseases both during acute and chronic administration (Perego *et al.*, 1982), but did not change it in hind-limbs of dogs with surgically limited vascular supply (Fujita *et al.*, 1984). It did increase oxygen tension, either by decreasing oxygen consumption (Sunder-Plassman *et al.*, 1981b) or by improving collateral circulation (Sunder-Plassman *et al.*, 1981a) but the data on its effect on muscle performance are variable (Clissold *et al.*, 1987).

Although muscle performance, measured mainly in terms of fatiguability in cases of peripheral vascular diseases, is related to blood supply, it can also be improved by a change in the composition of the muscle fibres. It is well known that muscles with a high proportion of oxidative fibres (type I or IIa) fatigue much less than those with a high proportion of glycolytic (type IIb) fibres (Burke *et al.*, 1971). Decrease in type I (slow oxidative) fibres was described in patients with muscle cramps (Telerman-Tuppet *et al.*, 1985); Clyne *et al.* (1982) did not find any change in the proportion of type I or IIa fibres, while Hammarsten *et al.*, (1980) described a higher proportion of type IIa fibres in patients who had intermittent claudication for at least one year. However, there are no data on the effect of drugs on fibre composition in muscles with limited blood supply.

The purpose of this study was to compare the long-term effect of several drugs, two of them (pentoxifylline, Hoechst, Werk Albert, and buflomedil, Abbot) routinely used in the clinic and one experimental drug (torbafylline, [7-ethoxymethyl-1-(5 hydroxy-5-methylhexyl)-3-methylxanthine], Gebert *et al.*, 1987; Okayayuz-Baklouti, 1988) on blood flow, fibre composition and capillary supply in skeletal muscles with limited blood supply.

Methods

Experiments were performed in four groups of male Sprague Dawley rats 480–550 g body weight. The right common iliac artery was ligated (under halothane anaesthesia and aseptic conditions) immediately below the bifurcation of the aorta in all animals. One group (8 animals) served as controls: four animals did not receive any treatment, four received 0.3 ml sterile saline solution intraperitoneally 3 times per day. The second group (7 animals) was injected following the same protocol with torbafylline 3 mg kg^{-1} body weight in 1% aqueous solution, the third group (6 animals) received 0.3 ml 1% (3 mg kg^{-1}) pentoxifylline and the fourth group (6 animals) 0.2 ml 1% (2 mg kg^{-1}) buflomedil. Treatment always started on the day after the operation, and the last injection was given the day before the final experiment, i.e. approximately 16 h before blood flow was estimated. All animals were treated for 5 weeks (7 days per week), with no obvious impairment of function (tested by the toe spreading reflex and walking) in the ligated leg under resting conditions. Animals on buflomedil showed a greater excitability after one week of treatment and thereafter.

Acute experiments were performed at the end of the treatment, under pentobarbitone anaesthesia. Blood flow at rest was measured using 15 μm microspheres labelled with ^{113}Stannum (Hudlická *et al.*, 1981) with injection into the left ventricle via the right carotid artery and withdrawal from one brachial artery. The contralateral brachial artery was cannulated to measure blood pressure. Samples of tibialis anterior

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(TA) and extensor digitorum longus (EDL) (whole cross section of the muscle in the mid-belly) were frozen in isopentane, precooled in liquid nitrogen. The rest of the muscles were used for blood flow estimations. The whole gastrocnemius muscle was also taken for blood flow measurements. To check adequate mixing of microspheres, blood flow was measured in the right and left kidney. The kidney flow (cortex) was $6.53 \pm 1.7\,ml\,g^{-1}\,min^{-1}$ in the control group, and was not affected by drug treatment. The difference between right and left kidney did not exceed 10%.

Sections (10 μm) from TA and EDL were cut on the cryostat, and stained for succinate dehydrogenase (Nachlas *et al.*, 1957) to show oxidative fibres, and for alkaline phosphatase using indoxyl-tetrazolium staining (Ziada *et al.*, 1984) to depict capillaries. In each muscle, 20 muscle bundles (300–400 muscle fibres) were counted for the number of muscle fibres and the number of capillaries, and the results were expressed as C/F ratio. In tibialis anterior, which is composed of a predominantly glycolytic superficial layer, and a predominantly oxidative core, counting was done separately in each part. Succinate dehydrogenase is present in minimal concentration in glycolytic fibres which appeared very pale. The percentage of these fibres was also counted in 20 bundles from each muscle, taking again the glycolytic and oxidative parts of the tibialis separately.

Student's *t* test (paired or unpaired, as appropriate) was used for statistical evaluation. Coefficient of variation (mean × 100)/s.d. was used to express variability of data.

Results

Body weight and muscle weight

All groups, with the exception of the animals treated with buflomedil, had similar body weights (Table 1). The slightly lower average body weight in animals on buflomedil was due to the fact that the animals were lighter at the beginning of the experiment. Similar increase in body weight (about 40 g) was observed in all groups over the period of five weeks. Tibialis anterior (TA) and extensor digitorum longus (EDL) muscles were significantly lighter in the ligated than in contralateral legs in control animals, and those treated with pentoxyfylline and buflomedil.

Blood pressure and blood flow

No significant differences among the groups were found in systolic, diastolic or mean blood pressure at rest (Table 2). Blood flow in gastrocnemius muscles did not seem to be affected by either drug treatment or the ligation of the common iliac artery. The average values of resting blood flow in contralateral TA and EDL muscles were higher in animals treated with torbafylline and pentoxifylline than in the untreated or the buflomedil-treated group, but the difference was not statistically significant using unpaired *t* test (Table 2). Similarly, long-term treatment with torbafylline and pentoxifylline slightly, but not significantly, increased resting flow in muscles with limited blood supply. Because of great variability of blood flow, particularly in torbafylline- and pentoxifylline-treated animals, flow in ligated muscles was calculated as a percentage of the flow in contralateral muscles, i.e. flow in the ligated leg was divided by flow in the contralateral leg. These values, although somewhat different from the proportions of means calculated from the data in Table 2, show more precisely the respective effect of individual drugs on the improvement of flow with respect to the contralateral muscles. Thus the mean of these values in ligated muscles represented $64.4 \pm 11.1\%$ of the value in the contralateral muscles in the untreated animals, being significantly ($P < 0.02$ against 1.0) lower. The relative decrease was smaller, $72.5 \pm 11.6\%$ ($P < 0.05$ against 1.0) in the torbafylline-treated group, and the values were not significantly different from 100% in the pentoxifylline ($73 \pm 13.8\%$, NS) and buflomedil ($85.7 \pm 19.5\%$, NS) groups, respectively. Thus, treatment with any of these drugs seemed to improve resting blood flow 5 weeks after the ligation of the common iliac artery. However, the lack of significant difference between the absolute values of flow in contralateral and ligated muscles, particularly in animals treated with pentoxifylline, was due to a tremendous variabil-

Table 1 Body weight (in g) and muscle weights (in g) of rats used in this study

Treatment	*Saline*	*Torbafylline*	*Pentoxifylline*	*Buflomedil*
Body weights	521 ± 8.7 (8)	536 ± 8.8 (7)	538 ± 4.0 (6)	507 ± 12 (6)
Muscle weights				
TA + EDL L	1.18 ± 0.01*	1.16 ± 0.06	1.12 ± 0.03*	0.98 ± 0.05**
C	1.25 ± 0.02	1.23 ± 0.03	1.27 ± 0.03	1.14 ± 0.05
Gastro L	2.79 ± 0.06	2.71 ± 0.11	2.86 ± 0.05	2.48 ± 0.04
C	2.85 ± 0.09	2.91 ± 0.08	2.82 ± 0.19	2.54 ± 0.05

Mean ± s.e.mean. Number of animals in parentheses.
L = ligated side, C = contralateral side, TA = tibialis anterior, EDL = extensor digitorum longus, Gastro = gastrocnemius.
* Significantly different from contralateral at $P < 0.005$.
** Significantly different from contralateral at $P < 0.05$.

Table 2 Mean blood pressure at rest (mmHg) and blood flow (ml $100\,g^{-1}$ × min) at rest and during contractions

Treatment	*Saline*	*Torbafylline*	*Pentoxifylline*	*Buflomedil*
BP systolic	139 ± 15	136 ± 11	128 ± 11	133 ± 5
diastolic	113 ± 13	112 ± 8	103 ± 9	108 ± 6
BP mean	124 ± 12.1 (8)	120.3 ± 7.4 (7)	111.0 ± 8.8 (6)	115.7 ± 4.6 (6)
Blood flow at rest				
TA + EDL L	2.54 ± 0.57 (6)*	3.99 ± 1.07 (7)	4.28 ± 1.36 (6)	2.27 ± 0.38 (6)
C	4.65 ± 0.78 (7)	7.2 ± 1.94 (7)	6.35 ± 1.46 (6)	3.23 ± 0.59 (6)
Gastro L	6.0 ± 2.2 (7)	3.3 ± 0.8 (7)	2.6 ± 0.5 (6)	1.4 ± 0.2 (6)
C	4.6 ± 2.4 (7)	4.2 ± 1.3 (7)	3.8 ± 0.9 (6)	1.2 ± 0.1 (6)

Number of measurements in each group in parentheses. L = muscles with common iliac artery ligated, C = contralateral muscles, TA = tibialis anterior, EDL = extensor digitorum longus, Gastro = gastrocnemius.
* Significantly different from contralateral muscles $P < 0.05$.

Table 3 Capillary supply in extensor digitorum longus (EDL) and subtypes of tibialis anterior (TA) muscles of the rat

	C/F ratio		
	EDL	*TA 'white'*	*TA 'red'*
Ligated + saline ($n = 7$)			
Control	2.01 ± 0.08	1.39 ± 0.08	2.47 ± 0.09
Ligated	2.04 ± 0.88	1.36 ± 0.08	2.48 ± 0.06
Ligated + torbafylline ($n = 7$)			
Control	2.11 ± 0.07	1.34 ± 0.03	2.40 ± 0.04
Ligated	2.07 ± 0.12	1.24 ± 0.03	2.44 ± 0.05
Ligated + pentoxyfylline ($n = 6$)			
Control	1.91 ± 0.08	1.24 ± 0.02*	2.43 ± 0.07
Ligated	2.05 ± 0.11	1.29 ± 0.05	2.46 ± 0.09
Ligated + buflomedil ($n = 6$)			
Control	1.95 ± 0.06	1.32 ± 0.04	2.37 ± 0.06
Ligated	1.95 ± 0.09	1.28 ± 0.03	2.36 ± 0.07

* $P < 0.05$ against control in ligated and saline.

ity in the development of collateral circulation: CV represented 60.4, 60.1, 117.8 and 51.5% in the control, torbafylline-, pentoxifylline- and buflomedil-treated animals, respectively.

Capillary supply

Capillary supply was estimated in TA and EDL as C/F ratio (Table 3). It was similar in all groups in control and ligated muscles, and no drug, with the exception of pentoxifylline, had any effect on C/F in either EDL or TA. There was a slightly but significantly lower C/F ratio in the glycolytic part of control tibialis in pentoxifylline-treated animals when compared to contralateral muscles in the control group.

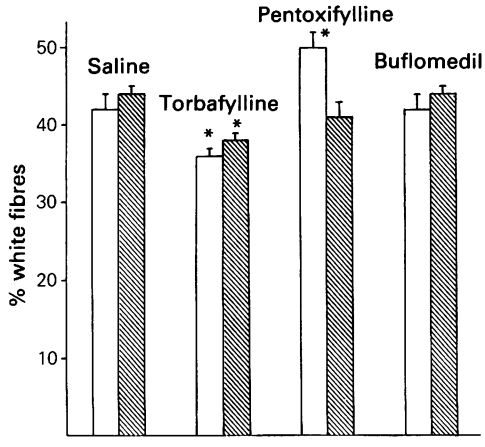


Figure 1 Proportion of white (glycolytic) fibres in rat extensor digitorum longus (EDL) with normal (control, open columns) and limited (ligated, hatched columns) blood supply. * Values significantly different from the control (saline) group.

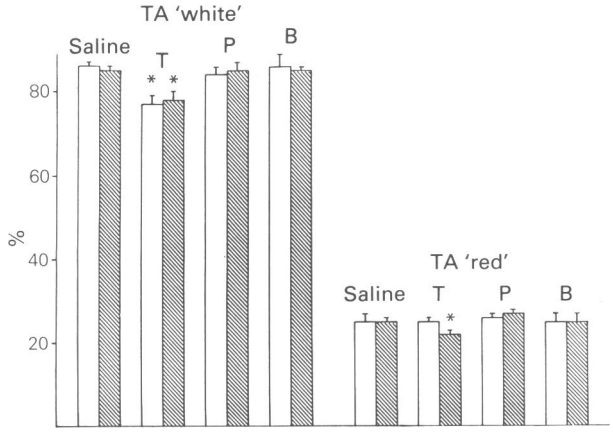


Figure 2 Proportion of glycolytic (white) fibres in the glycolytic cortex ('white') and oxidative core ('red') tibialis anterior (TA) muscles with normal (control, open columns) and limited (ligated, hatched columns) blood supply: T, torbafylline; P, pentoxifylline; B, buflomedil-treated rats. * Values significantly different from saline-treated (control) animals.

Proportion of glycolytic fibres

The proportion of glycolytic fibres was 42 ± 1% and 44 ± 1% in control and ligated EDL in the control group and in animals treated with buflomedil. Animals treated with torbafylline showed a significantly lower proportion of glycolytic fibres in both control and ligated muscles (36 ± 1 and 38 ± 1 respectively) while extensor digitorum longus in rats treated with pentoxifylline showed a higher proportion of glycolytic fibres (50 ± 2%) in control muscles and 41 ± 3% in the ligated ones (Figure 1). The proportion of glycolytic fibres in the tibialis anterior was higher in the predominantly glycolytic cortex (86 ± 1 and 85 ± 1), in the contralateral and ligated muscles in the control group with similar values in animals treated with pentoxifylline and buflomedil. It became significantly lower (77 ± 2% in contralateral and 78 ± 2% in ligated) in animals treated with torbafylline. This effect of torbafylline in the oxidative core of TA was seen in ligated muscles only (Figure 2) where the proportion of glycolytic fibres decreased from 25 to 22 ± 1%.

Discussion

The data in this paper show that circulation in rat skeletal muscles 5 weeks after the ligation of the common iliac artery was sufficient to maintain normal blood flow so that there was no loss of weight in gastrocnemius which is a muscle involved in the maintenance of posture. Fast contracting tibialis anterior and extensor digitorum longus showed a small degree of atrophy and a significantly lower flow. These findings are seemingly contradictory to the data of Nicholson *et al.* (1985) and Angersbach *et al.* (1988) who found lower blood flow in the rat gastrocnemius muscle 6–12 weeks after the ligation of the femoral artery. However, faster development of collateral circulation, judged by blood flow measurements during contractions, was described by Elander *et al.* (1985) 4 days after ligation of the common iliac artery in soleus (another postural muscle) than in EDL. Our previous findings (Hudlická & Torres, 1989) also showed a faster spontaneous development of collateral circulation in soleus. The discrepancies may be due to the methods used, ^{133}xenon clearance in Nicholson's experiments and microspheres in those by Elander and in this study. Administration of xenon into the muscle, particularly into a muscle as complex as gastrocnemius, may vary according to the fibre types, and it may be that if the isotope were placed in the glycolytic part of the muscle, flow would be found to be lower, in agreement with our results on EDL and TA.

Nevertheless, this would not explain the different results found in the present study on muscle weights. It is possible that the development of collateral circulation is different, particularly with respect to various muscles, depending on the site of ligation. However, this discrepancy can only be resolved by direct comparisons in the same strain of animals preferably in the same laboratory.

The administration of various drugs did not have any dramatic effect on either muscle atrophy or muscle blood flow. The weights of TA and EDL after ligation were not significantly different from the weights of contralateral muscles in animals treated with torbafylline (albeit the effect was very small) but were still smaller in animals treated with pentoxifylline or buflomedil. The difference between the resting blood flow in muscles with ligated common iliac arteries and contralateral muscles was no longer significant after drug treatment. This was due to a small, though insignificant, elevation of flow in the case of torbafylline and pentoxifylline, particularly in muscles with limited blood supply, but to a decrease in blood flow in contralateral muscles in the case of buflomedil.

Previous results have shown that torbafylline and pentoxifylline increase blood flow during acute infusion of $3\,mg\,kg^{-1}$ to a similar degree, both at rest and during muscle contractions in ischaemic muscles, but only torbafylline significantly diminished muscle fatigue. Torbafylline, but not pentoxifylline, also increased force of contraction in ischaemic muscles in a dose of up to $5\,mg\,kg^{-1}\,min^{-1}$ and increased surface Po_2 tension of ischaemic muscles. Further increase in dosage did not produce any greater changes, and it actually resulted in a decrease in Po_2 in animals treated with pentoxifylline, very probably due to decreased blood pressure. When these two drugs were given to rats for 16 days at the same dose as in the present experiments, torbafylline but not pentoxifylline, prolonged significantly the time the animals could run (Okyauz-Baklouti, 1988). It was therefore important to compare these doses of both drugs to find out whether the difference in improved performance could be explained either by better capillary supply or changes in fibre composition.

Capillary supply was not affected either by ligation or by drug treatment. Mäkitie (1977) described higher capillary/fibre ratio in gastrocnemius muscles in patients with intermittent claudication, but Henriksson *et al.* (1980) did not see any differences and Clyne *et al.* (1985) found lower values. It is difficult to find an explanation for such controversial results. Capillary supply is usually adapted to the level of oxidative metabolism, but it is still not quite clear whether this is decreased (e.g. Henriksson *et al.*, 1980) or increased (e.g. Bylund-Fellenius *et al.*, 1988) in muscles with limited blood supply. Moreover, increased capillarisation was found in muscles of animals treated with the xanthine derivative HWA 285, but the fibre composition was not changed and neither was muscle performance (Ziada *et al.*, 1984). The lack of increased capillarisation in muscles with intact blood supply after long-term administration of various drugs is in agreement with the lack of higher blood flow: increased capillarisation was usually found only in muscles of animals treated with drugs that produced a longlasting vasodilatation (Tornling *et al.*, 1980; Ziada *et al.*, 1984; Hudlická *et al.*, 1984). The fact that there was no capillary loss in muscles with limited blood supply would indicate that the collateral circulation and perfusion pressure were sufficient stimulus for the maintenance of the capillary structure.

The most pronounced difference in the effect of treatment with various drugs was observed on muscle fibre composition. Rat EDL is composed of approximately 40% glycolytic fibres (Ariano *et al.*, 1973), and this proportion was not changed by ligation. Neither was there a change in fibre composition in the predominantly glycolytic cortex or predominantly oxidative core of the tibialis anterior. These results are in agreement with the data on fibre type distribution in gastrocnemius muscles of patients with peripheral vascular disease (Henriksson *et al.*, 1980; Clyne *et al.*, 1982; Jansson *et al.*, 1988), but are at variance with findings reported by Hammarsten *et al.* (1980) who described a higher proportion of IIa (oxidative) fibres in the muscles of patients with intermittent claudication. Zeman *et al.* (1988) described a slight increase in the percentage of fast fibres and their hypertrophy in rat soleus and EDL after long-term treatment with a β_2-adrenoceptor agonist, clenbuterol. However, there are no data available on the effects of drugs on fibre composition in muscles with limited blood supply. In the present study, neither pentoxifylline nor buflomedil had any effect, while torbafylline significantly decreased the proportion of glycolytic fibres in all ligated muscles; it also decreased it in muscles or muscle parts with intact blood supply with a high proportion of glycolytic fibres. It may be argued that a higher dose of e.g. pentoxifylline could have had a similar effect to torbafylline. However, since the proportion of glycolytic fibres in normal muscles was significantly increased using the same dose of pentoxifylline as that of torbafylline, it is highly unlikely that any increase in dosage could reverse the effect of this drug.

Conversion of fibre types towards the oxidative form has so far only been described during endurance training (e.g. Saltin & Gollnick, 1983) and chronic electrical stimulation (e.g. Brown *et al.*, 1976). Increased oxidative capacity has also been achieved by administration of thyroid hormones (e.g. Winder *et al.*, 1980). Each of these conditions is very probably initiated by different signals and it would be premature to consider whether torbafylline may act by similar or different mechanisms.

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CGP 37849 and CGP 39551: novel and potent competitive N-methyl-D-aspartate receptor antagonists with oral activity

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1 The pharmacological properties of CGP 37849 (DL-(E)-2-amino-4-methyl-5-phosphono-3-pentenoic acid; 4-methyl-APPA) and its carboxyethylester, CGP 39551, novel unsaturated analogues of the N-methyl-D-aspartate (NMDA) receptor antagonist, 2-amino-5-phosphonopentanoate (AP5), were evaluated in rodent brain *in vitro* and *in vivo*.

2 Radioligand binding experiments demonstrated that CGP 37849 potently (K_i 220 nM) and competitively inhibited NMDA-sensitive L-[^{3}H]-glutamate binding to postsynaptic density (PSD) fractions from rat brain. It inhibited the binding of the selective NMDA receptor antagonist, [^{3}H]-(±)-3-(2-carboxypiperazin-4-yl)propyl-1-phosphonate (CPP), with a K_i of 35 nM, and was 4, 5 and 7 fold more potent than the antagonists ((±)-*cis*-4-phosphonomethylpiperidine-2-carboxylic acid) (CGS 19755), CPP and D-AP5, respectively. Inhibitory activity was associated exclusively with the *trans* configuration of the APPA molecule and with the D-stereoisomer. CGP 39551 showed weaker activity at NMDA receptor recognition sites and both compounds were weak or inactive at 18 other receptor binding sites.

3 CGP 37849 and CGP 39551 were inactive as inhibitors of L-[^{3}H]-glutamate uptake into rat brain synaptosomes and had no effect on the release of endogenous glutamate from rat hippocampal slices evoked by electrical field stimulation.

4 In the hippocampal slice *in vitro*, CGP 37849 selectively and reversibly antagonized NMDA-evoked increases in CA1 pyramidal cell firing rate. In slices bathed in medium containing low Mg^{2+} levels, concentrations of CGP 37849 up to 10 μM suppressed burst firing evoked in CA1 neurones by stimulation of Schaffer collateral-commissural fibres without affecting the magnitude of the initial population spike; CGP 39551 exerted the same effect but was weaker. *In vivo*, oral administration to rats of either CGP 37849 or CGP 39551 selectively blocked firing in hippocampal neurones induced by ionophoretically-applied NMDA, without affecting the responses to quisqualate or kainate.

5 CGP 37849 and CGP 39551 suppressed maximal electroshock-induced seizures in mice with ED_{50}s of 21 and 4 mg kg^{-1} p.o., respectively.

6 CGP 37849 and CGP 39551 are potent and competitive NMDA receptor antagonists which show significant central effects following oral administration to animals. As such, they may find value as tools to elucidate the roles of NMDA receptors in brain function, and potentially as therapeutic agents for the treatment of neurological disorders such as epilepsy and ischaemic brain damage in man.

Introduction

N-methyl-D-aspartate (NMDA) receptor mechanisms have been implicated in the pathophysiology of a number of neurological disorders and a great deal of interest has centred on the therapeutic potential of agents which block the actions of this excitatory amino acid receptor sub-type (see collection of articles edited by Cavalheiro *et al.*, 1988). Many studies have demonstrated that activation of the NMDA receptor is involved in the generation of epileptiform activity and in hypoxic-ischaemic neuronal damage, and conversely that NMDA receptor antagonists are anticonvulsant and cerebroprotective in animal models of epilepsy and stroke (see reviews by Meldrum, 1985; Rothman & Olney, 1986; Choi, 1988; Patel *et al.*, 1988; Iversen *et al.*, 1989; Albers *et al.*, 1989). Such findings have led to the prediction that NMDA receptor antagonists with appropriate potency, selectivity and bioavailability may form novel agents for the treatment of seizure disorders and ischaemic neuropathologies in man (Meldrum, 1985; Rothman & Olney, 1986; Albers *et al.*, 1989; Iversen *et al.*, 1989).

Antagonism of NMDA receptor mechanisms potentially may be achieved by a number of different approaches. In addition to the transmitter recognition site, the NMDA receptor complex comprises an allosteric regulatory site and a channel binding domain (Foster & Fagg, 1987b), and possibly also sites defined by the actions of Zn^{2+}, polyamines, tricyclic antidepressants, ifenprodil and CGP 31358 (see Lodge, 1989; Baud *et al.*, 1989). At present, however, the two most well characterized sites are (1) the transmitter recognition site, at which substances such as D-2-amino-5-phosphonopentanoate (D-AP5), (±)-3-(2-carboxypiperazin-4-yl) propyl-1-phosphonate (CPP) (±)-*cis*-4-phosphonomelthylpiperidine-2-carboxylic acid (CGS 19755) competitively antagonize the actions of excitatory amino acids (Evans *et al.*, 1982; Davies *et al.*, 1986; Lehmann *et al.*, 1987; 1988), and (2) a channel site at which non-competitive antagonists such as phencyclidine (PCP) and (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d] cyclohepten-5,10-imine maleate (MK 801, Wong *et al.*, 1986; Lodge *et al.*, 1988) bind to function as open-channel blockers.

The potential therapeutic advantage of competitive antagonists is that, following systemic administration, their beneficial effects may be discriminated from their side-effects (Meldrum, 1985; Lehmann *et al.*, 1987; 1988; Ferkany *et al.*, 1988; France *et al.*, 1989; Tricklebank *et al.*, 1989), and therefore they may prove to be suitable for the treatment of disorders in man requiring either acute or chronic drug administration. The disadvantage of currently-available substances, however, is that they are not active following oral administration. Here we describe a novel analogue of AP5 which exhibits high potency and selectivity for the NMDA receptor *in vitro* and is centrally-active following oral administration *in vivo*. As

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Figure 1 Chemical structures of (left) CGP 37849 (DL-(E)-2-amino-4-methyl-5-phosphono-3-pentenoic acid; or 4-methyl-APPA) and (right) CGP 39551.

detailed elsewhere (Fagg *et al.*, 1989a; Schmutz, Portet, Jekes, Klebs, Vassout, Allgeier, Heckendorn, Fagg, Olpe & van Riezen, unpublished observations), this compound (CGP 37849; DL-(E)-2- amino-4-methyl-5-phosphono-3-pentenoic acid, or 4-methyl-APPA) and its carboxy-ethylester (CGP 39551) (for structures, see Figure 1) show potent oral anticonvulsant properties which can be separated from their behavioural side-effects and hence are potential candidates for novel anticonvulsant therapy in man. Preliminary results of this study have been presented in abstract form (Fagg *et al.*, 1988; 1989; Schmutz *et al.*, 1989).

Methods

Radioligand binding assays

NMDA receptor assays Subcellular fractions enriched in postsynaptic densities (PSDs) were isolated from the brains of adult male Tif:RAIf(SPF) rats (180–250 g) as previously described (Foster & Fagg, 1987a). In brief, synaptic plasma membranes were isolated by sucrose density gradient centrifugation, treated with 0.4% Triton X-100, subjected to one freeze-thaw cycle, and washed 4 times by resuspension and centrifugation in 0.5 mM HEPES-KOH buffer (pH 7.0). The resultant crude PSDs were stored as a suspension at −80°C until assay, at which time they were thawed, washed once more and resuspended in assay buffer.

The binding of L-[3,4-^{3}H]-glutamic acid (44.1 Ci mmol^{-1}; Dupont-New England Nuclear) (Foster & Fagg, 1987a), [1,2-^{3}H]-CPP (20 Ci mmol^{-1}; Tocris Neuramin) (Olverman *et al.*, 1986; Murphy *et al.*, 1987) and [2-^{3}H]-glycine (43.5 Ci mmol^{-1}; Dupont-New England Nuclear) (Bristow *et al.*, 1986; Kessler *et al.*, 1989) was determined essentially according to previously published procedures. Aliquots (40–50 μg protein) of crude PSDs were incubated in triplicate with radioligand (50 nM L-[^{3}H]-glutamate; 25 nM [^{3}H]-CPP; 20 nM [^{3}H]-glycine) in a final volume of 0.5 ml 50 mM Tris-acetate buffer (pH 6.9 for L-[^{3}H]-glutamate; pH 7.8 for [^{3}H]-CPP; pH 7.5 for [^{3}H]-glycine), and membrane-bound radioactivity was measured after centrifugation and aspiration of the supernatant.

Incubations were 30 min at 32°C for L-[^{3}H]-glutamate and [^{3}H]-CPP, and 20 min at 4°C for [^{3}H]-glycine. Non-specific binding was determined in the presence of 0.5 mM L-glutamate, 0.1 mM CPP or 0.5 mM glycine, and was routinely about 15%, 30% and 25% of total binding, respectively. Potential inhibitors were added to the incubation tubes prior to the addition of radioligand. IC_{50} values were determined by fitting data to the logistic equation using an iterative curve fitting procedure (Research System 1; Bolt, Beranek & Newman Inc.; see Foster & Fagg, 1987a) operated on a VAX 8600 computer, and K_i values were calculated from the relationship: $K_i = IC_{50}/(1 + F/K_d)$, where F is the free concentration of radioligand and K_d its equilibrium dissociation constant (K_d values were determined by saturation analyses of the type illustrated in Figure 2; mean values used here were 250 nM for L-[^{3}H]-glutamate, and 130 nM for [^{3}H]-CPP).

Other receptors Radioreceptor assays were conducted by use of procedures which have been established and documented previously (Foster *et al.*, 1981; Honore & Nielsen, 1985; Lehmann *et al.*, 1988). Assays (and radioligands) used were as follows: quisqualate (AMPA), kainate (kainate), $GABA_A$ (muscimol), benzodiazepine (flunitrazepam), $GABA_B$ (CGP 27492), adenosine (cyclohexyladenosine), muscarinic cholinoceptor (diozalane and QNB), α_1-adrenoceptor (prazosin), α_2-adrenoceptor (clonidine), β-adrenoceptor (dihydroalprenolol), 5-HT_1 (5-HT), 5-HT_2 (ketanserin), histamine H_1 (doxepine), histamine H_2 (thiotidine), substance P (substance P) and neurotensin (neurotensin). All assays were validated using standard reference compounds.

Transport studies

The Na^+-dependent uptake of L-[3,4-^{3}H]-glutamate (52.6 Ci mmol^{-1}; Dupont-New England Nuclear) into crude synaptosomal (P_2) fractions isolated from rat brain was investigated by the method described by Yunger *et al.*, (1984) for [^{3}H]-GABA uptake with minor modifications. In brief, the P_2 fraction (recovered as a pellet in 0.32 M sucrose) was suspended in 0.32 M glucose and diluted with Krebs-Ringer bicarbonate buffer (composition in mM: NaCl 118, KCl 4.8, $CaCl_2$ 2.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, final glucose concentration 8, equilibrated with 5% CO_2 in O_2) containing 0.1 mM isoniazid and L-methionine sulphoximine to inhibit the metabolism of L-[^{3}H]-glutamate. Aliquots of this P_2 suspension were preincubated in duplicate (with test compounds as appropriate) at 37°C for 15 min before addition of L-[^{3}H]-glutamate (final concentration 1 μM, except for K_m determinations). 'Blank' tubes were incubated on ice. Incubations were continued for a further 3 min, and were terminated by rapid filtration (Millipore, 0.45 μm pore size) and washing twice with ice-cold buffer. Radioactivity retained on the filter was determined by liquid scintillation counting.

Transmitter release experiments

The electrically-evoked release of endogenous glutamate from rat hippocampal slices was determined by a slight modification of the method described by Waldmeier *et al.* (1988) for the release of endogenous GABA. Cross-chopped slices (about 0.36 × 0.36 × 1 mm) were suspended in a Krebs-Ringer bicarbonate buffer (composition as for Transport studies above, except that the final concentrations of $CaCl_2$ and D-glucose were 1.8 mM and 10 mM, respectively) equilibrated with 5% CO_2 in O_2, and aliquots were transferred to superfusion chambers with platinum ring electrodes above and below the tissue slices. Slices were superfused with buffer (0.25 ml min^{-1}, 37°C) containing 0.5 mM dihydrokainate (to inhibit reuptake) for 1 h before collecting 1.25 ml fractions for amino acid analysis. The glutamate content of the superfusate and of the slices (collected at the end of each experiment) were determined by high performance liquid chromatography (h.p.l.c.) after pre-column derivatization with *o*-phthalaldehyde, and the glutamate released in each fraction was expressed as a percentage of the tissue content at the time of collection (% fractional release).

Previous studies have shown that, in this system, the evoked release of endogenous glutamate is frequency-dependent over the range 2–40 Hz and is entirely Ca^{2+}-dependent (Thedinga *et al.*, 1989). For the experiments described here, two trains of monophasic pulses (10 Hz, 20 mA, 2 ms, 2 min duration) were delivered during fractions 3 (S_1) and 9 (S_2); the first stimulation was always in superfusion buffer alone, and the second was either in the absence or the presence of test compounds (added to the buffer 20 min previously). Drug effects on evoked release were determined by comparing the ratio S_2/S_1 in drug-treated and control slices (Waldmeier *et al.*, 1988). All experiments were conducted in triplicate.

Electrophysiological investigations

In vitro Experiments were performed on transverse hippocampal slices (450 μm) prepared from male Tif:RAIf(SPF) rats (150–200 g weight) as previously described (Olpe & Lynch, 1982). Slices were allowed to stabilize in a humidified interface chamber, and were subsequently perfused (6 ml min^{-1}; equivalent to 2 chamber volumes per min) with a physiological salt solution (composition in mM: NaCl 124,

KCl 2.5, $CaCl_2$ 2.5, $MgSO_4$ 2, KH_2PO_4 1.25, $NaHCO_3$ 26, D-glucose 10; gassed with 5% CO_2 in O_2) at 32°C. Extracellular recordings were made from the stratum pyramidale region of area CA1 by use of glass microelectrodes filled with 4 M NaCl.

Two series of experiments were performed: (1) Agonist-antagonist interactions were investigated by applying submaximal concentrations (10 μM) of NMDA, quisqualate or kainate at intervals of 5–10 min in the presence and absence of test blockers, and by plotting the firing rate of single neurones (action potentials in 5–10 s bins) on a chart recorder. All substances were applied via the perfusion system to submerged hippocampal slices.

(2) The ability of test drugs to block synaptically-evoked epileptiform activity was evaluated using an approach employed by Coan & Collingridge (1985). Stable single population spikes were first elicited in CA1 neurones by stimulation of Schaffer collateral-commissural fibres (submerged slices), and the Mg^{2+} concentration of the perfusion medium was then decreased from 2 mM to nominally 0.1 mM (conditions which reduce the voltage-dependent blockade of the NMDA receptor ion channel). Subsequent sub-maximal stimulation (at a frequency of 0.125 Hz) evoked bursts of population spikes, which previously have been shown to be sensitive to blockade by NMDA receptor antagonists (Coan & Collingridge, 1985; Lester *et al.*, 1988).

In vivo Male rats (Tif:RAIf(SPF), 280–320 g weight) were anaesthetized with chloral hydrate (400 mg kg^{-1}, i.p.) and the tips of four-barrelled micropipettes were stereotaxically positioned in the region of the hippocampal CA1 pyramidal neurones. Three barrels of the pipette contained NMDA, quisqualate and kainate (0.01 M, pH 10) and the fourth recording barrel 4 M NaCl. Excitatory amino acids were applied ionophoretically (currents 20–30 nA; adjusted to yield similar response amplitudes) for periods of 20–60 s at constant intervals of 2–3 min (retaining currents of 20 nA were employed between drug applications). The number of action potentials in 5–10 s bins was plotted on a chart recorder, and responses to ionophoretically-applied agonists were monitored for up to 8 h. Antagonists were dissolved in physiological saline and were administered cumulatively (3 h intervals) via a canula in the oesophagus.

Anticonvulsant evaluation

Convulsions in mice were elicited by maximal electroshock (Bernasconi *et al.*, 1986). Brief current pulses (16 mA, 50 Hz, 0.2 s) were applied via corneal electrodes and the number of animals displaying tonic hind limb extension seizures after saline or drug administration (5 animals per dose group) was determined. Test anticonvulsants were administered orally (via an oesophageal cannula) 2–4 h before electroshock. ED_{50} values (dose which prevented seizures in 50% of animals) were determined by the method of Spearman-Kaerber.

Results

Receptor binding profiles of CGP 37849 and CGP 39551

The high affinity of CGP 37849 for the NMDA receptor was identified on the basis of its activity at NMDA-sensitive L-[^{3}H]-glutamate binding sites in crude PSDs (Foster & Fagg, 1987a; Fagg & Baud, 1988) and subsequently was confirmed using the selective radioligand, [^{3}H]-CPP (Olverman *et al.*, 1986; Murphy *et al.*, 1987). Systematic analyses of a series of ω-phosphono amino acid analogues revealed that CGP 37849 was a highly potent inhibitor of L-[^{3}H]-glutamate binding, with a K_i similar to that of L-glutamate itself. Inhibitory activity was found to be associated with the *trans* configuration of the APPA molecule (not with the naturally-occurring *cis*-isomer) and, as in the case of AP5 (Table 1; Evans *et al.*, 1982) and other NMDA receptor antagonists (Watkins *et al.*, 1990), with the D-stereoisomer (CGP 40116). Saturation analyses indicated that the interaction of CGP 37849 with the L-[^{3}H]-glutamate binding site was of a competitive nature; increasing concentrations of the blocker did not alter the maximum number of binding sites labelled by the radioligand, but progressively decreased the apparent affinity of L-[^{3}H]-glutamate for the receptor (Figure 2).

CGP 37849 inhibited the binding of the selective NMDA receptor antagonist, [^{3}H]-CPP, to rat brain PSDs with a K_i

Table 1 Comparative potencies of CGP 37849, CGP 39551 and related substances as inhibitors of NMDA receptor binding and of maximal electroshock-induced seizures in mice

Substance	K_i (μM), and radioligand: L-Glutamate	CPP	ED_{50} (mg kg^{-1} p.o.) Electroshock
CGP 37849	0.22 ± 0.04	0.035 ± 0.020	21
CGP 40116	0.11 ± 0.04	0.019 ± 0.007	7
CGP 40117	>10	3.2 ± 0.6	>60
trans-APPA	0.39 ± 0.03	NT	107
cis-APPA	32 ± 9	NT	>25
CGP 39551	8.4 ± 3.4	0.31 ± 0.03	4
DL-AP5	1.5 ± 0.2	NT	>600
D-AP5	0.88 ± 0.45	0.26 ± 0.05	NT
L-AP5	29 ± 9	4.9 ± 0.7	NT
DL-AP7	8.9 ± 1.5	NT	>300
CGS 19755	0.84 ± 0.39	0.13 ± 0.02	140[a]
CPP	0.82 ± 0.05	0.17 ± 0.02	73[b]
L-Glutamate	0.20 ± 0.03	0.64 ± 0.05	NT
NMDA	5.3 ± 0.9	7.7 ± 0.4	NT

K_i values for the inhibition of NMDA-sensitive L-[^{3}H]-glutamate and [^{3}H]-CPP binding are means ± s.e.mean of data from 3–5 separate experiments, each conducted in triplicate. ED_{50}s for the suppression of maximal electroshock-induced seizures in mice were determined 2 or 4 h after oral drug administration (5 animals per group; method of Spearman-Kaerber). Data from [a]Lehmann *et al.* (1988) and [b]Lehmann *et al.* (1987; NMDA-evoked seizures in mice). CGP 40116 and CGP 40117 are respectively the D- and L-isomers of CGP 37849; APPA is DL-(E)-2-amino-5-phosphono-3-pentenoic acid; AP7 is 2-amino-7-phosphonoheptanoate; see text for other abbreviations. NT, not tested.

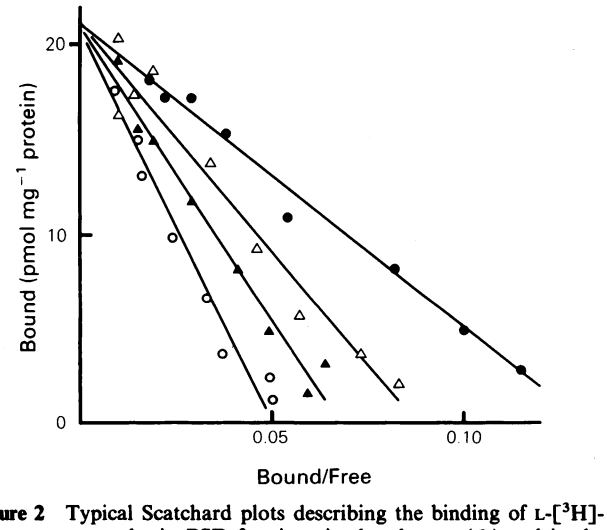


Figure 2 Typical Scatchard plots describing the binding of L-[^{3}H]-glutamate to rat brain PSD fractions in the absence (●) and in the presence of 50 nM (△), 200 nM (▲) and 500 nM (○) CGP 37849. Ordinate scale, pmol L-[^{3}H]-glutamate bound/mg PSD protein; abscissa scale, amount bound/concentration of L-[^{3}H]-glutamate (25–2000 nM). Values are means of triplicate observations.

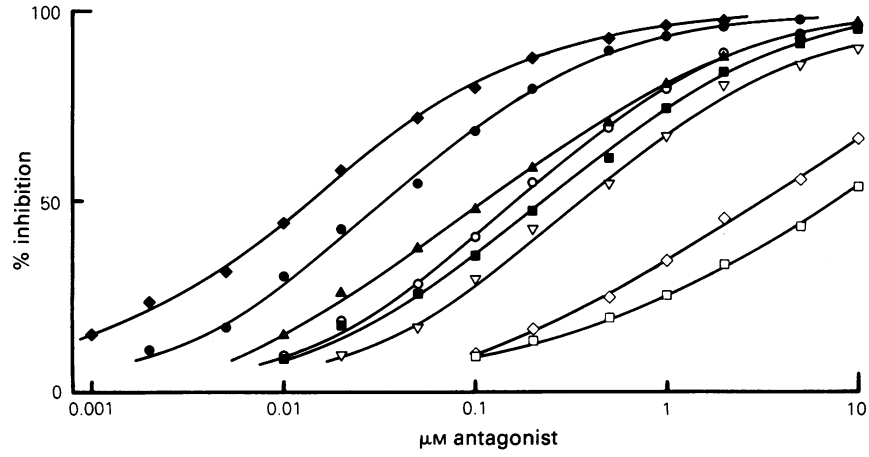


Figure 3 Composite log concentration-response curves illustrating the inhibition of [^{3}H]-CPP binding to rat brain PSD fractions by CGP 40116 (◆), CGP 37849 (●), CGS 19755 (▲), CPP (○), D-AP5 (■), CGP 39551 (▽), CGP 40117(◇) and L-AP5 (□). Values are means of data from 3–5 independent experiments. Mean K_i values (±s.e.mean) determined from these data are shown in Table 1.

of 35 nM (Figure 3 and Table 1), and was thus some 4, 5 and 7 fold more potent than the antagonists CGS 19755, CPP and D-AP5, respectively. As observed when using L-[^{3}H]-glutamate as radioligand, activity was found to reside essentially exclusively in the D-isomer (CGP 40116, K_i 19 nM; Table 1), which showed an affinity roughly 150 fold greater than that of the L-enantiomer (CGP 40117). The ethylester, CGP 39551, showed measurable inhibitory activity at both L-[^{3}H]-glutamate (K_i 8.4 μM) and [^{3}H]-CPP binding sites (K_i 0.31 μM), but was weaker than the free acid, CGP 37849 (Table 1).

Both CGP 37849 and CGP 39551 were weak or inactive in receptor binding assays for 18 other neurotransmitters or modulators. At a concentration of 50 μM, CGP 37849 inhibited strychnine-insensitive [^{3}H]-glycine binding by 26 ± 2% and CGP 39551 by 22 ± 2% (means ± s.e.mean, n = 3). In the remaining 17 receptor assays used (see Methods), both compounds showed 20% inhibition of binding or less at the test concentration of 10 μM. CGP 37849 thus is minimally 300 fold more selective for the NMDA receptor recognition site than for any of the other sites examined.

Effects of CGP 37849 and CGP 39551 on the uptake and release of L-glutamate

Alterations of synaptic function elicited by CGP 37849 and CGP 39551 (see below) potentially might result not simply from their interaction with postsynaptic NMDA receptors, but also from modifications of glutamate uptake or release. In experiments to evaluate these possibilities, no effects of the compounds were observed. As shown by other investigators (Davies & Johnston, 1976; Johnston *et al.*, 1979), L-[^{3}H]-glutamate was accumulated with high affinity (K_m 11 μM) by rat brain synaptosomes and its uptake was inhibited by the reference compounds, D-aspartate and dihydrokainate (IC_{50}s 7 μM and 315 μM, respectively; mean values, 2 separate experiments). However, neither CGP 37849 nor CGP 39551, at concentrations of 1–1000 μM, impaired L-[^{3}H]-glutamate uptake. For example, in the presence of 10 μM CGP 37849 or CGP 39551, L-[^{3}H]-glutamate uptake was respectively 104% and 100% of control values, and in the presence of 1 mM drug, 102% and 106%, respectively (means, 2 separate experiments, each in duplicate).

Similarly, neither CGP 37849 nor CGP 39551 modified the electrically-evoked release of endogenous glutamate from rat hippocampal slices. In these experiments, basal glutamate release routinely was in the range 0.2–0.4% of the tissue content per fraction, and this was increased 2.5 ± 0.2 times by 10 Hz electrical field stimulation. Under control conditions, the ratio S_2/S_1 (release evoked by the second stimulation/release evoked by the first stimulation) was close to unity (1.2), and this ratio was not altered by inclusion of 10 μM CGP 37849 (1.1) or CGP 39551 (1.0) in the superfusion medium (mean values, 2 separate experiments, each in triplicate).

Neuropharmacological actions of CGP 37849 and CGP 39551 in vitro

Agonist-antagonist interaction Electrophysiological studies using the hippocampal slice preparation indicated that CGP 37849 showed no excitatory activity, but at concentrations as low as 0.3 μM antagonized the increases in CA1 pyramidal cell firing rate induced by sub-maximal concentrations of NMDA (Figure 4a). This effect was rapid in onset and in offset, and was agonist-selective, in that responses evoked by quisqualate and kainate were unaffected. Thus, the potency and selectivity of CGP 37849 as an antagonist of NMDA receptor responses is in good agreement with its mechanism of action as determined from radioligand binding studies.

Epileptiform activity Stimulation of Schaffer collateral-commissural fibres in hippocampal slices bathed in low Mg^{2+}-containing medium previously has been shown to evoke multiple population spikes in CA1 pyramidal neurones that are reminiscent of an epileptiform burst (Coan & Collingridge, 1985; Lester *et al.*, 1988; see Figure 4b,i). Bath application of CGP 37849 at concentrations up to 10 μM markedly suppressed this type of repetitive firing, while having no effect on the first population spike of the burst (Figure 4b,ii). This effect was rapidly reversible, with the control pattern of bursting being restored within 10 min following drug wash-out (Figure 4b,iii). CGP 39551 also suppressed stimulus-induced burst firing in this experimental paradigm, but was weaker than CGP 37849 (not shown).

Anticonvulsant properties of CGP 37849 and CGP 39551

In contrast to AP5, AP7, CPP and CGS 19755, which were ineffective anticonvulsants when given orally, CGP 37849 suppressed maximal electroshock-induced seizures in mice following oral administration with an ED_{50} of 21 mg kg^{-1} (Table 1). In agreement with radioligand binding studies at the NMDA receptor (see above), anticonvulsant activity was found to reside in the D-stereoisomer (CGP 40116; ED_{50} 7 mg kg^{-1}). Unlike the investigations *in vitro*, however, the ethylester of CGP 37849 (CGP 39551; ED_{50} 4 mg kg^{-1}) was more potent than the free acid. Details of the anticonvulsant properties of CGP 37849 and CGP 39551 will be published elsewhere.

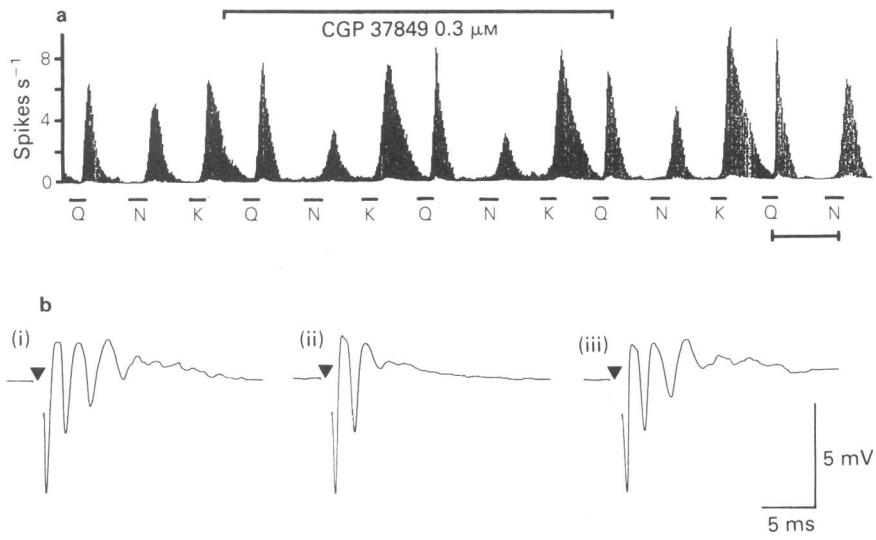


Figure 4 (a) Ratemeter record showing the effect of 0.3 μM CGP 37849 on the firing of CA1 pyramidal neurones in the hippocampal slice in response to 10 μM NMDA (N), quisqualate (Q) and kainate (K). Time bar corresponds to 10 min. (b) Multiple population spikes recorded extracellularly from CA1 neurones in the hippocampal slice (low Mg^{2+}-containing medium) in response to low-frequency stimulation of Schaffer collateral-commissural fibres: (i) control; (ii) 10 min after addition of 2 μM CGP 37849 to the bathing medium; (iii) after drug wash-out. Arrow-heads indicate the position of the stimulus artefact, which has been removed for clarity. Both experiments were repeated 5 times with similar results.

Antagonism of NMDA-evoked responses by CGP 37849 and CGP 39951 in vivo

Since CGP 37849 and CGP 39551 exhibited anticonvulsant activity following oral administration, it was of interest, in terms of understanding their mechanism of action *in vivo*, to determine whether they antagonized NMDA-evoked responses after such treatments. Figure 5 shows that this is indeed the case. At doses some 5–7 times greater than their oral ED_{50}s as anticonvulsants, both drugs selectively blocked the responses of hippocampal neurones to ionophoretically-applied NMDA, without affecting responses to quisqualate or kainate (or acetylcholine, not shown). For CGP 37849, the onset of this effect was 30–40 min after administration, with almost complete blockade of NMDA-evoked responses after 75–85 min. CGP 39551 showed a longer latency to onset;

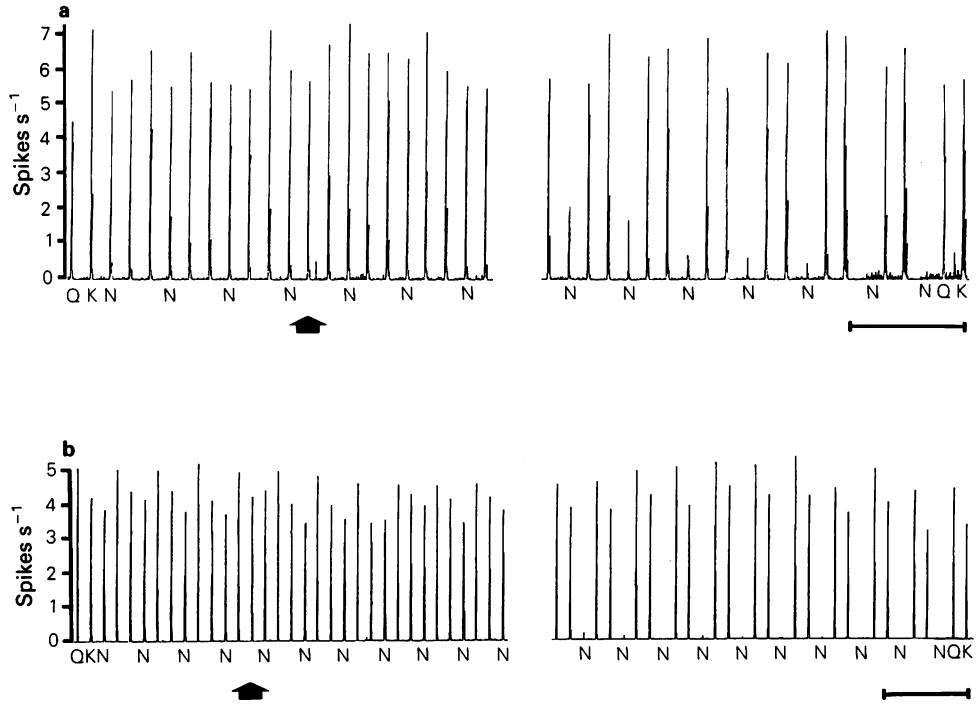


Figure 5 Ratemeter records showing the effects of (a) CGP 37849 100 mg kg^{-1} and (b) CGP 39551 30 mg kg^{-1} on the firing of rat hippocampal neurones *in vivo* in response to cycles of ionophoretically-applied NMDA (N), quisqualate (Q) and kainate (K). For clarity, only the application of NMDA (N) is indicated after the first cycle. Drugs were administered via an oesophagoeal cannula at the arrows. The breaks in the traces correspond approximately to (a) 30 min and (b) 2.5 h. Time bars represent 10 min. The experiments were repeated (a) 4 times and (b) 5 times with similar results.

clear antagonism of NMDA responses was first observed 80–90 min after oral dosage, and responses were essentially abolished after 110–120 min.

Discussion

CGP 37849 (or 4-methyl-APPA) is a novel competitive antagonist at the NMDA-preferring sub-type of excitatory amino acid receptor and, together with its carboxyethylester (CGP 39551), is the first agent of this pharmacological class reported to show significant central effects following oral administration in animals. The NMDA receptor-active properties of CGP 37849 were identified on the basis of its high affinity for L-[^{3}H]-glutamate binding sites in rat brain PSDs (predominantly NMDA-sensitive sites, see Foster & Fagg, 1987a; Fagg & Baud, 1988) and were confirmed using the selective NMDA receptor radioligand, [^{3}H]-CPP (Olverman *et al.*, 1986; Murphy *et al.*, 1987). In the hippocampal slice preparation, it showed no agonist activity, but antagonized NMDA-evoked increases in neuronal firing at concentrations within the range shown to inhibit radioligand binding to the NMDA receptor recognition site; in addition, its ability to suppress burst firing in CA1 pyramidal neurones without affecting the fast excitatory postsynaptic potential (e.p.s.p.) or population spike is in accordance with the properties of NMDA receptor antagonists as established by other investigators (Collingridge *et al.*, 1983; Coan & Collingridge, 1985; Lester *et al.*, 1988). The selectivity of CGP 37849 was indicated by its negligible interactions with binding sites for a range of other neurotransmitter receptors, including those for the quisqualate and kainate excitatory receptor sub-types, while its weak activity at strychnine-insensitive glycine binding sites provided further evidence that its primary locus of action on the NMDA receptor was indeed the transmitter recognition site. Additional demonstrations of its selectivity were obtained from electrophysiological studies in the hippocampus *in vitro* and *in vivo*, in which it failed to affect neuronal responses to applied quisqualate or kainate at doses which reduced or abolished responses to NMDA. With a K_i of 35 nM at [^{3}H]-CPP binding sites, CGP 37849 thus shows a higher affinity and selectivity for the NMDA receptor recognition site than any compound described to date.

NMDA receptor antagonists have been shown to exhibit anticonvulsant activity following central or systemic (i.p. or i.v.) administration to a variety of animal species (see Meldrum, 1985; Lehmann *et al.*, 1987; 1988; Patel *et al.*, 1988), and the oral efficacy of CGP 37849 was initially discerned from its ability to prevent maximal electroshock-induced seizures in mice. Its potency in this respect (ED_{50} 21 mg kg^{-1}, p.o.) is within a therapeutically-useful dose-range as compared with established anticonvulsant drugs (see Meldrum & Porter, 1986), and was further augmented by esterification of the carboxyl moiety (yielding CGP 39551, ED_{50} 4 mg kg^{-1}, p.o.) (detailed anticonvulsant profiles for CGP 37849 and CGP 39551 will be published elsewhere). That these compounds are indeed acting centrally as NMDA receptor antagonists following oral administration was suggested by their observed enantiomeric selectivity (D-isomer active, as for all known NMDA receptor antagonists; see Watkins *et al.*, 1990), and was more directly demonstrated by microionophoretic experiments in the rat hippocampus *in vivo*. At doses some 5–7 fold higher than their oral ED_{50}s as anticonvulsants, both compounds were shown to abolish neuronal responses to NMDA, without affecting those to quisqualate, kainate or acetylcholine. The onset of action of CGP 39551 in this regard was somewhat slower than that of CGP 37489. This observation, coupled with the weaker action of CGP 39551 *in vitro* and current understanding of structure-activity relationships at the NMDA receptor (Fagg & Baud, 1988; Watkins *et al.*, 1990), may indicate that the esterified compound functions as a more-readily absorbed pro-drug of CGP 37849 *in vivo*. However, definitive proof of the precursor-product relationship of these two substances *in vivo* is required to verify this hypothesis.

CGP 37849 and CGP 39551 are the first competitive NMDA receptor antagonists reported to show significant central effects following oral administration in animals, and hence are candidates for the treatment of neurological disorders in man which require either acute or chronic drug administration. Whilst extensive discussion is outside the realms of this paper, it is appropriate to consider some of the issues which must be resolved before this goal can be realized. Major questions have centred on the therapeutic window of NMDA receptor antagonists (loss of muscle tone occurs at sufficient dosage; see Turski *et al.*, 1988), their possible psychotomimetic effects (based on experience in man with non-competitive blockers such as PCP and ketamine; see Domino & Luby, 1981; Lodge *et al.*, 1988), and their potentially detrimental effects on learning (Morris *et al.*, 1986; Collingridge & Bliss, 1987). In this respect, recent evidence indicates that competitive NMDA receptor antagonists may have significant advantages over non-competitive blockers (Meldrum, 1985; Ferkany *et al.*, 1988; Leander *et al.*, 1988; France *et al.*, 1989; Tricklebank *et al.*, 1989). Studies on the novel competitive NMDA receptor antagonists described here indicate, especially in the case of CGP 39551, a therapeutic window at least as great as clinically-used anticonvulsant agents (Fagg *et al.*, 1989a; Schmutz *et al.*, unpublished observations), no direct impairments of learning performance in the anticonvulsant dose-range (Mondadori, Buerki & Petschke, unpublished), and no generalization to the discriminative stimulus effects of ketamine or MK801 in rhesus monkeys (France & Woods, personal communication). Pending favourable toxicological profiles, they are thus leading candidates for clinical testing in man.

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Inhibition of neutrophil and eosinophil induced chemotaxis by nedocromil sodium and sodium cromoglycate

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1 Neutrophils and eosinophils infiltrate the airways in association with the allergen-induced late phase asthmatic reaction. Mobilization of these cells takes place via lipid-like and protein-like chemotactic factors. In this study platelet-activating factor (PAF), leukotriene B_4 (LTB_4), zymosan-activated serum (ZAS) and N-formyl-methionyl-leucyl-phenylalanine (FMLP) were used as illustrative examples of both groups. Chemotaxis was studied in human neutrophils and eosinophils. The inhibitory effects of nedocromil sodium and sodium cromoglycate were evaluated.

2 All chemotactic factors tested attracted neutrophils with the following rank order of activity: ZAS > PAF ≡ FMLP ≡ LTB_4. Eosinophils were only mobilized by PAF, LTB_4 and ZAS with the following rank order of activity: ZAS > PAF > LTB_4.

3 Nedocromil sodium and sodium cromoglycate were equally active as the PAF antagonist BN 52021 in inhibiting the PAF-induced chemotaxis of neutrophils ($IC_{50} \approx 10^{-8}$ M). Both drugs were also equally active in inhibiting the chemotaxis of neutrophils induced by ZAS ($IC_{50} \approx 10^{-7}$–10^{-6} M), FMLP ($IC_{50} \approx 10^{-7}$ M) and LTB_4 ($IC_{50} \approx 10^{-6}$ M).

4 Nedocromil sodium significantly inhibited the chemotaxis of eosinophils induced by PAF ($IC_{50} \approx 10^{-6}$ M) and LTB_4 ($IC_{50} \approx 10^{-7}$ M). The inhibitory potency of BN 52021 was similar to that of nedocromil sodium on the PAF-induced chemotaxis of eosinophils. Sodium cromoglycate was incapable of eliciting significant inhibition of these chemotactic responses. However, sodium cromoglycate significantly inhibited the chemotaxis of eosinophils induced by ZAS ($IC_{50} \approx 10^{-7}$ M), whereas nedocromil sodium was ineffective.

Introduction

When allergic asthmatic individuals are challenged with allergen an early phase asthmatic reaction (within 30 min of challenge) and a late phase reaction (4–6 h after challenge) may develop. The cellular events taking place during this late phase reaction are considered highly relevant to the pathogenesis of asthma (Durham & Kay, 1985; de Monchy, 1986). Bronchoalveolar lavage (BAL) studies of the cellular changes during this response have demonstrated the presence of inflammatory cells (e.g. eosinophils and neutrophils) (de Monchy *et al.*, 1985; Metzger *et al.*, 1985; Diaz *et al.*, 1987), indicating active migration of these cells into the bronchial lumen. Inflammatory cells may be mobilized by chemotactic agents of protein- or lipid-nature such as neutrophil chemotactic factor, eosinophil chemotactic factor, complement fragments such as C5a, leukotriene B_4 (LTB_4) and platelet-activating factor (PAF) (Shaw *et al.*, 1981; Atkins & Wasserman, 1983). Although none of these factors is specifically chemotactic for either neutrophils or eosinophils, evidence has been put forward that PAF is a more selective chemotactic agent for eosinophils (Wardlaw *et al.*, 1986). Most studies comparing the chemotactic activity of neutrophils and eosinophils have used eosinophils from hypereosinophilic donors (Udén *et al.*, 1983; Wardlaw *et al.*, 1986). Since it has been shown that these eosinophils have an altered metabolic activity they may also show a different chemotactic behaviour (Pincus *et al.*, 1981; Udén *et al.*, 1983; Prin *et al.*, 1984; Wardlaw *et al.*, 1986). Recently we have developed an isolation procedure for eosinophils from the blood of healthy volunteers which gives relatively high recovery and purity (Koenderman *et al.*, 1988). This enabled us to compare the chemotactic response of neutrophils and eosinophils from the same healthy volunteers to the following chemotactic compounds: PAF, zymosan-activated serum (ZAS), N-formyl-methionyl-leucyl-phenylalanine (FMLP) and LTB_4. In our hands ZAS was the most powerful chemoattractant for neutrophils and eosinophils. With the exception of FMLP which showed hardly any chemoattractant activity towards eosinophils, the other chemotaxins had lower activity than ZAS but were approximately equiactive with each other. The neutrophils always showed a much stronger chemotactic response than the eosinophils. In addition, we evaluated the effects of nedocromil sodium and sodium cromoglycate on migratory responses elicited by optimum concentrations of the chemotaxins.

Methods

Purification of granulocytes

Granulocytes were isolated as follows. Platelet-rich plasma was removed after centrifugation of citrated blood (15 min, 275 g_{max}, room temperature). After collection of the buffy coat, granulocytes were separated from lymphocytes and monocytes by centrifugation on Ficoll-Paque (20 min, 1000 g_{max}, room temperature). The remaining erythrocytes were removed by ammonium chloride lysis at 0°C and subsequent centrifugation. Thereafter the cells were allowed to recover for 30 min at 37°C in minimal essential medium containing 10% foetal calf serum. After centrifugation the mixed granulocyte preparation was resuspended in buffer A (composition: phosphate buffered saline/trisodium citrate (38 g l^{-1})/human pasteurised plasma protein (protein: 40 g l^{-1}; sodium caprylate: 4 mmol l^{-1}; sodium: 130–160 mmol l^{-1}) = 80/10/10, v/v/v) at a concentration of 75×10^6 cells ml^{-1}.

Purification of neutrophils

One millilitre of the mixed granulocytes (containing 75×10^6 cells) was carefully layered on 4 ml of an isotonic Percoll solution (density: 1.082 g ml^{-1}) and centrifuged (10 min, 1000 g_{max},

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room temperature). Neutrophils (about 98% pure, recovery > 85%), separated at the top of the 1.082 g ml^{-1} Percoll solution. These neutrophils were collected, washed in buffer A and resuspended in Gey's buffer (composition: Gey's balanced salt solution/human serum albumin (5%, w/v)/heparin (10 iu ml^{-1})/glucose (1 mg ml^{-1})).

Purification of eosinophils

The remainder of the mixed granulocyte preparation (containing 75 × 10^6 cells ml^{-1} in buffer A) was first incubated with FMLP (10 nM) in buffer A for 10 min at 37°C before 1 ml of cell suspension was layered on 4 ml of an isotonic Percoll solution (density: 1.082 g ml^{-1}). To prevent contamination of the eosinophils with cell debris and remaining erythrocytes, 1 ml of Percoll (density: 1.100 g ml^{-1}) was brought under the Percoll layer of 1.082 g ml^{-1}. After centrifugation (10 min, 1000 g_{max}, room temperature) eosinophils with a purity of over 85% (recovery > 50%) were present at the interface of both Percoll layers. These eosinophils were collected, washed with buffer A and resuspended in Gey's buffer. As described elsewhere (Koenderman *et al.*, 1988), the eosinophils isolated in this way are not 'primed' or 'activated'. The cell viability of both neutrophils and eosinophils was >99%.

Chemotaxis assay

Chemotaxis was measured with a 48-well microchemotaxis chamber (Neuro Probe, Cabin John, MD, U.S.A.). Chemoattractants or Gey's buffer (30 μl) were placed in the lower wells. Two filters were placed between the upper and lower compartment. The lower filter had a pore width of 0.45 μm (Millipore) and the upper filter had a pore width of 8 μm (Sartorius). The filters had previously been soaked in Gey's buffer. Purified neutrophils and eosinophils were placed in the upper compartment of the control and test wells (10^5 cells in 25 μl for each well). Concentration ranges of the chemotactic agents PAF, lyso-PAF, ZAS, FMLP and LTB_4 were tested using the buffer as a control. At optimal concentrations of these chemotactic agents, dose-response inhibition curves for nedocromil sodium and sodium cromoglycate were constructed. In the case of PAF-induced chemotaxis, dose-response inhibition curves for BN 52021 were also included. The cells were preincubated for 30 min with the drugs before the chemotaxis assay was carried out. The chambers were incubated for 1.5 h at 37°C for neutrophils and for 2.5 h at 37°C for eosinophils. Thereafter, the filters were removed, fixed in butanol/ethanol (20/80, v/v) for 10 min and stained with Weigerts solution (composition: 1% haematoxylin (v/v) in 95% ethanol (v/v) and an acidic $FeCl_3$-solution (70 mM) mixed in a volume ratio of 1:1). The filters were dehydrated with ethanol, made transparent with 100% xylene (v/v) and fixed upside down. The number of cells per high power field (hpf) was determined with light microscopy (magnification 400×). In this way, the number of cells that had passed the upper filter was determined.

Statistical analysis

All data are presented as mean ± s.e.mean. Before statistical analysis the buffer controls were subtracted from the data. Thereafter statistical analysis was performed using the Student's *t* test for paired samples. *P* values < 0.05 were considered significant. Drug IC_{50} values were calculated with a polynomial fit programme.

Materials

1-O-hexadecyl/octadecyl-2-O-acetyl-sn-glycero-3-phosphorylcholine (PAF) and 1-O-hexadecyl-sn-glycero-3-phosphorylcholine (lyso-PAF) were purchased from Bachem (Bubendorf, Switzerland) and were stored at −20°C in a toluene/ethanol (1:1, v/v) solution under N_2. Zymosan A, N-formyl-methionyl-leucyl-phenylalanine (FMLP) and leukotriene B_4 (LTB_4) were purchased from Sigma (St. Louis, MO, U.S.A.). Synthetic LTB_4 was also obtained from Dr J. Rokach (Merck-Frosst Laboratories, Pointe Claire-Dorval, Quebec, Canada). The purity of both LTB_4 products was checked (>98%) by means of reversed phase h.p.l.c. as described by Verhagen *et al.* (1984). Zymosan activated serum (ZAS) was prepared as described previously (Goldstein *et al.*, 1975). Ficoll-Paque (1.077 g ml^{-1}) and Percoll (1.129 g ml^{-1}) were obtained from Pharmacia (Uppsala, Sweden). Nedocromil sodium and sodium cromoglycate were provided by Fisons plc (Loughborough, Leicestershire). BN 52021 was a kind gift of Dr P. Braquet (Institut Henri Beaufour, Le Plessis Robinson, France). All other materials were reagent grade. Human blood was obtained from healthy volunteers at the Red Cross Bloodbank Foundation (Utrecht).

Results

Dose-response curves for chemotaxis of neutrophils and eosinophils

The concentration-dependency of PAF-, lyso-PAF-, ZAS-, FMLP- and LTB_4-induced chemotaxis of isolated neutrophils and eosinophils is depicted in Figures 1–4. In the case of neutrophils all compounds except lyso-PAF induced significant chemotaxis. In the case of eosinophils, all compounds with the exception of lyso-PAF and FMLP induced significant chemotaxis. The optimal chemotactic concentrations for neutrophils were for PAF: 1 μM, for ZAS: a dilution of 1/3, for FMLP: 10 nM and for LTB_4: 1 μM. With eosinophils the optimal chemotactic concentrations were for PAF: 1 μM, for ZAS: a dilution of 1/3 and for LTB_4: 1 μM. For neutrophils the order of activity determined by the maximum response to the tested chemotactic compounds was: ZAS > PAF ≡ FMLP ≡ LTB_4. For eosinophils the order of activity was: ZAS > PAF > LTB_4. In our first series of experiments ZAS was a very powerful chemoattractant for eosinophils, whereas in later experiments it had a much weaker chemotactic potency (see Figures 2 and 6). Since the shape of the ZAS concentration curve with both ZAS batches was similar, we used all data obtained. This however resulted in large standard errors of the mean (see Figure 2). The dose-response curve for the FMLP-induced chemotaxis of eosinophils (see Figure 3) suggests significant chemotaxis of eosinophils. This measured chemotaxis however can be completely attributed to neutrophil contamination of the eosinophil cell preparation. It is noteworthy that all chemotactic agents tested attracted neutrophils more powerfully than eosinophils. At the optimal chemotactic concentration this ratio was generally 2–3.

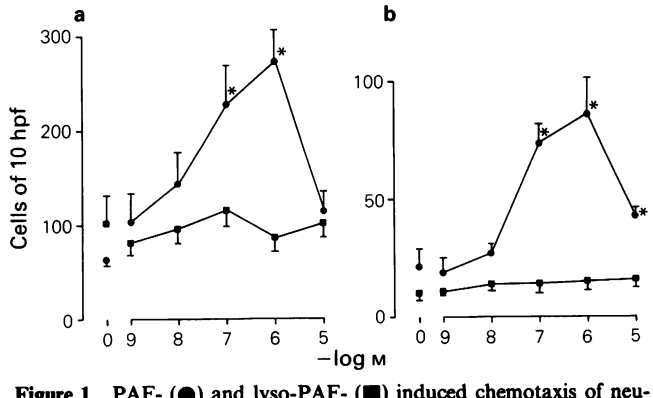


Figure 1 PAF- (●) and lyso-PAF- (■) induced chemotaxis of neutrophils (a) and eosinophils (b) with buffer used as a control ($n = 12$; $^*P < 0.05$). The cell purities were 98 ± 1% for neutrophils and 90 ± 2% for eosinophils. In this and subsequent figures, hpf = high power field.

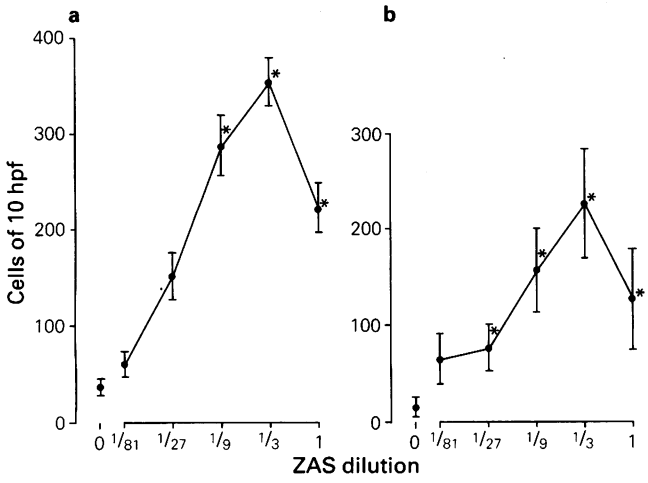


Figure 2 Zymosan-activated serum (ZAS)-induced chemotaxis of neutrophils (a) and eosinophils (b) with buffer used as a control ($n = 12$; $^*P < 0.05$). The cell purities were 98 ± 2% for neutrophils and 89 ± 2% for eosinophils. In cases where heat-inactivated serum was used instead of buffer, similar control values were obtained.

Inhibition experiments with nedocromil sodium and sodium cromoglycate

Inhibition with PAF as chemotaxin In further experiments we investigated the inhibitory activities of sodium cromoglycate and nedocromil sodium against optimum concentrations of the leucocyte chemotaxins. In addition, the effect of the PAF antagonist BN 52021 was also evaluated.

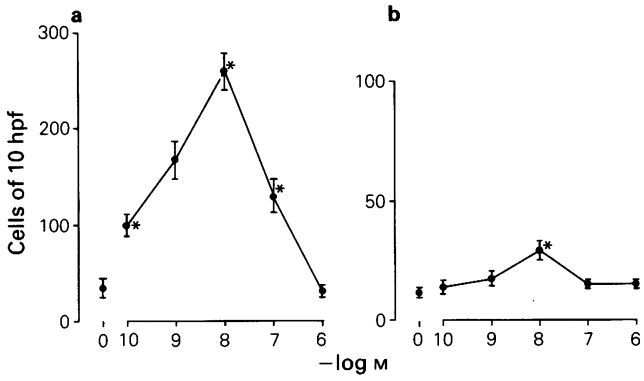


Figure 3 N-formyl-methionyl-leucyl-phenylalanine (FMLP)-induced chemotaxis of neutrophils (a) and eosinophils (b) with buffer used as a control ($n = 12$; $^*P < 0.05$). The cell purities were 98 ± 1% for neutrophils and 90 ± 2% for eosinophils.

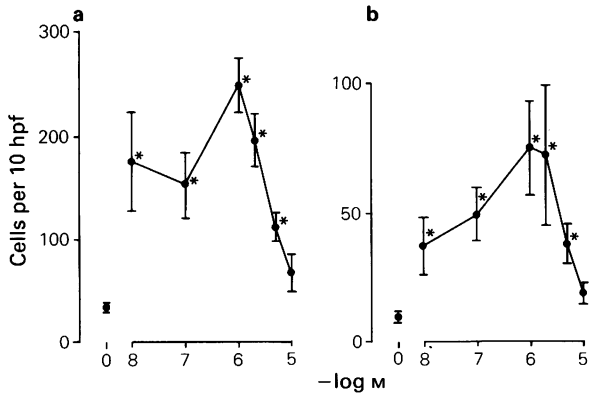


Figure 4 Leukotriene B_4 (LTB_4)-induced chemotaxis of neutrophils (a) and eosinophils (b) with buffer used as a control ($n = 8$; $^*P < 0.05$). The cell purities were 98 ± 1% for neutrophils and 90 ± 1% for eosinophils.

The inhibition of the PAF-induced chemotactic response of neutrophils and eosinophils by nedocromil sodium, sodium cromoglycate and BN 52021 is shown in Figure 5. All drugs tested significantly inhibited the PAF-induced chemotactic response of neutrophils. The IC_{50} for nedocromil sodium was 7.3×10^{-9} M, for sodium cromoglycate 1.8×10^{-8} M and for BN 52021 9.0×10^{-9} M. In the case of eosinophils, sodium cromoglycate hardly inhibited the PAF-induced chemotaxis. The IC_{50} for nedocromil sodium was 1.3×10^{-6} M, for BN 52021 1.3×10^{-6} M.

Inhibition with zymosan-activated serum as chemotaxin The inhibition of the ZAS-induced chemotaxis of neutrophils and eosinophils by nedocromil sodium and sodium cromoglycate is shown in Figure 6. Both drugs significantly inhibited the ZAS-induced chemotactic response of neutrophils. The IC_{50} for nedocromil sodium was 7.8×10^{-8} M for sodium cromoglycate 1.2×10^{-6} M. Only sodium cromoglycate inhibited the ZAS-induced chemotaxis of eosinophils significantly. The IC_{50} for sodium cromoglycate was 1.5×10^{-7} M.

Inhibition with FMLP as chemotaxin The inhibition of the FMLP-induced neutrophil and eosinophil chemotaxis by nedocromil sodium was 2.4×10^{-7} M, and for sodium cro-
Figure 7. Both drugs significantly inhibited the FMLP-induced chemotactic response of neutrophils. The IC_{50} for nedocromil sodium was 2.4×10^{7} M, and for sodium cromoglycate 2.5×10^{-7} M. In the case of eosinophils the FMLP-induced chemotactic response could be accounted for by the presence of contaminating neutrophils.

Inhibition with leukotriene LTB_4 as chemotaxin The inhibition of the LTB_4-induced chemotaxis of neutrophils and eosinophils by nedocromil sodium and sodium cromoglycate is shown in Figure 8. Both drugs significantly inhibited the LTB_4-induced chemotactic response of neutrophils. The IC_{50} for nedocromil sodium was 1.1×10^{-6} M and for sodium cromoglycate 7.3×10^{-7} M. With eosinophils there was little inhibition of LTB_4-induced chemotaxis by sodium cromoglycate,

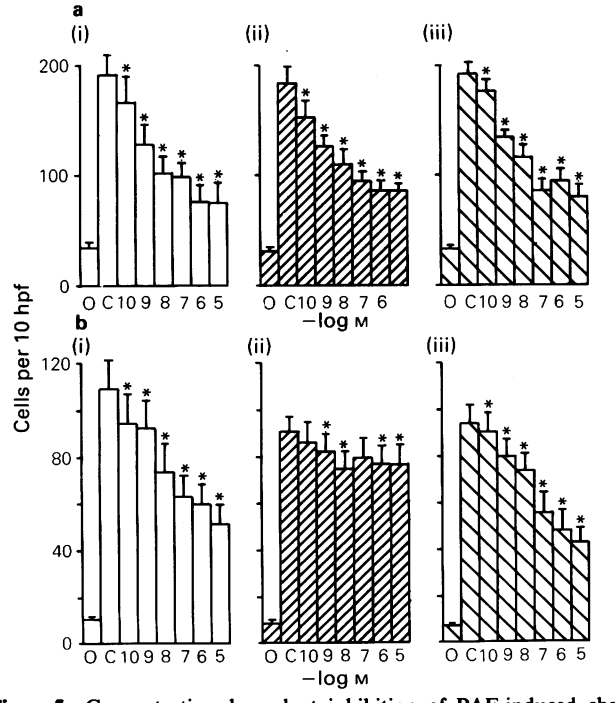


Figure 5 Concentration-dependent inhibition of PAF-induced chemotaxis of neutrophils (a) and eosinophils (b) by nedocromil sodium (i), sodium cromoglycate (ii) and BN 52021 (iii). O: buffer; C: chemotaxis obtained with PAF (1 μM) ($n = 11$; $^*P < 0.05$ when compared with the control value). The cell purities were 97 ± 1% for neutrophils and 85 ± 3% for eosinophils.

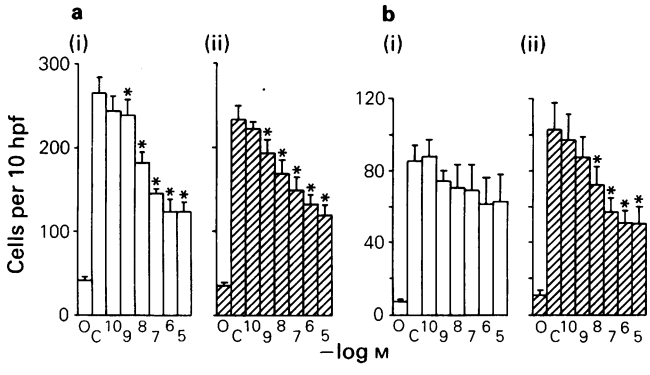


Figure 6 Concentration-dependent inhibition of zymosan-activated serum (ZAS)-induced chemotaxis of neutrophils (a) and eosinophils (b) by nedocromil sodium (i) and sodium cromoglycate (ii). O: buffer; C: chemotaxis obtained with a ZAS dilution 1/3 ($n = 6$; * $P < 0.05$ when compared with the control value). The cell purities were 96 ± 2% for neutrophils and 90 ± 2% for eosinophils. In cases where heat-inactivated serum was used instead of buffer, similar control values were obtained.

whereas nedocromil sodium was active with an IC_{50} of 9.4×10^{-8} M.

In order to ensure that the inhibitory action of the drugs was due to their interaction with the cells and not with the chemoattractant, experiments were carried out in which the drug was added to the compartment which contained the chemoattractants. For all chemoattractants tested there was no influence on the chemotactic response (data not shown).

Furthermore, we tested whether a preincubation time of 30 min of the cells with the drugs under study was sufficient to obtain maximal inhibition. Generally an inhibition of the chemotactic response of over 80% was reached after this preincubation time. Since a plateau was reached, a preincubation time of 30 min was used in all subsequent studies.

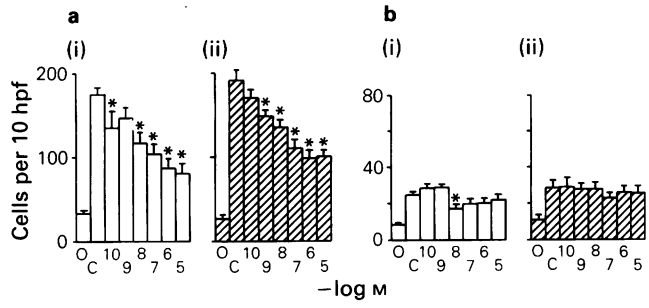


Figure 7 Concentration-dependent inhibition of N-formyl-methionyl-leucyl-phenylalanine (FMLP)-induced chemotaxis of neutrophils (a) and eosinophils (b) by nedocromil sodium (i) and sodium cromoglycate (ii). O: buffer; C: chemotaxis obtained with FMLP (10 nM) ($n = 6$; * $P < 0.05$ when compared with the control value). The cell purities were 96 ± 2% for neutrophils and 84 ± 4% for eosinophils.

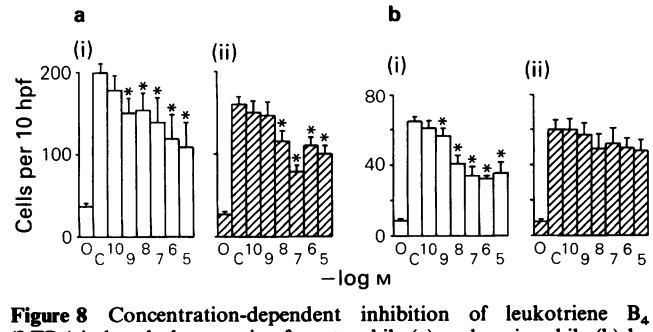


Figure 8 Concentration-dependent inhibition of leukotriene B_4 (LTB_4)-induced chemotaxis of neutrophils (a) and eosinophils (b) by nedocromil sodium (i) and sodium cromoglycate (ii). O: buffer; C: chemotaxis obtained with LTB_4 (1 μM) ($n = 6$; $P < 0.05$ when compared with the control value). The cell purities were 96 ± 1% for neutrophils and 87 ± 2% for eosinophils.

Discussion

The influx of inflammatory cells such as neutrophils and eosinophils into the lung during the allergen-induced late phase asthmatic responses is considered to be of great importance for the pathogenesis of asthma. The factors responsible for this influx are not yet fully characterized. However, most probably they are chemotactic factors of lipid- or protein-nature. In this study we have investigated the *in vitro* induced chemotactic response of purified neutrophils and eosinophils from human blood towards PAF, ZAS, FMLP and LTB_4 as representatives of some of those factors. FMLP proved to be a rather specific chemotactic factor for human neutrophils. Eosinophils of healthy volunteers do not seem to respond to FMLP as a chemoattractant, possibly because these cells lack FMLP binding sites (Roberts & Gallin, 1985). All the other chemoattractants induced significant neutrophil and eosinophil chemotaxis. Generally, the neutrophil-induced chemotaxis was two to three fold higher than the eosinophil-induced chemotactic response. In case of neutrophils the rank order of chemotactic activity was: ZAS > PAF ≡ FMLP ≡ LTB_4; for eosinophils the rank order of potency was: ZAS > PAF > LTB_4.

The results of our study contrast with those of Wardlaw *et al.* (1986), who found that PAF was a specific and powerful chemotactic agent for eosinophils with a similar chemotactic potency for both eosinophils and neutrophils. They also showed that LTB_4 had hardly any chemotactic activity towards eosinophils. In our hands, PAF and LTB_4 were almost equally potent in attracting eosinophils. This difference may be due to the fact that eosinophils from donors with eosinophilia were used by Wardlaw and colleagues. These eosinophils may have undergone membrane changes resulting in an increased expression of high affinity PAF receptor subtypes responsible for chemotaxis (Hwang, 1988; Fukuda *et al.*, 1989) and a decreased expression of the high affinity LTB_4 receptor subtype responsible for chemotaxis. The latter may have been compensated for by an increased low affinity LTB_4 receptor subtype expression responsible for chemiluminescence (Palmblad *et al.*, 1984; Goldman & Goetzl, 1984). Therefore we feel that our results once more underline that eosinophils from normal healthy volunteers may have completely different characteristics from those of individuals with moderate or severe eosinophilia.

The inhibition data of the PAF-induced chemotaxis of neutrophils with nedocromil sodium, sodium cromoglycate and BN 52021 are in agreement with those previously obtained (Bruijnzeel *et al.*, 1989). In the case of eosinophils, nedocromil sodium and BN 52021 showed similar inhibitory capacity, whereas sodium cromoglycate was hardly active. These results contrast with those of Kurihara *et al.* (1988), who found that the PAF-induced chemotaxis of neutrophils and eosinophils could only be inhibited by BN 52021 and not by nedocromil sodium and sodium cromoglycate. The inhibition of LTB_4-induced chemotaxis of neutrophils and eosinophils by nedocromil sodium and sodium cromoglycate were similar to those with the PAF-induced chemotaxis. Therefore, these results suggest that nedocromil sodium may be a powerful inhibitor of the PAF- and LTB_4-induced mobilization of both neutrophils and eosinophils, whereas sodium cromoglycate is only active on neutrophils. Interestingly, the inhibition of ZAS-induced mobilization of neutrophils and eosinophils by nedocromil sodium and sodium cromoglycate indicated that both drugs were active on neutrophils whereas only sodium cromoglycate was active on eosinophils. Thus, our results could suggest that nedocromil sodium acts on eosinophil chemotaxis by lipid factors like PAF and LTB_4, whereas sodium

cromoglycate acts on eosinophil chemotaxis induced by protein-like factors like C3a and C5a. This will have to be further substantiated by *in vivo* investigations.

The inhibition of FMLP-induced neutrophil chemotaxis did not show any difference in inhibitory activity between the two drugs.

Our experiments do not indicate the mechanism of action of the cromones, but we can exclude the possibility that they act by inhibiting the rise in cytosolic free Ca^{2+} elicited by PAF or ZAS (Bruijnzeel *et al.*, 1989). However, these findings do not contradict the earlier view that both drugs interfere with post-receptor events such as at the level of protein kinase C activity (Bradford & Rubin, 1986; Kay, 1987; Lucas & Shuster, 1987).

The *in vitro* chemotaxis results described extend our earlier observations on neutrophils (Bruijnzeel *et al.*, 1989) with those on eosinophils. Since our results suggest that nedocromil sodium and sodium cromoglycate seem to have a different mode of action on eosinophil chemotaxis *in vitro*, *in vivo* data are needed to substantiate these findings further.

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α_2-Adrenoceptor subtypes and imidazoline-like binding sites in the rat brain

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1 The binding of [^{3}H]-yohimbine and [^{3}H]-idazoxan to rat cortex and hippocampus is rapid, reversible and of high affinity. Saturation data indicate that a single population of binding sites exist for [^{3}H]-yohimbine in the cortex (B_{max} 121 ± 10 fmol mg^{-1}, protein; K_d 5.2 ± 0.9 nM) and hippocampus (B_{max} 72 ± 6 fmol mg^{-1} protein; K_d 5.8 ± 0.7 nM). [^{3}H]-idazoxan labels one site in the cortex (B_{max} 87 ± 8 fmol mg^{-1} protein; K_d 4.1 ± 0.9 nM) and hippocampus (B_{max} 30 ± 6 fmol mg^{-1} protein; K_d 3.5 ± 0.5 nM), when 3 μM phentolamine is used to define non-specific binding. A second distinct [^{3}H]-idazoxan binding site (B_{max} 110 ± 21 fmol mg^{-1} protein; K_d 3.6 ± 0.07 nM) is identified in rat cortex if 0.3 μM cirazoline is used to define non-specific binding and 3 μM yohimbine is included to prevent binding to α_2-adrenoceptors.

2 Displacement studies indicate that the α_1-adrenoceptor antagonist prazosin and the 5-HT$_1$ ligands 8-OH-DPAT, RU 24969 and methysergide differentiate [^{3}H]-yohimbine binding into two components; a high and low affinity site. In contrast the displacement of [^{3}H]-idazoxan by each ligand was monophasic.

3 The affinities of 8-OH-DPAT, RU 24969 and methysergide determined against [^{3}H]-idazoxan binding to the cortex and hippocampus correlate significantly with the binding site displaying low affinity for prazosin and previously designated α_{2A}. In contrast, a poor correlation exists for the high affinity site for prazosin designated α_{2B}.

4 [^{3}H]-idazoxan, in the presence of 3 μM yohimbine, labels a site that displays high affinity towards cirazoline, naphazoline and guanabenz, but low affinity towards clonidine, *p*-aminoclonidine, adrenaline, noradrenaline and the α_2-adrenoceptor antagonists yohimbine, rauwolscine, WY 26703 and BDF 6143.

5 The results of this study indicate that [^{3}H]-yohimbine labels two sites; the α_{2A}- and α_{2B}-adrenoceptors whereas [^{3}H]-idazoxan labels an α_2-adrenoceptor with a profile consistent with the α_{2A}-adrenoceptor subtype. In addition, [^{3}H]-idazoxan labels an imidazoline binding site in the rat cortex that is pharmacologically distinct from α_2-adrenoceptors. The low affinity of clonidine and *p*-aminoclonidine indicates that the imidazoline-like binding site in rat cortex is different from the site labelled by [^{3}H]-clonidine and [^{3}H]-*p*-aminoclonidine in human, rat and bovine brain stem, providing evidence of potential heterogeneity within this class of binding sites.

Introduction

Evidence indicates that α_2-adrenoceptors are heterogeneous. Prazosin inhibits [^{3}H]-yohimbine binding in the rat CNS in a biphasic manner (Bylund, 1985), with an approximately 50 fold separation between the high affinity site (4–20 nM) and the low affinity site (200–1000 nM). This moderately high affinity of prazosin has led to some hesitation in defining the binding site labelled by [^{3}H]-yohimbine as the α_2-adrenoceptor subtype (Yamada *et al.*, 1980). However, the low affinity exhibited by yohimbine for truly 'high' affinity [^{3}H]-prazosin (0.01–0.05 nM) sites suggests that [^{3}H]-yohimbine binding is not to an α_1-adrenoceptor site. This also means that the affinity ratio between yohimbine and prazosin is not as useful for differentiating between α_1- and α_2-adrenoceptor subtypes as originally suggested (Bylund & U'Prichard, 1983). A moderately high potency of prazosin at rat α_2-adrenoceptors has also been demonstrated in functional studies of noradrenaline release in the rat submandibular gland (Turner *et al.*, 1984). On the basis of the high and low affinities for prazosin against [^{3}H]-yohimbine, two subtypes of α_2-adrenoceptors have been suggested; α_{2A} (yohimbine = rauwolscine ⋙ prazosin) and α_{2B} (rauwolscine > yohimbine ⩾ prazosin) (Nahorski *et al.*, 1985). Although originally proposed as a non-rodent (α_{2A}), rodent (α_{2B}) classification, both subtypes have been shown to exist in the rat cerebrum (Bylund, 1985) and human caudate (Petrash & Bylund, 1986). 'Selective tissues', such as human platelet (α_{2A}) (Nahorski *et al.*, 1985), neonatal rat lung (α_{2B}) (Latifpour *et al.*, 1982) and selective cell lines HT29 (α_{2A}) and NG108-15 (α_{2B}) have also been described (Bylund *et al.*, 1988) which may be useful in further studies of the proposed subtypes.

Recent autoradiographical and pharmacological evidence has been presented suggesting that [^{3}H]-idazoxan labels two distinct α_2-adrenoceptor populations in the rat brain with high affinity, only one of which is labelled by [^{3}H]-rauwolscine (Boyajian *et al.*, 1987; Boyajian & Leslie, 1987). A non-α_2-adrenoceptor site labelled by [^{3}H]-idazoxan has been reported to exist in a number of peripheral tissues (Yablonsky *et al.*, 1988; Langin & Lafontan, 1989; Michel *et al.*, 1989; MacKinnon *et al.*, 1989). A central imidazoline preferring binding site labelled by [^{3}H]-clonidine and [^{3}H]-*p*-aminoclonidine has been identified in human, rat and bovine brainstem (Ernsberger *et al.*, 1987; Bricca *et al.*, 1989).

In this study we have examined the binding of [^{3}H]-yohimbine and [^{3}H]-idazoxan to α_2-adrenoceptors in rat cerebrocortical and hippocampal membranes and [^{3}H]-idazoxan binding to imidazoline-like binding sites in the rat cerebral cortex. The results suggest that the α_2-adrenoceptor labelled by [^{3}H]-idazoxan in the rat cortex and hippocampus correlates with the α_{2A}-adrenoceptor subtype, and that the imidazoline-like binding site in rat cortex is distinct from the imidazoline preferring binding site identified in human, rat and bovine brain stem. In addition, the affinities of the 5-HT$_{1A}$ ligands 8-hydroxy-2-(di n-propylamino) (8-OH-DPAT), RU 24969 and methysergide were examined as we have shown previously that these 5-HT$_{1A}$ ligands show selectivity for an α_2-adrenoceptor site labelled by [^{3}H]-yohimbine (Brown *et al.*, 1990).

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Methods

Membrane preparation

Male Sprague-Dawley rats (150–200 g) were killed by cervical dislocation, the brains rapidly removed and dissected on ice. Cerebral cortices or hippocampi (5 hippocampi/membrane preparation) were homogenized in 25 volumes of Tris buffer (50 mM Tris HCl, 5 mM EDTA: pH 7.4 at 25°C) using a polytron PT 10 (setting 10; 2 × 10 s bursts). The homogenate was then centrifuged at 49,000 *g* for 15 min at 4°C. After a second wash, the pellet obtained was resuspended in Tris assay buffer (50 mM Tris HCl, 0.5 mM EDTA; pH 7.4 at 25°C) before undergoing two further washes. The final pellet was resuspended in assay buffer for direct use in binding assays or stored under liquid nitrogen.

α_2-Adrenoceptor binding assays

[^{3}H]-yohimbine binding to rat cerebral membranes α_2-Adrenoceptor competition binding assays were performed by incubating washed rat cerebral membranes (500 μg protein) with [^{3}H]-yohimbine (1.5 nM) in the presence or absence of a range of 12 concentrations of the competing ligand in a total volume of 500 μl Tris assay buffer (50 mM Tris HCl, 0.5 mM EDTA; pH 7.4 at 25°C). Non-specific binding was defined as the concentration of bound ligand in the presence of 10 μM phentolamine. Specific binding represented 60–70% of total binding at 1.5 nM [^{3}H]-yohimbine. Following equilibrium (30 min at 25°C), bound radioactivity was separated from free by vacuum filtration over Whatman GF/B glass fibre filters using a Brandel cell harvester and determined by liquid scintillation spectrometry. Saturation assays were performed under similar conditions using a range of concentrations of [^{3}H]-yohimbine (0.1–15 nM).

[^{3}H]-idazoxan binding to rat cerebral membranes Experiments were performed under identical conditions to [^{3}H]-yohimbine binding assays, using a Tris HCl buffer system (50 mM Tris HCl, 0.5 mM EDTA; pH 7.4 at 25°C). Membranes were incubated with [^{3}H]-idazoxan (1 nM) to equilibrium (15 min) at 25°C in a final volume of 1 ml. Specific binding was determined using 3 μM phentolamine and represented 50–57% of total binding at 1 nM [^{3}H]-idazoxan (total d.p.m. 1975; non-specific d.p.m. 900). Saturation experiments were carried out under identical conditions with a range of concentrations of [^{3}H]-idazoxan (0.1–12 nM). Membranes were collected through Whatman GF/B filters using a Brandel cell harvester and the radioactivity determined as described above. [^{3}H]-idazoxan binding to non-adrenoceptor imidazoline binding sites was performed as described previously for the binding of [^{3}H]-idazoxan with the exception that 3 μM yohimbine was included to block binding to α_2-adrenoceptors. In these assays non-specific binding was determined by use of 0.3 μM cirazoline. In the presence of 3 μM yohimbine total d.p.m. was reduced from 1975 to approximately 1000 d.p.m. and non-specific to 150 d.p.m.

Data analysis

Saturation analysis Fitting of data to the appropriate models was achieved with the Scafit non-linear regression analysis programme for LIGAND (Munson & Rodbard, 1980). Comparisons between different binding models were made using the extra sum of the squares principle as outlined by Munson & Rodbard (1980), and shown in equation 1.

$$F = \frac{(SS_1 - SS_2)/(d.f._1 - d.f._2)}{(SS_2/d.f._2)} \quad (1)$$

SS and d.f. refer to the residual sum of the squares and degrees of freedom associated with the two fits being compared. The subscript 1 is designated to the fit with highest number of degrees of freedom (i.e. the most complicated model of analysis). Statistical significance of the F value was determined from a table of the percentage of the F distribution using $d.f._1 - d.f._2$ and $d.f._1$ degrees of freedom as the numerator and denominator respectively.

Competition analysis The inhibition of the radioligands by competing ligands was analysed to estimate the IC_{50} (concentration of competitor displacing 50% of specifically bound radioligand), using a non-linear least squares programme (Michel & Whiting, 1988) specially designed for the interpretation of sigmoidal concentration curves in terms of total and non-specific binding as well as inhibition constants and curve steepness. When Hill coefficients were not significantly different from unity the concentration of competitor displacing 50% of specific binding (IC_{50}) was converted to an affinity constant (K_i) using the expression derived by Cheng & Prusoff (1973) and shown in equation 2. In this expression [L] and K_d represent the radioligand concentration and dissociation constant respectively.

$$K_i = \frac{IC_{50}}{1 + ([L]/K_d)} \quad (2)$$

All data were initially analysed assuming a one site model of radioligand binding. The data, with Hill coefficients less than unity, were then analysed assuming a two site model, and the results of the curves fitting were statistically compared with those of the one site fit by an F test. The two site model was accepted if the observed fit was significantly better ($P < 0.05$) than the one site fit.

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

Drugs

Reagents used were of the highest analytical grade. Compounds were kindly donated by their manufacturers, synthesized at Syntex (Palo Alto) (indicated by an asterisk) or purchased. The following compounds were used: [^{3}H]-idazoxan (60 Ci mmol^{-1}) Amersham, U.K.; [^{3}H]-yohimbine (89 Ci mmol^{-1}) Du Pont, U.K.; amiloride hydrochloride (Sigma); p-aminoclonidine hydrochloride (Research Biochemicals); BDF 6143 (4-chloro-2-[2-imidazoline-2-ylamino]-isoindoline) hydrochloride*; clonidine hydrochloride (Sigma); cirazoline*; 8-OH-DPAT (8-hydroxy-2 (di n-propylamino)-tetralin hydrobromide (Research Biochemicals); guanabenz acetate (Wyeth); guanfacine hydrochloride (Sandoz); 5-HT creatinine sulphate (Sigma); idazoxan hydrochloride (Reckitt & Colman); imiloxan hydrochloride (Syntex); methysergide bimaleate (Sandoz); naphazoline hydrochloride (Sigma); (−)-noradrenaline bitartrate (Sigma); phentolamine mesylate (Ciba); prazosin hydrochloride (Pzifer); rauwolscine hydrochloride (Carl Roth); RU 24969 (5-methoxy-3-(1,2,3,6,tetra hydropyridin-4-yl)-1H-indole (Roussel); WB4101 (2-[2,6-dimethoxy-phenoxyethyl]aminomethyl-1,4-benzodioxan dihydrochloride (Ward Blenkinsop); WY 26703 (N-methyl-N-(1,3,4,6,7,11b-hezahydro-2H-benzo-$\alpha a\beta$-quinolizin-2-yl)-s-butane-sulphonamide hydrochloride (Wyeth); yohimbine hydrochloride (Sigma).

Results

Saturation experiments carried out in the presence of 3 μM or 10 μM phentolamine, respectively, to define non-specific binding, were consistent with [^{3}H]-idazoxan and [^{3}H]-yohimbine each labelling a single population of high affinity sites in rat cortex and hippocampus (Table 1). Significant differences were observed in each tissue between the maximum number of [^{3}H]-idazoxan and [^{3}H]-yohimbine binding sites. The density of α_2-adrenoceptors was higher with [^{3}H]-yohimbine in each tissue. The affinity of each ligand however, did not differ between tissues. Saturation experiments repeated in

Table 1 Specific binding of [^{3}H]-yohimbine and [^{3}H]-idazoxan to rat cerebral cortex and rat hippocampus membranes

	[^{3}H]-yohimbine		[^{3}H]-idazoxan	
	K_d	B_{max}	K_d	B_{max}
Cortex	5.2 ± 0.9	121 ± 10	4.1 ± 0.9	87 ± 8
			3.6 ± 0.7	110 ± 21*
Hippocampus	5.8 ± 0.7	72 ± 6	3.5 ± 0.5	36 ± 6

Assays were performed as described in the methods section. Non-specific binding was defined with 10 μM phentolamine for [^{3}H]-yohimbine binding and 3 μM phentolamine for [^{3}H]-idazoxan binding to α_2-adrenoceptors. To determine [^{3}H]-idazoxan binding to the imidazoline-like binding site, *saturation experiments were carried out in the presence of 3 μM yohimbine and non-specific binding at each ligand concentration defined with 0.3 μM cirazoline. Values shown were obtained using the non-linear iterative curve programme 'Ligand' (Munson & Rodbard, 1980), and are the mean ± s.e.mean of at least three experiments.

the presence of 3 μM yohimbine to block [^{3}H]-idazoxan binding to α_2-adrenoceptors, revealed a second component of binding in the rat cortex, that was saturable, reversible and of high affinity (Table 1). The density of α_2-adrenoceptors and imidazoline-like binding sites labelled by [^{3}H]-idazoxan in the rat cerebral cortex was not significantly different.

Displacement of [^{3}H]-idazoxan binding from cortical and hippocampal membranes by prazosin was of low affinity and with Hill slopes close to unity (Figure 1). In contrast, inhibition of [^{3}H]-yohimbine binding by prazosin was complex, with Hill slopes considerably less than unity (Figure 2). Computer analysis of the displacement curves indicated that prazosin displaced [^{3}H]-yohimbine binding to hippocampal membranes with high (α_{2B}; pK_i 8.10 ± 0.3) and low (α_{2A}; pK_i 5.94 ± 0.08) affinity. The failure of prazosin to differentiate [^{3}H]-idazoxan binding is thus unlikely to be due to the binding conditions employed but most likely represents an inherent difference in the two ligands.

[^{3}H]-idazoxan and [^{3}H]-yohimbine binding to rat cortex

The α_2-adrenoceptor antagonists examined inhibited [^{3}H]-idazoxan and [^{3}H]-yohimbine binding to the rat cortex with Hill slopes not significantly different from unity. A comparison of the affinities of α_2-adrenoceptor antagonists indicated that yohimbine, rauwolscine, WY 26703 and BDF 6143 displayed higher affinity if determined against [^{3}H]-yohimbine than against [^{3}H]-idazoxan (Table 2). The difference in affinity was less marked for idazoxan and imiloxan.

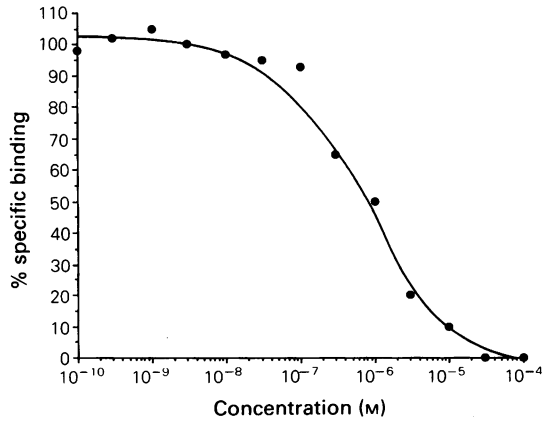


Figure 1 Inhibition studies of [^{3}H]-idazoxan binding to rat hippocampal membranes were conducted over a concentration-range of 1×10^{-10}–1×10^{-4} M prazosin. Incubations were performed for 15 min at 25°C in a Tris HCl buffer (50 mM Tris HCl, 0.5 mM EDTA; pH 7.4). Non-specific binding was determined in the presence of 3 μM phentolamine. The data describe a single experiment performed in duplicate. Computer-assisted curve fitting procedures showed a one site fit.

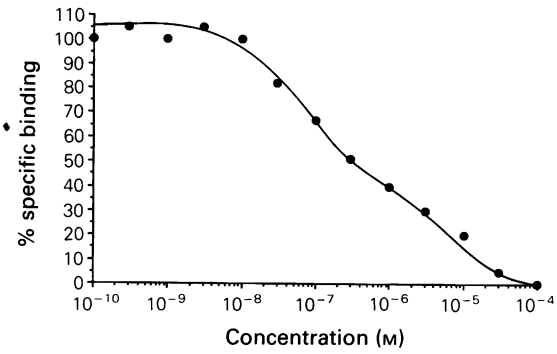


Figure 2 Inhibition studies of [^{3}H]-yohimbine binding to rat hippocampal membranes were conducted over a concentration-range of 1×10^{-10}–1×10^{-4} M prazosin. Incubations were performed for 30 min at 25°C in a Tris HCl buffer (50 mM Tris HCl, 0.5 mM EDTA; pH 7.4). Non-specific binding was determined in the presence of 10 μM phentolamine. The data describe a single experiment performed in duplicate. Computer-assisted curve fitting procedures showed a two site fit, $P < 0.001$. (1 site, SS_1 62882; 2 site, SS_2 2965; F value 91.1).

Displacement of [^{3}H]-idazoxan binding to rat cortex by the 5-HT$_1$ ligands 8-OH-DPAT, RU 24969, methysergide and 5-HT, was not complex, having Hill slopes not significantly different from unity. Inhibition data indicated that each ligand had low affinity towards the [^{3}H]-idazoxan binding site. This contrasts with the complex displacement curves observed for the displacement of [^{3}H]-yohimbine binding. Methysergide, RU 24969 and 8-OH-DPAT but not 5-HT showed biphasic displacement curves with a greater than two log order difference in affinity between their high affinity and low affinity sites. The rank order of potency for the higher affinity sites was methysergide > RU 24969 > 8-OH-DPAT.

Noradrenaline showed moderate affinity for [^{3}H]-idazoxan binding (pIC_{50} 6.70) and [^{3}H]-yohimbine binding at both sites, α_{2B} (pIC_{50} 5.73) and α_{2A} (pIC_{50} 6.58). In each case the Hill slopes were significantly less than unity.

In contrast to the high affinity displayed by yohimbine, rauwolscine, WY 26703 and BDF 6143 towards the α_2-adrenoceptor labelled by [^{3}H]-yohimbine and [^{3}H]-idazoxan, these compounds were weak at displacing [^{3}H]-idazoxan from the imidazoline binding site in the rat cortex. The low affinity of noradrenaline, adrenaline and 5-HT suggests that this site is neither an adrenoceptor nor a 5-HT binding site. The imidazoline related compounds, cirazoline, guanabenz and naphazoline displaced [^{3}H]-idazoxan from this site with high affinity whereas guanfacine, clonidine, *p*-aminoclonidine and amiloride possessed only modest affinity. Cirazoline and guanabenz, under these conditions, displayed approximately three and one log order selectivity for the imidazoline binding site over α_2-adrenoceptors. The pharmacological profile of the imidazoline binding site is thus distinct from either the α_{2A}- or α_{2B}-adrenoceptor subtypes identified with [^{3}H]-yohimbine or the α_{2A}-subtype labelled by [^{3}H]-idazoxan.

[^{3}H]-idazoxan and [^{3}H]-yohimbine binding to rat hippocampus

The overall pharmacological profile of the compounds in displacing [^{3}H]-yohimbine and [^{3}H]-idazoxan binding to α_2-adrenoceptors in rat hippocampus was similar to that obtained for the binding to rat cortex. The imidazoline α_2-adrenoceptor antagonists idazoxan, BDF 6143 and the imidazole, imiloxan, were more potent at displacing [^{3}H]-idazoxan than [^{3}H]-yohimbine, whereas the non-imidazoline antagonists displayed higher affinity if determined against [^{3}H]-yohimbine than [^{3}H]-idazoxan (Table 3). The 5-HT$_1$ ligands 8-OH-DPAT, RU 24969 and methysergide, although

Table 2 Inhibition of [^{3}H]-yohimbine and [^{3}H]-idazoxan binding to rat cortex

	pK_i			
	[^{3}H]-*yohimbine*		[^{3}H]-*idazoxan*	
Postulated sites	α_{2B} %	α_{2A} %	α_{2A}	Imidazoline
Yohimbine	7.90 ± 0.04		7.25 ± 0.07	5.28 ± 0.20
Rauwolscine	8.15 ± 0.08		7.09 ± 0.03	4.97 ± 0.15
WY 26703	8.04 ± 0.07		7.53 ± 0.03	4.79 ± 0.20
Idazoxan	8.04 ± 0.04		8.12 ± 0.05	8.37 ± 0.03
Imiloxan	7.10 ± 0.10		6.65 ± 0.12	5.18 ± 0.10
BDF 6143	9.22 ± 0.07		8.65 ± 0.03	5.59 ± 0.2
Oxymetazoline	6.92 ± 0.13 (40)	8.25 ± 0.20 (60)	7.50 ± 0.05	4.45 ± 0.05
Prazosin	7.30 ± 0.11 (50)	6.00 ± 0.12 (50)	6.40 ± 0.06	4.84 ± 0.13
5-HT	<4		<4	<4
8-OH-DPAT	6.43 ± 0.04 (40)	4.97 ± 0.04 (60)	6.07 ± 0.08	5.24 ± 0.13
RU 24969	6.70 ± 0.03 (40)	5.20 ± 0.07 (60)	6.02 ± 0.05	5.75 ± 0.13
Methysergide	7.17 ± 0.08 (45)	5.35 ± 0.11 (55)	5.13 ± 0.05	4.05 ± 0.09
Adrenaline	7.12 ± 0.13*		7.49 ± 0.11*	4.47 ± 0.16
Noradrenaline	5.73 ± 0.20*	6.58 ± 0.25*	6.70 ± 0.13*	<4
Amiloride	4.80 ± 0.1		5.05 ± 0.2	6.25 ± 0.19*
Guanfacine	6.95 ± 0.05		7.00 ± 0.15	5.57 ± 0.15*
Guanabenz	7.55 ± 0.15		7.70 ± 0.1	8.21 ± 0.17
Naphazoline	7.21 ± 0.05		7.50 ± 0.2	7.33 ± 0.1*
Cirazoline	6.03 ± 0.06		6.30 ± 0.05	8.59 ± 0.10
Clonidine	7.85 ± 0.01*		8.00 ± 0.08*	6.27 ± 0.23*
p-Aminoclonidine	8.11 ± 0.06*		8.35 ± 0.08*	5.23 ± 0.16*

* nH < 1

The affinity values were obtained for specific [^{3}H]-yohimbine and [^{3}H]-idazoxan binding to rat cortex. Experiments were performed using 1–2 nM [^{3}H]-yohimbine or [^{3}H]-idazoxan and the concentration of drug that inhibited specific binding by 50% (IC_{50}) was obtained and converted to a K_i value using the relationship $K_i = IC_{50}/(1 + [L]/K_d)$, where [L] represents the free ligand concentration and K_d = the equilibrium dissociation constant of the radioligand. Values shown are the mean pK_i ± s.e.mean of at least three experiments performed in duplicate. Results were evaluated by computer-assisted curve fitting procedures described under methods. A two site fit was accepted only if it was statistically better than a one site fit.

Where high and low affinity sites are found they are placed in the α_{2A} and α_{2B} categories on the basis of 1. The affinity of prazosin, 2, whether binding of the compound survives occlusion of the high affinity site by 10^{-7} prazosin (Brown *et al.*, 1990).

failing to differentiate [^{3}H]-idazoxan binding to the hippocampus, clearly gave rise to biphasic displacement of [^{3}H]-yohimbine binding. The low density of [^{3}H]-idazoxan binding in the rat hippocampus made it difficult to characterize the imidazoline binding site in this tissue.

The K_i values of the compounds derived from the displacement of [^{3}H]-idazoxan were compared with the values obtained for the sites labelled by [^{3}H]-yohimbine (Figure 3). A significant correlation was found between the affinity of the compounds at the [^{3}H]-idazoxan site and the α_{2A}-adrenoceptor subtype in both the cortex ($r = 0.837$; $P < 0.001$) and hippocampus ($r = 0.821$; $P < 0.01$). In contrast, a poor correlation was found for [^{3}H]-yohimbine binding to the α_{2B}- site and the [^{3}H]-idazoxan binding site in the cortex ($r = 0.550$) and hippocampus ($r = 0.614$). These results suggest that [^{3}H]-idazoxan labels a site consistent with the α_{2A}-adrenoceptor subtype.

Discussion

The present study examining the binding of [^{3}H]-idazoxan and [^{3}H]-yohimbine provides additional evidence for heterogeneity of α_2-adrenoceptors and the existence of an imidazoline-like binding site in rat brain. Initial studies in rat cortex and hippocampal membranes show that the pK_i values for displacing [^{3}H]-idazoxan or [^{3}H]-yohimbine binding differ between the non-imidazoline and imidazoline related α_2-adrenoceptor antagonists. The non-imidazoline compounds show higher affinity for [^{3}H]-yohimbine binding whereas the imidazolines show higher affinity for [^{3}H]-idazoxan binding, suggesting differences in the α_2-adrenoceptor populations labelled by the two radioligands. Before this is

Table 3 Inhibition of [^{3}H]-yohimbine and [^{3}H]-idazoxan binding to rat hippocampus

	K_i (nM)		
	[^{3}H]-*yohimbine*		[^{3}H]-*idazoxan*
	α_{2B} %	α_{2A} %	
Yohimbine	7.87 ± 0.07		7.29 ± 0.07
Rauwolscine	7.86 ± 0.06		7.15 ± 0.08
WY 6703	7.94 ± 0.07		7.70 ± 0.05
Idazoxan	7.67 ± 0.06		8.70 ± 0.05
Imiloxan	6.34 ± 0.07		—
BDF 6143	8.96 ± 0.04		9.59 ± 0.07
Prazosin	8.10 ± 0.3 (50)	5.94 ± 0.08 (50)	6.59 ± 0.37
5-HT	<5		
8-OH-DPAT	6.68 ± 0.08 (55)	4.79 ± 0.09 (45)	5.83 ± 0.15
RU 24969	7.50 ± 0.13 (50)	5.13 ± 0.07 (50)	6.04 ± 0.08
Methysergide	7.15 ± 0.06 (50)	4.69 ± 0.20 (50)	4.95 ± 0.05

The affinity values were obtained for specific [^{3}H]-yohimbine and [^{3}H]-idazoxan binding to rat hippocampus. Experiments were performed using 1–2 nM [^{3}H]-yohimbine or [^{3}H]-idazoxan and the concentration of drug that inhibited specific binding by 50% (IC_{50}) was obtained and converted to a K_i value using the relationship $K_i = IC_{50}/(1 + [L]/K_d)$, where [L] represents the free ligand concentration and K_d = the equilibrium dissociation constant of the radioligand. Values shown are the mean pK_i ± s.e.mean of at least three experiments performed in duplicate. Results were evaluated by computer-assisted curve fitting procedures described in methods. A two site fit was accepted only if it was significantly better than a one site fit.

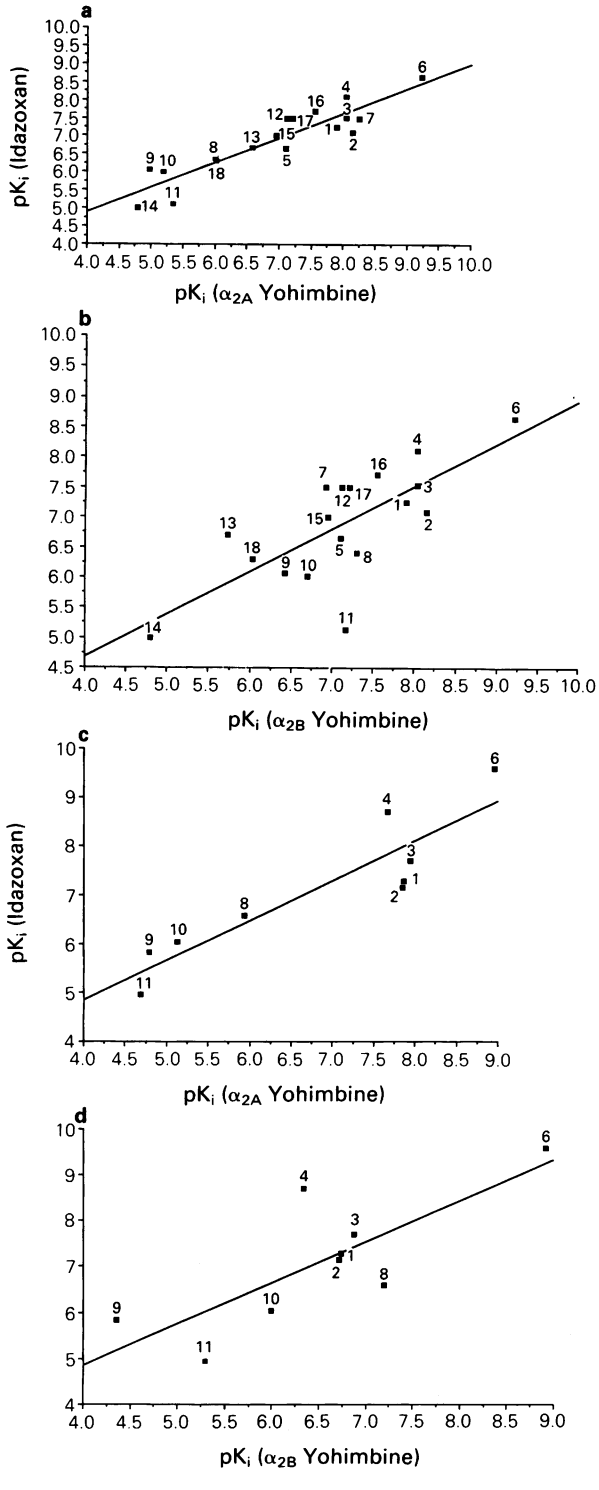


Figure 3 Pharmacological profile of the α_2-adrenoceptor subtypes. The pK_i values of a number of compounds for α_{2A}- and α_{2B}-adrenoceptor subtypes are compared with the values obtained for the displacement of [^{3}H]-idazoxan: (1) yohimbine; (2) rauwolscine; (3) WY 26703; (4) idazoxan; (5) imiloxan; (6) BDF 6143; (7) oxymetazoline; (8) prazosin; (9) 8-OH-DPAT; (10) RU 24969; (11) methysergide; (12) adrenaline; (13) noradrenaline; (14) amiloride; (15) guanfacine; (16) guanabenz; (17) naphazoline; (18) cirazoline. Correlations between the pK_i values for [^{3}H]-idazoxan and the subtypes are as follows; r = 0.837, $P < 0.001$ for α_{2A} in cortex (3a); r = 0.550 for α_{2B} in cortex (3b); r = 0.821, $P < 0.01$ for α_{2A} in hippocampus (3c); r = 0.614 for α_{2B} in hippocampus (3d).

accepted as evidence for multiple sites several factors must be considered.

The range of densities of α_2-adrenoceptor binding sites in the rat cortex reported in the literature is wide (90–242 fmol mg^{-1} protein, Bylund & U'Prichard, 1983; Lane *et al.*, 1983), a finding probably related to different assay conditions and to membrane preparations being purified to different extents. In order to compare the binding of [^{3}H]-idazoxan and [^{3}H]-yohimbine, identical conditions were employed with regard to both membrane preparations and ligand binding. Under the present conditions (crude membrane preparation and Tris HCl buffer system: 50 mM Tris HCl, 0.5 mM EDTA; pH 7.4 at 25°C) the specific binding of [^{3}H]-yohimbine (1–15 nM) and [^{3}H]-idazoxan (1–12 nM) to membranes from rat cerebrum was rapid, reversible, saturable and of high affinity. Extensive washing of the membrane preparation with EDTA was used to remove endogenous Mg^{2+} and other divalent cations which can reduce the affinity of the radioligand and complicate analysis of displacement curves generated by competing drugs (Cheung *et al.*, 1982).

The α_2-adrenoceptor has been shown to exist in different affinity states modulated by the presence of mono- and divalent cations and guanine nucleotides. ^{3}H-agonist radioligands are affected whereas ^{3}H-antagonist binding is generally little affected by these modulators. Lane *et al.* (1983) suggested that the binding of the antagonist [^{3}H]-idazoxan is increased markedly in the presence of a high concentration of sodium ions (physiological buffer containing 119.2 mM sodium). However, they used a crude synaptosomal P_2-fraction prepared in sucrose followed by resuspension in physiological buffer. Apparent maximal [^{3}H]-yohimbine binding to cortical membranes prepared in a similar manner was also increased in the presence of Na^+ ions (Woodcock & Murley, 1982). Further investigation of this membrane preparation (Cheung *et al.*, 1984) revealed it to be comprised of a high proportion of intact synaptosomes containing endogenous noradrenaline, which could interfere with the subsequent α_2-adrenoceptor binding assay. Sensitivity of [^{3}H]-yohimbine binding to guanine nucleotides and mono- and divalent ions is not as marked with membranes prepared in hypotonic buffers.

In rabbit cerebral cortex, [^{3}H]-yohimbine has been reported to label almost twice as many binding sites as does [^{3}H]-idazoxan yet both ligands are reported to label an 'α_2-adrenoceptor' binding site (Hamilton *et al.*, 1988). In this study both yohimbine and idazoxan bind to sites classifiable as an α_2-adrenoceptor. The values obtained for [^{3}H]-idazoxan binding were similar to those described for a rat P_2 cerebral membrane (Lane *et al.*, 1983). All the competing antagonists for the displacement of [^{3}H]-idazoxan showed monophasic inhibition with Hill slopes not significantly different from unity. Prazosin, methysergide, 8-OH-DPAT and RU 24969 all showed low affinity towards the α_2-adrenoceptor labelled by [^{3}H]-idazoxan. However, the saturation studies indicated that [^{3}H]-idazoxan labelled fewer binding sites in the rat cortex and hippocampus than did [^{3}H]-yohimbine. Differences in the density (B_{max}) of α_2-adrenoceptors labelled by the two ligands has been cited as evidence for heterogeneity within this class of receptor. The difference in B_{max} values obtained with the radioligands is consistent with the view that idazoxan binds to only one of the two sites labelled by [^{3}H]-yohimbine. A number of compounds have been identified that show biphasic inhibition curves for the displacement of [^{3}H]-yohimbine binding. Interestingly these compounds display monophasic displacement curves and with the exception of oxymetazoline only displace [^{3}H]-idazoxan binding with low affinity. Thus affinities of these compounds for inhibiting [^{3}H]-idazoxan binding show a good correlation with [^{3}H]-yohimbine binding to the α_{2A}-subtype. Displacement studies suggest that α_{2A} and α_{2B}-adrenoceptors exist in approximately equal proportions in the hippocampus and 60:40% in favour of the α_{2A} in the cerebral cortex. Saturation studies demonstrated that [^{3}H]-yohimbine labels twice as many sites as [^{3}H]-idazoxan in the hippocampus consistent with [^{3}H]-yohimbine labelling α_{2A}- and α_{2B}-adrenoceptors and [^{3}H]-idazoxan binding to only the α_{2A}-adrenoceptor subtype. Similarly, the number of sites labelled by [^{3}H]-idazoxan relative to [^{3}H]-yohimbine in the cerebral cortex is in broad agreement with [^{3}H]-idazoxan labelling the α_{2A}-adrenoceptor subtype.

Although [^{3}H]-idazoxan labels only a subpopulation of high affinity α_2-adrenoceptors, it also labels a second site that is saturable, reversible and pharmacologically distinct from α_2-adrenoceptors. In competition experiments specific binding of 1 nM [^{3}H]-idazoxan to α_2-adrenoceptors in rat cerebral cortex varied between 50–57%. The remaining binding, minus a small percentage of non-specific binding, represents binding to the imidazoline-like sites and should approximate the number of α_2-adrenoceptors. The identification of more selective imidazoline-like antagonists with low affinity at α_2-adrenoceptors and the reverse will be required if accurate determination of the density of these two sites is to be achieved.

The observation that [^{3}H]-idazoxan labels two distinct populations of binding sites in rat brain (Boyajian *et al.*, 1987; Boyajian & Leslie, 1987) is often used as evidence for heterogeneity within α_2-adrenoceptors. These studies were carried out using a Na^+/K^+ buffer system. A possible explanation which could account for the large percentage of [^{3}H]-idazoxan binding reported by Boyajian *et al.* (1987), is that, under the conditions used, [^{3}H]-idazoxan binds preferentially to a proposed imidazoline-like site, similar to that reported to exist in rabbit adipocytes (Langin & Lafontan, 1989), rabbit urethral tissue (Yablonsky *et al.*, 1988; Yablonsky & Dausse, 1989) and hamster adipocytes (MacKinnon *et al.*, 1989). The low affinity of clonidine and *p*-aminoclonidine at the imidazoline-like binding sites in rat cerebral cortex indicates that this site is distinct from the imidazoline-like site labelled by [^{3}H]-clonidine and [^{3}H]-*p*-aminoclonidine in human, rat and bovine brain stem (Ernsberger *et al.*, 1987; Bricca *et al.*, 1988; 1989). Thus it is unlikely that the endogenous imidazoline substance, clonidine displacing substance (CDS) considered to act through the imidazoline-like binding site in the brain stem (Atlas *et al.*, 1987; Bousquet & Feldman, 1987) will act at the imidazoline-like site labelled by [^{3}H]-idazoxan in rat cerebral cortex. The functional importance of the imidazoline-like binding site identified by [^{3}H]-idazoxan has still to be determined. Doxey *et al.* (1983) demonstrated that idazoxan antagonized guanabenz-induced contraction of the rat anococcygeus muscle which was not blocked by phentolamine or piperoxan. Further studies will be required to determine if the anococcygeus muscle represents a functional model for this imidazoline site.

The results of this study support the view that α_2-adrenoceptor binding sites are not homogeneous. [^{3}H]-yohimbine labels two sites in the rat brain both of which have the overall profile of an α_2-adrenoceptor, although a number of significant differences were observed between the sites in the hippocampus and cortex. [^{3}H]-yohimbine binding to the α_{2A}-subtype correlates with the binding of [^{3}H]-idazoxan. The pharmacological profile obtained for the displacement of both ^{3}H-ligands to this site is similar; moderate affinity for NA, high affinity for the α_2-adrenoceptor antagonists, yohimbine, rauwolscine and the imidazolines, idazoxan and imiloxan but low affinity for prazosin, 8-OH-DPAT, RU 24969 and methysergide.

The second binding site labelled by [^{3}H]-yohimbine, (α_{2B}), shows high affinity for the α_2-adrenoceptor antagonists yohimbine and rauwolscine but lower affinity than the α_{2A}-subtype for NA although this is still within a range considered indicative of α_2-adrenoceptors. The α_{2B}-subtype is further characterized as having high affinity towards 8-OH-DPAT, RU 24969 and methysergide. Recently 8-OH-DPAT has been shown to be a postsynaptic α_2-adrenoceptor antagonist (Crist & Surprenant, 1987; pA_2 6.9–7.2).

Further investigations of the properties of α_2-adrenoceptor subtypes in both membrane and soluble populations are needed. It will also be important to establish whether the existence of α_2-adrenoceptor subtypes and the identification of a distinct non-α_2-adrenoceptor imidazoline-like binding site in rat cortex are reflected by appropriate functional responses.

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The actions of human atrial natriuretic factor on hepatic arterial and portal vascular beds of the anaesthetized dog

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1 The vascular actions of atrial natriuretic factor (ANF) have been assessed with other vasoactive agents on the hepatic arterial and portal vascular beds of the anaesthetized dog.

2 Intra-arterial bolus injections of ANF (0.1–50 nmol) caused graded increases in hepatic arterial blood flow representing a vasodilatation of relatively short duration. Vasoconstriction was never observed.

3 The maximum increase in hepatic arterial blood was the same for ANF and isoprenaline (Iso) i.e. approximately 60–70% increase over control flow.

4 On a molar basis, ANF was less potent than Iso although over the higher dose range (10^{-9}–10^{-7} mol) its vasodilator activity exceeded that of the endogenous vasodilator adrenaline.

5 Intraportal bolus injections (1.0–50 nmol) of ANF did not alter portal inflow resistance since no changes in portal inflow pressure occurred when the portal circuit was perfused at constant inflow volume.

6 This differential action of ANF on the hepatic arterial and portal vascular beds may provide a change in total liver blood flow in favour of the arterial component.

7 ANF, by altering hepatic haemodynamics to favour formation of trans-sinusoidal fluid exchange, may provide a temporary expansion of the extravascular fluid reservoir to buffer any increased venous pressure. However, chronically elevated plasma levels of ANF would encourage the formation of ascitic fluid.

Introduction

Mammalian atrial myocytes synthesize and release the 28 amino acid residue peptide, atrial natriuretic factor (ANF). Raised venous pressure, leading to atrial distension, is one of the factors which releases ANF into the systemic circulation (Anderson *et al.*, 1986; Gerbes *et al.*, 1986). In addition to promoting natriuresis and diuresis the peptide causes hypotension, due principally to a fall in peripheral vascular resistance (see reviews by Genest & Cantin, 1988; Gerbes *et al.*, 1987). However, the regional distribution of its vasodilator activity has not been fully examined.

Elevated central venous pressure also leads to raised hepatic venous pressure with profound consequences for hepatic haemodynamics and trans-sinusoidal fluid exchange (Laine *et al.*, 1979). Enlargement of the liver with movement of fluid across the hepatic capsule into the peritoneal space may be a normal, although temporary compensating mechanism to expand the extravascular fluid space. However, chronic elevation of hepatic venous pressure leads to the persistent presence of excess peritoneal (ascitic) fluid. A significant fraction of the raised hepatic venous pressure may be transmitted through the liver to elevate portal pressure (Bennett & Rothe, 1981) causing enlargement of the spleen (Withrington *et al.*, 1980), together with venous congestion and oedema of the gastrointestinal tract.

The aim of the present experiments was to characterize the actions of ANF on the primary intra-hepatic resistance sites in the hepatic arterial and portal vascular beds which control total liver blood inflow and, in addition, to assess its molar potency relative to other vasoactive agents. In this way some conclusions could be drawn about the role of ANF in the regulation of total liver blood flow and its distribution between the two inflow circuits both under normal conditions and in those clinical situations where the systemic circulating levels of ANF deviate significantly from normal. A preliminary account of some of these results has been published (Dhume *et al.*, 1988).

Methods

The experiments were performed on 10 dogs (mean weight 21.9 ± 2.0 kg, range 10.0–29.0 kg) anaesthetized with an intravenous mixture of chloralose and urethane (50 and 500 mg kg^{-1} respectively) after induction with methohexitone sodium (6 mg kg^{-1}). The trachea was cannulated although respiration was spontaneous throughout the experiment. The right femoral vein was cannulated to administer additional anaesthetic when appropriate. The right femoral artery was cannulated to provide hourly blood samples for analysis of pH, Po_2 and Pco_2 (Blood Gas Analyser, Instrumental Laboratory, Model 1302); $NaHCO_3$ was administered i.v. (1.0 mmol min^{-1}) when necessary, to maintain a normal arterial pH. The left carotid artery was cannulated and connected to a strain gauge transducer (Statham P23Gb), to provide a continuous registration of phasic systemic blood pressure from which a continuous recording of heart rate was derived electronically. Body temperature was maintained within normal limits, as indicated by a buccal thermometer, by either table heaters or overhead lamps. The animals were heparinised (500 iu kg^{-1}) once perfusion had started and half this dose was administered hourly.

The perfusion circuits and surgery required for hepatic arterial and portal perfusion have been described in detail previously (Corder & Withrington, 1988) and in all essential details the same procedure was adopted for the present series. Briefly, the hepatic artery was cannulated after ligation of major side tributaries, and perfused with arterial blood from the cannulated left femoral artery. The hepatic periarterial innervation remained intact. Incorporated into the arterial perfusion circuit was a strain gauge pressure transducer (Statham P23Gb) and a cannulating electromagnetic flow probe (Cardiovascular Instruments) to measure perfusion pressure and flow respectively. These signals were heavily

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damped electronically to provide mean values which were continuously monitored on a chart recorder (Devices M19). In addition, the signals were digitised (Data Translation DT-2801) and processed by an IBM PC-XT computer programmed to calculate and store the absolute values of hepatic arterial vascular resistance (mean pressure/mean flow) and the changes induced by any experimental procedure. A 'T' piece was included in the perfusion circuit so that vasoactive substances could be administered by bolus injection directly into the hepatic arterial circuit without necessarily entering the systemic circuit. This avoided either altering the conditions of the perfusion (i.e. constant pressure) or eliciting reflex changes in hepatic sympathetic tone.

In the portal perfusion experiments the hepatic portal vein was cleared of all attachments and principal side branches ligated. The spleen was removed and a wide bore polythene tube passed retrogradely down the splenic vein to the portal vein. The portal vein was then tied cranially just beyond the entrance of the superior mesenteric and splenic veins so that the venous drainage from the gastrointestinal tract was diverted along the splenic vein cannula to a reservoir. The hepatic end of the portal vein was then cannulated and blood from the mesenteric drainage reservoir pumped, by means of a roller pump (Watson-Marlow MHRE 1000), into the intrahepatic portal vascular bed. The pump was set to deliver the same volume flow as the mesenteric venous drainage and, once set, remained constant throughout any experiment. Incorporated into the portal perfusion circuit between the pump and liver was a low pressure strain-gauge transducer and an electromagnetic flow probe to measure continuously hepatic portal venous pressure (HPVP) and to check the pump outflow (HPVF) respectively. A long, narrow bore, polythene cannula was passed up the inferior vena cava (IVC) from the right femoral vein to a point just below the diaphragm and opposite the entry of the hepatic veins. After suitable electronic damping this provided a continuous recording of IVC pressure (IVCP). The signals from these pressure transducers were heavily damped electronically to provide mean values which were continually monitored on a pen recorder. Again the values were digitised and fed into a personal IBM computer programmed to calculate hepatic portal mean inflow resistance [(HPVP – IVCP)/HPVF] and also to store the maximum changes in portal pressure and portal inflow resistance induced by any experimental procedure. Incorporated into the portal circuit was a 'T' piece for injection of vasoactive substances directly into the portal inflow.

The flow probes were calibrated with whole blood at the end of each experiment. The liver was weighed at the end of each experiment so that hepatic arterial and portal flows and resistances could be expressed in terms of 100 g liver weight.

Drugs used and vehicles

Vasoactive substances were washed in with saline (0.9% w/v NaCl solution) to give a total injectate of 2.0 ml. ANF was obtained from three sources (Sigma, Novabiochem, Bissendorf) and made up in sterile saline which contained human serum albumin (10 mg ml^{-1}, Elstree) and polypep (2.5 mg ml^{-1}; low viscosity; Sigma). Close arterial or intraportal injection of this vehicle did not alter either hepatic arterial or portal inflow resistance. The human serum albumin and polypep were used to reduce non-specific binding of ANF onto plastic surfaces. (±)-Isoprenaline hydrochloride (Pharmax Ltd.) and (−)-noradrenaline acid tartrate (Winthrop) were diluted immediately before injection in normal saline and the solutions maintained in ice.

Statistics

Results are presented as mean ± standard errors of the mean (s.e.mean). Tests for significance refer to Students unpaired *t* test.

Results

Control values

The mean liver weight was 502 ± 33 g representing 2.39 ± 0.16% of the body weight. In the 6 hepatic arterial perfusion experiments the initial mean hepatic arterial blood flow was 187 ± 12.8 ml min^{-1}. The mean hepatic arterial perfusion pressure was 151 ± 7.0 mmHg and the mean calculated hepatic arterial vascular resistance was 0.81 ± 0.05 mmHg ml^{-1} min. These values agree with those previously obtained in this laboratory.

In the four hepatic portal perfusion experiments the mean initial systemic blood pressure, once portal perfusion had started, was 115 ± 3.3 mmHg. The mean hepatic portal blood flow was 178 ± 38 ml min^{-1} or 36.7 ± 7.4 ml min^{-1} 100 g. The mean hepatic portal venous pressure was 8.50 ± 0.74 mmHg and the mean inferior vena cava pressure 0.71 ± 0.36 mmHg; giving a mean hepatic portal perfusion pressure gradient (i.e. HPVP – IVCP) of 7.82 ± 0.30 mmHg. The mean calculated hepatic portal inflow resistance was 0.23 ± 0.045 mmHg ml^{-1} min 100 g. These values are similar to those reported previously from this laboratory.

Hepatic arterial vascular responses to intra-arterial injections

Isoprenaline The non-selective β-adrenoceptor agonist isoprenaline (Iso) was administered in all the hepatic arterial preparations as a bolus injection over a range of doses (0.1–50 nmol) to construct, in each experiment, a complete dose-response curve. The only vascular response observed to i.a. Iso was an increase in hepatic arterial blood flow [HABF] which was graded with dose and of relatively short duration (Figure 1). This increase in blood flow reflects, at constant hepatic arterial perfusion pressure [HAPP], a decrease in hepatic arterial vascular resistance [HAVR] and therefore vasodilatation. Previous studies have shown this vasodilator response to be due to the activation of β_2-adrenoceptors within the hepatic arterial circuit since it is blocked by prop-

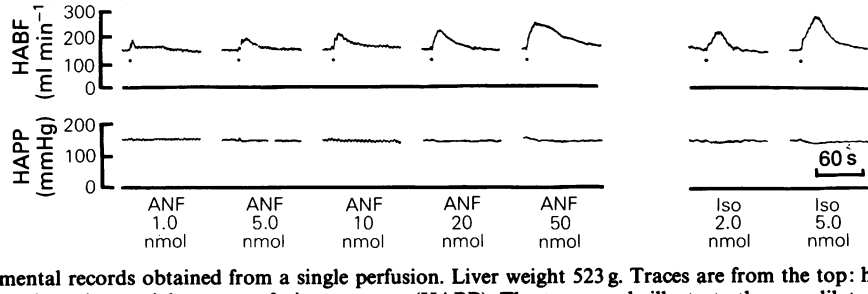


Figure 1 Experimental records obtained from a single perfusion. Liver weight 523 g. Traces are from the top: hepatic arterial mean blood flow (HABF), hepatic arterial mean perfusion pressure (HAPP). The two panels illustrate the vasodilator responses to intra-arterial bolus injections of atrial natriuretic factor (ANF; 1.0–50 nmol) and isoprenaline (Iso; 2.0 and 5.0 nmol). The black dots indicate the point of injection.

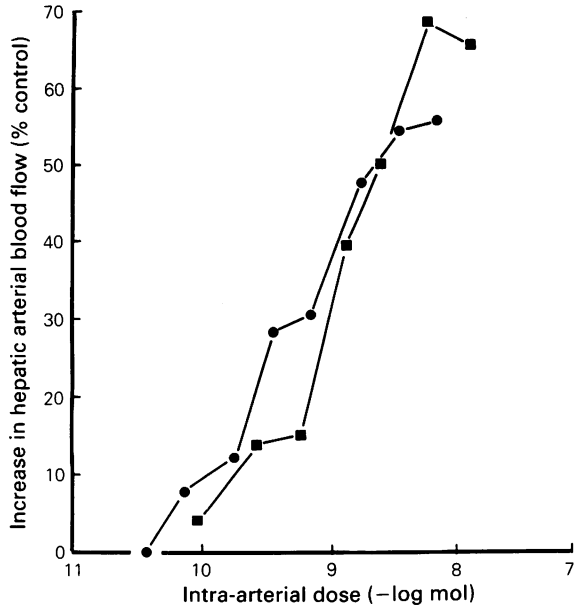


Figure 2 Dose-response curves relating the hepatic arterial vasodilator responses (expressed as the percentage increase in hepatic arterial blood flow compared to the pre-injection control) to intra-arterial bolus injections of atrial natriuretic factor (■) and isoprenaline (●). All the data were obtained in the same perfusion experiment (liver weight 294 g) under comparable conditions of vascular tone. Mean control hepatic arterial vascular resistance was 0.98 ± 0.09 mmHg ml^{-1} min.

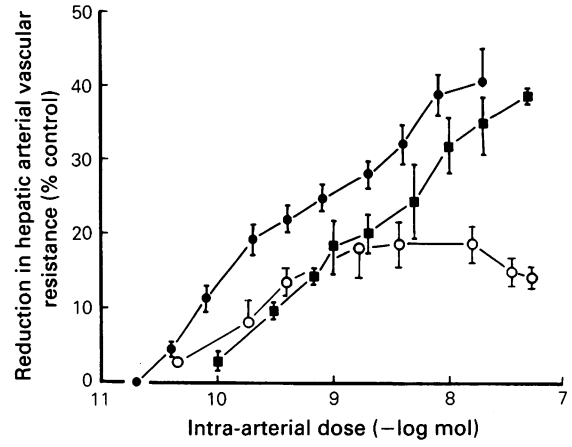


Figure 3 Relationship between the intra-arterial molar dose of isoprenaline (●), atrial natriuretic factor (■) and adrenaline (○) and the decrease in hepatic arterial vascular resistance (hepatic arterial vasodilatation) expressed as a percentage of the control resistance prior to the injection. The points represent the means and the vertical bars the s.e. of at least 6 observations. The data for isoprenaline and atrial natriuretic factor were from the current series whilst the adrenaline results come from a previous series of similar preparations (see Richardson & Withrington, 1977; Figure 2).

ranolol (Richardson & Withrington, 1977) and the selective β_2-adrenoceptor antagonist, ICI 118,551 (Withrington, 1987). The threshold dose was usually between 0.1 and 0.5 nmol and the maximum vascular response achieved at either 10 or 20 nmol. In the current series of perfusion experiments the mean maximum increase in blood flow to Iso was 72 ± 16% of the control flow whilst the mean molar dose of Iso to decrease the hepatic arterial vascular resistance by 50% of the maximum was 0.42 ± 0.13 nmol.

Atrial natriuretic factor In the 6 arterial perfusion preparations ANF was administered as a bolus injection (on 54 occasions), in doses from 0.1–50 nmol to construct 8 dose-response curves, in most cases from the threshold value to maximum effect. The threshold dose of ANF to obtain a hepatic arterial response was usually 0.1–0.5 nmol whilst the maximum response was elicited at either 20 or 50 nmol. The only hepatic arterial vascular response to i.a. ANF was an increase in arterial blood flow, graded with dose, and of the same time course as that observed with i.a. Iso (Figure 1). These increases in arterial flow at constant perfusion pressure reflect hepatic arterial vasodilatation. A vasoconstrictor response to intra-arterial ANF was never observed. In any individual experiment (e.g. Figure 2) the dose-response curve relating the molar dose of ANF to the increase in hepatic arterial blood flow always lay to the right of that for Iso; i.e. it was less potent hepatic arterial vasodilator.

The mean maximum increase in hepatic arterial blood flow to ANF was 61 ± 11% of the control value; this is not significantly different ($P > 0.5$) from the maximum vasodilator effect to i.a. Iso. However there is a difference in the position of the mean dose-response curve (Figure 3) relating the molar dose of Iso or ANF to the decrease in hepatic arterial vascular resistance. A consequence of this is that the mean molar ED_{50} to ANF i.e. the mean molar dose of ANF to reduce the vascular resistance to 50% of the maximum response, was 2.78 ± 0.87 nmol; a value significantly greater than the ED_{50} for Iso (0.42 nmol) in the same experiments ($P < 0.01$) i.e. on a molar basis ANF was less potent than Iso. In a previous series of identical experiments (Richardson & Withrington, 1977) the dose-response curve for adrenaline was constructed and this is included in Figure 3 for comparison (see Discussion).

Hepatic portal responses to intraportal injections of atrial natriuretic factor and noradrenaline

ANF was injected as a bolus into the portal circuit on 22 occasions in four perfusion preparations in doses ranging from 1.0–50 nmol. The results were unequivocal in that no change in portal inflow pressure was observed on any occasion (Figure 4). These observations clearly indicate that, at constant inflow volume, no change in portal inflow resistance was elicited with the range of doses of ANF administered in this series. In 3 of the 4 experiments the higher intraportal bolus doses of ANF were observed to cause systemic hypotension indicating passage of the peptide through the liver into the systemic circulation and peripheral vasodilatation thus confirming the biological activity of the injectate. In the same experiments noradrenaline (NA) was administered (6.0–600 nmol) intraportally to elicit graded increases in portal pressure and portal vasoconstriction confirming the reactivity of the preparation.

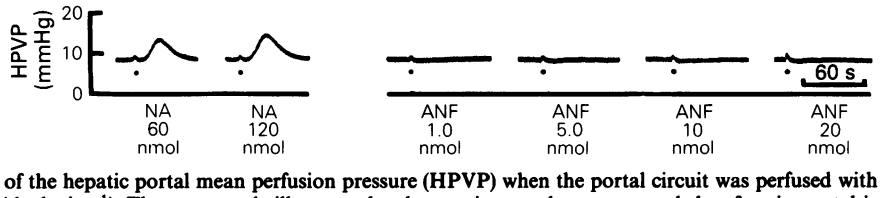


Figure 4 Records of the hepatic portal mean perfusion pressure (HPVP) when the portal circuit was perfused with mesenteric blood at constant flow (146 ml min^{-1}). The two panels illustrate the changes in portal pressure, and therefore in portal inflow resistance in response to intraportal bolus injections of noradrenaline (NA, 60 and 120 nmol) or atrial natriuretic factor (ANF 1.0, 5.0, 10 and 20 nmol). Liver weight 588 g.

Discussion

The infusion of ANF (0.5 $\mu g\,min^{-1}$) into normal man causes a 20% reduction in apparent liver blood flow (Biollaz *et al.*, 1986) as assessed by iodocyanin green clearance technique. However, such a fall in flow may arise from the reflex adjustments of the hepatic circulation to maintain systemic blood pressure. In addition such information does not provide data on any differential flow changes in the two hepatic inflow circuits. We have been concerned to establish any selective actions of ANF on those parameters of the hepatic circulation which control total liver blood flow and its distribution between the arterial and portal inflow circuits.

Histochemical studies have indicated the presence of ANF binding sites along the hepatic arterial and portal vascular territories. Using [I^{125}]-ANF, Bianchi *et al.* (1985), demonstrated labelling in the endothelium and smooth muscle of both arterial and portal inflow circuits of the rat. No binding sites were observed along the mesenteric artery.

Close-arterial injection of ANF avoids any of the reflex changes in the peripheral circulation which inevitably follow systemic administration with consequent falls in mean BP. It also mimics the normal route of access of ANF into the liver via the arterial supply. In the current experiments, ANF caused a marked hepatic arterial vasodilatation of short duration with a maximum percentage increase in blood flow not significantly different from that elicted by the non-selective β-adrenoceptor agonist, Iso. However, the molar potency of ANF, as assessed by the molar ED_{50}, was significantly less than Iso.

However, Iso is not a natural endogenous agent so that a more meaningful comparison, to assess any physiological role of ANF, is with the mixed α- and β-adrenoceptor agonist adrenaline (Ad). The hepatic arterial responses to i.a. ADR are complex (Figure 3) since they represent the overlapping excitation of both α- and β_2-adrenoceptors (Richardson & Withrington, 1977) with opposing vascular effects of vasoconstriction and vasodilatation respectively. At the lower bolus dose range (less than 10^{-9} mol) then ANF and Ad are equipotent as hepatic arterial vasodilators. As the bolus dose is increased the dilator activity of Ad declines presumably because its α-agonist vasoconstrictor properties become dominant. Therefore at doses above 10^{-9} mol, ANF is the more potent hepatic vasodilator. Since different systemic situations provoke the release of ANF and Ad, this different pattern of responses of the hepatic arterial vasculature may be appropriate. Clearly a next step in the evaluation of any potential vascular role of ANF is to examine the hepatic vascular interactions between ANF and other systemic vasoactive agents.

The effect of ANF on portal haemodynamics is more difficult to assess. In the present experiments ANF caused no change in portal inflow resistance when the bed was perfused with mesenteric venous blood at constant flow. In this series no experimental information was obtained about ANF on the other principal determinant of portal haemodynamics, mesenteric flow. Employing chronically implanted pulsed Doppler flow probes in the conscious normotensive rat, Gardiner *et al.* (1988) observed a fall in mesenteric flow and vasoconstriction following bolus i.v. ANF. Garcia *et al.* (1984) described the relative insensitivity of isolated mesenteric resistance vessels to the relaxant properties of ANF compared to preparations from other vascular territories. Tentative conclusions from these results might explain the fall in total hepatic flow reported in man by Biollaz *et al.* (1986) since the change in total flow would depend upon the respective extents of arterial dilatation and the fall in portal inflow from the mesenteric circuit. ANF appears to have a unique spectrum of pharmacological activity since it causes marked hepatic arterial dilatation yet without either mesenteric or hepatic portal vasodilatation. It may therefore change total liver blood flow in favour of the arterial component. This differential activity of ANF may suggest some therapeutic potential.

It is clear, however, that in terms of its absolute molar potency, ANF is amongst the least potent vasodilator substances present in the hepatic inflow circuits. Many other vasoactive substances, particularly peptides of gastrointestinal origin (see review, Withrington & Richardson, 1989) enter the liver in the portal inflow principally during digestion. It is now established that vasoactive substances present in the portal inflow alone may nevertheless have access to hepatic arterial resistance sites by 'transhepatic routes' (Richardson & Withrington, 1978; Lautt *et al.*, 1984). It remains an important aspect of hepatic circulatory physiology to establish any interactions, on the liver vasculature, between substances of primarily systemic origin (i.e. ANF) with those of GI origin (i.e. substance P). In this way the extent to which hepatic haemodynamics is related to, and integrated with, systemic and GI events, may be evaluated.

The physiological and pathophysiological role of ANF in liver haemodynamics remains to be fully elucidated. In the absence of other changes then hepatic arterial vasodilatation to ANF would increase the extent of fluid formation within the liver by increasing mean capillary pressure, enlarge the liver and promote movement of fluid across the liver capsule into the peritoneal space. In this way ANF may contribute towards buffering the effects of increased venous pressure by temporary occupation of an enlarged extravascular space. In the chronic condition this movement of fluid would lead to ascites and the presence of ANF in ascitic fluid (Gerbes *et al.*, 1988) confirms the access of ANF to the hepatic microvasculature in conditions of hepatic cirrhosis and therefore its potential role as a factor contributing towards those hepatic circulatory changes characteristic of cirrhosis.

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Use-dependent effects of pirmenol on $\dot{V}_{max}$ and conduction in guinea-pig ventricular myocardium

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1 The use-dependent effects of pirmenol, a new antiarrhythmic drug, on the maximal rate of rise ($\dot{V}_{max}$) of the action potential, conduction velocity, and their corresponding recovery kinetics were studied in isolated papillary muscles of guinea-pig. Standard microelectrode techniques were used to monitor the conduction and action potential characteristics of the muscles.

2 Pirmenol decreased $\dot{V}_{max}$ and the overshoot of action potentials in a dose-dependent fashion. Also, doses of pirmenol greater than 1 mM abolished the generation of action potentials. Low concentrations of pirmenol (3 and 10 μM) prolonged the action potential duration, while concentrations greater than 0.1 mM shortened it markedly.

3 The resting block of $\dot{V}_{max}$ in the presence of 10 and 30 μM pirmenol was 9.48 ± 3.12 and 20.36 ± 3.61%, and that of conduction velocity 2.87 ± 1.52 and 6.58 ± 2.09%, respectively.

4 The degree of use-dependent block induced by 10 and 30 μM pirmenol during 0.2, 1, 2 and 3 Hz stimulations was dose- and rate-dependent.

5 In the presence of 30 μM pirmenol, mean values of time constants for the onset of the use-dependent inhibition of $\dot{V}_{max}$ and conduction velocity during a 2 Hz stimulation were 1.32 ± 0.15 and 1.28 ± 0.09 s, respectively. The recovery time constants averaged 15.83 ± 2.14 (for $\dot{V}_{max}$) and 27.80 ± 8.74 (for conduction velocity) s in the presence of 30 μM pirmenol.

6 These results showed that the characteristics of the use-dependent inhibition of $\dot{V}_{max}$ and conduction velocity induced by pirmenol are similar to those of slow kinetic drugs such as disopyramide rather than of fast ones.

Introduction

Pirmenol, *cis*-(±)-α-[3-(2,6-dimethyl-1-piperidinyl)propyl]-α-phenyl-2-pyridinemethanol monohydrochloride, is a new antiarrhythmic agent which has been studied in cardiac muscle *in vitro* (Reder *et al.*, 1980; Dukes *et al.*, 1986; Nakaya *et al.*, 1988). Furthermore, pirmenol has already undergone some experimental (Mertz & Steffe, 1980; Steffe *et al.*, 1980; Mertz & Kaplan, 1982; Lynch *et al.*, 1986; Hashimoto *et al.*, 1988) as well as clinical studies to assess its antiarrhythmic potential *in vivo* (Chang, 1987; Estes *et al.*, 1987; Farnham, 1987; Nieminen *et al.*, 1987). Pharmacological studies have indicated that pirmenol is a class I antiarrhythmic drug (Reder *et al.*, 1980; Nakaya *et al.*, 1988) and resembles disopyramide (Dukes *et al.*, 1986) in its electrophysiological effects on the maximum rate of rise of the action potential ($\dot{V}_{max}$) and the action potential duration of the myocardium. Class I drugs block the rapid inward sodium current and depress the $\dot{V}_{max}$ of the fast response.

Recent studies based on antiarrhythmic drug interactions with myocardial Na^+ channel have shown that class I drugs act in various ways to produce use-dependent suppression of $\dot{V}_{max}$ (Hondeghem & Katzung, 1977; 1980; Courtney, 1980a,b; Kohlhardt, 1982; Campbell, 1983a,b; Kohlhardt *et al.*, 1983; Grant *et al.*, 1984; Varro *et al.*, 1985; Hondeghem, 1987). From their use-dependent kinetics these drugs have been divided into two (Courney, 1980a) or three (Campbell, 1983b) subclasses. However, the kinetics of action of pirmenol remain to be elucidated.

Conduction velocity is influenced by changes not only in $\dot{V}_{max}$ but also in other factors such as membrane capacitance, intracellular resistance, and the height of the action potential. Buchannan *et al.* (1985) have shown that changes in the steady-state conduction velocity produced by antiarrhythmic drugs are proportional to the square root of $\dot{V}_{max}$. If the use-dependent effect of pirmenol on $\dot{V}_{max}$ is not also seen on conduction velocity, a difference between quantitative analysis of drug effect on $\dot{V}_{max}$ *in vitro* and clinical data on conduction may be expected.

The experiments described in this paper were designed to evaluate the use-dependency of the effect of pirmenol on $\dot{V}_{max}$ and conduction velocity in guinea-pig ventricular papillary muscles.

Methods

Papillary muscles (2.5 to 5 mm long and 0.5 to 1.0 mm in diameter) from the right ventricle of 15 guinea-pigs were superfused with aerated (95% O_2, 5% CO_2) Tyrode solution (pH 7.4 ± 0.05) at 36.0 ± 0.2°C. The composition of the normal Tyrode solution in mM was as follows: NaCl 132, KCl 4, $NaHCO_3$ 12, NaH_2PO_4 0.4, $CaCl_2$ 1.8, $MgCl_2$ 1 and glucose 10. The flow rate was kept constant at a rate of 3 ml min^{-1} by using a micro-tube pump (Tokyo Rikakikai, MP-3). The basal part of the muscle was pinned to a silicon rubber floor in the bath by means of a bipolar stimulating electrode which was connected to a stimulator (Nihon Kohden SEN-3201) via an isolator (Nihon Kohden SS-102J). The rate of stimulation was varied as described in the protocol. The duration of the pulses was 1 ms and the voltage was twice the threshold.

Membrane potentials were measured by microelectrodes filled with 3 M KCl (resistance 10–20 Ω) and connected to amplifiers (Nihon Kohden MEZ-7101). Membrane potentials were monitored on an oscilloscope (Nihon Kohden VC-10), photographed by a camera (Nihon Kohden RLG-6201), and recorded simultaneously on a recticorder (Nihon Kohden RJG-4122). Analogue differentiation of the action potentials was obtained with an electronic differentiator that was linear from 0 to 500 V s^{-1} (time constant 10 μs).

To measure the conduction velocity, two separate microelectrode impalements were made in a line along the longitudinal axis of the preparation. Conduction time was defined as the time between the peaks of the two separate $\dot{V}_{max}$ signals.

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Conduction velocity was calculated by dividing the inter-electrode distance, measured by using a calibration graticule in the eyepiece of a binocular microscope, by the conduction time. Values of $\dot{V}_{max}$ of action potentials led from the proximal microelectrode were used for the analysis of the relation between $\dot{V}_{max}$ and conduction velocity.

To assess the effects of pirmenol on the action potential characteristics at a steady state, action potentials were recorded at a stimulation rate of 0.5 Hz in both the absence and presence of pirmenol. The drug was successively applied from lower to higher concentrations at intervals of 30 min or more. The use-dependent block of $\dot{V}_{max}$ was measured by a procedure similar to that previously described (Courtney, 1980a; Kohlhardt, 1982; Campbell, 1983a,b; Kohlhardt *et al.*, 1983; Grant *et al.*, 1984). The preparation was stimulated at rates of 0.2, 1, 2 and 3 Hz for 30–60 s. These trains of stimuli were applied after a rest period of 90 s, which was sufficient for recovery from the use-dependent decrease in $\dot{V}_{max}$ and conduction velocity. To analyse the kinetics of recovery from drug action, the preparation was stimulated by a rapid (2 Hz) train of impulses for 30 s after a 90 s rest period. Stimulation was then terminated and an extra stimulus was applied. The coupling intervals following termination of the trains were progressively increased from 0.5 to 1, 3, 5, 10, 20 and 30 s.

A computer was used to obtain the onset and recovery time constants of $\dot{V}_{max}$ and conduction velocity from the use-dependent effects of the drug, and the slope of the regression line in the relationship between the logarithm of drug-induced changes and time was calculated. The degree of use-dependent block was defined as % decrease of $\dot{V}_{max}$ or conduction velocity compared to the value of the first action potential to the steady-state level. The resting block was defined as % decrease of $\dot{V}_{max}$ or conduction velocity, in the presence of a drug, compared to the values of the first action potential in the control. All experimental results were obtained from a single continuous impalement.

Pirmenol hydrochloride was donated by Warner-Lambert Inc. Values are given as mean ± s.e.mean. Statistical analysis was performed by Student's t test, and P values less than 0.05 were considered significant.

Results

Effects of pirmenol on steady-state action potentials

The effects of pirmenol on the characteristics of action potentials at a stimulation rate of 0.5 Hz ($n = 7$) are shown in Figure 1. Pirmenol decreased $\dot{V}_{max}$ (B) and the overshoot (D) of action potentials in a dose-related manner, and abolished any generation of action potentials at doses greater than 1 mM. Low concentrations of pirmenol (3 and 10 μM) prolonged the action potential duration measured at 90% repolarisation, while concentrations greater than 0.1 mM shortened it significantly (C). The resting potential of the membrane was not affected by applications of pirmenol (D).

Resting block of $\dot{V}_{max}$ and conduction velocity

The mean values of resting block induced by 10 and 30 μM pirmenol were 9.48 and 20.36% (for $\dot{V}_{max}$, $P < 0.005$), and 2.87 and 6.58% (for conduction velocity, $P < 0.05$), respectively (Table 1). The extent of resting block increased in a concentration-dependent manner and, in the presence of 30 μM

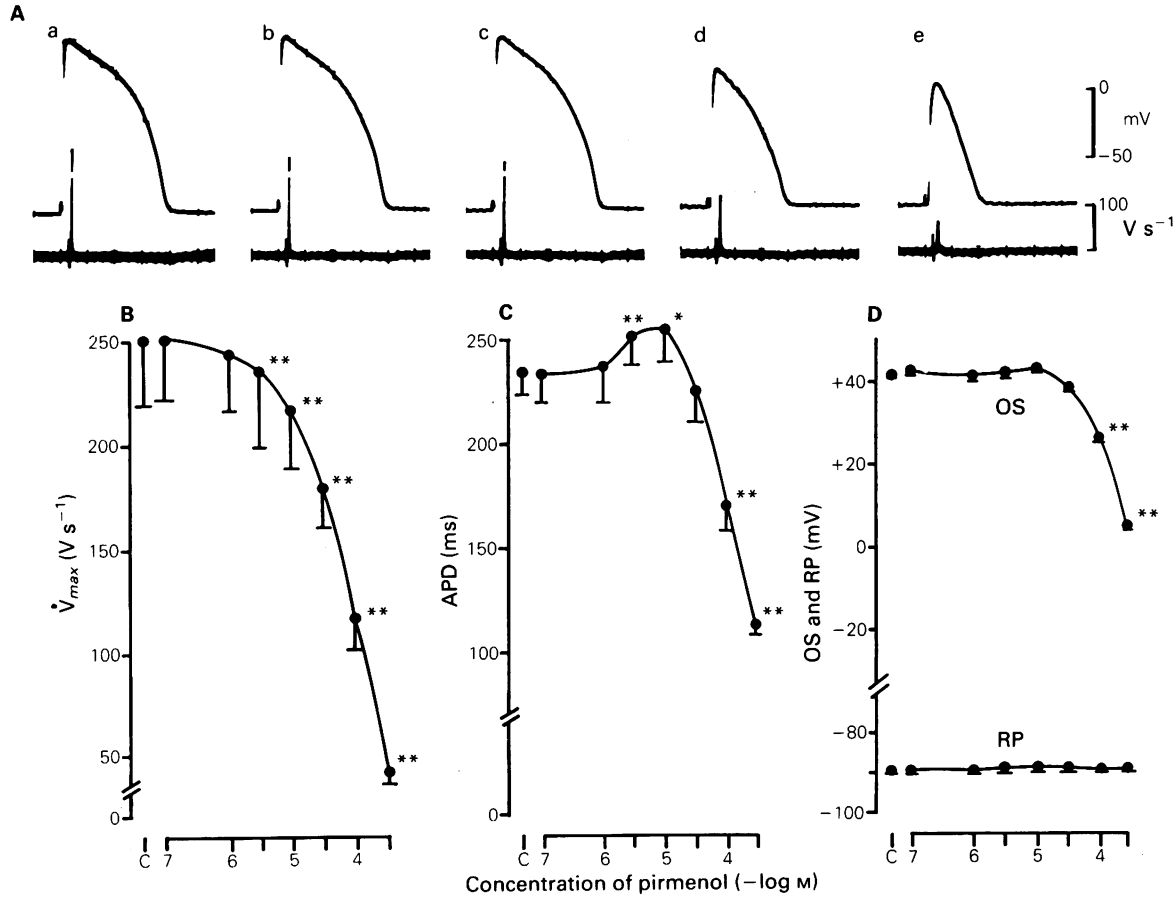


Figure 1 The depressant effect of pirmenol on the steady-state action potential. Action potentials and $\dot{V}_{max}$ stimulated at a constant rate of 0.5 Hz in control (A,a), in the presence of 10 (A,b), 30 (A,c), 100 (A,d) and 300 (A,e) μM pirmenol are shown. Dose-response relationships on $\dot{V}_{max}$ (B), duration (C) and overshoot (D, OS) of action potentials and resting potential (D, RP) are illustrated. Each point is the mean value of 7 experiments, vertical bars show s.e.mean. $\dot{V}_{max}$: maximum rate of rise of action potential. APD: action potential duration measured at 90% repolarisation. The C on the abscissa scales indicate the control value. * $P < 0.05$, ** $P < 0.01$.

Table 1 Resting and use-dependent block induced by pirmenol (%)

	Resting block	*Use-dependent block* 0.2 Hz	1 Hz	2 Hz	3 Hz
$\dot{V}_{max}$ (n = 7, 8)					
10 μM	9.48 ± 3.12 ***	8.47 ± 1.56 ****	15.40 ± 1.77 **	21.44 ± 1.37 ****	26.48 ± 1.39 ****
			**	***	***
30 μM	20.36 ± 3.60 *	18.77 ± 2.44	33.25 ± 4.64 *	36.36 ± 3.32 ***	42.78 ± 4.21 ****
CV (n = 4)					
10 μM	2.87 ± 1.52 *	6.00 ± 1.37	7.66 ± 2.28 *	9.92 ± 2.99 *	12.93 ± 3.94 *
				*	
30 μM	6.58 ± 2.09	14.25 ± 4.12	16.58 ± 4.52	19.30 ± 4.69 *	22.65 ± 6.03 *

Values are mean ± s.e.mean. CV: conduction velocity; $\dot{V}_{max}$: maximal rate of rise of action potential. The control values of $\dot{V}_{max}$ and CV were 211.6 ± 18.0 Vs^{-1} and 0.716 ± 0.083 ms^{-1}, respectively. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$, **** $P < 0.001$.

pirmenol the effect on $\dot{V}_{max}$ was more prominent than that on conduction velocity ($P < 0.05$).

Onset of use-dependent effect of pirmenol on $\dot{V}_{max}$

After resting block of $\dot{V}_{max}$ induced by pirmenol, a further decline of $\dot{V}_{max}$ to a new steady-state level during the train of stimuli indicated use-dependent block. Pirmenol-induced use-dependent inhibition of $\dot{V}_{max}$ depended on the concentration ($P < 0.01$–$P < 0.005$) and the stimulation frequency ($P < 0.05$–$P < 0.001$). Typical experimental results are shown in Figure 2. Development kinetics of use-dependent block at different stimulation rates can be defined as either a time- or an event-dependent process. In the presence of 10 μM pirmenol, the mean values for the time constant of onset of the use-dependent block (τ_{on}, s) during a 1, 2 and 3 Hz stimulation were 5.90, 3.25 and 1.67, respectively. In the presence of 30 μM pirmenol, values for τ_{on} (s) were 13.44 (0.2 Hz), 3.03 (1 Hz), 1.32 (2 Hz) and 1.05 (3 Hz) (n = 7, Table 2).

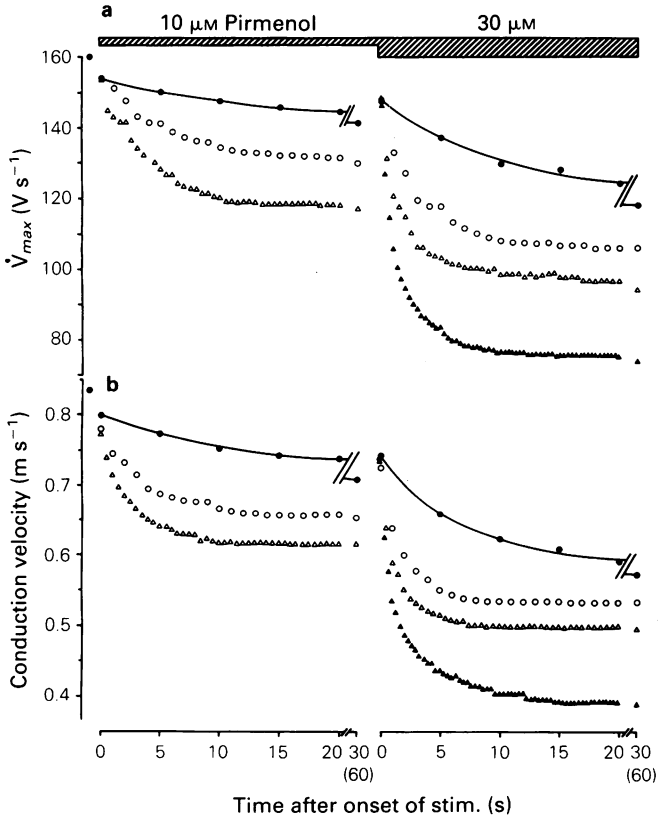


Figure 2 Onset rates of use-dependent block of $\dot{V}_{max}$ and conduction velocity. The kinetics of development of the use-dependent block of $\dot{V}_{max}$ (a) and conduction velocity (b) at a stimulation rate of 0.2 Hz (●), 1 Hz (○), 2 Hz (△) and 3 Hz (▲) for 30 (60 for 0.2 Hz) s in the presence of 10 and 30 μM pirmenol are shown. The initial reduction from the control value represents the resting block induced by pirmenol, and the further decline shows the onset of a use-dependent block. $\dot{V}_{max}$ and conduction velocity show similar reduction rates.

Onset of use-dependent effect of pirmenol on conduction velocity

The use-dependent suppression of conduction by pirmenol is shown in Figure 2. According to the reduction of $\dot{V}_{max_1}$ ($\dot{V}_{max}$ of action potential measured at a proximal site), repetitive stimulations suppressed $\dot{V}_{max_2}$ ($\dot{V}_{max}$ of action potential measured at a distal site) and prolonged the distance between $\dot{V}_{max_1}$ and $\dot{V}_{max_2}$ (Figure 3).

The extent of use-dependent block induced by pirmenol increased in a stimulation frequency- ($P < 0.05$, $1 < 2 < 3$ Hz) and concentration-dependent manner ($P < 0.05$ at 1 and 2 Hz stimulations) (Table 1). The degree of suppression of conduction velocity by pirmenol was less than that of $\dot{V}_{max}$ ($P < 0.05$–0.005 at 1, 2 and 3 Hz stimulations) as expected from its quadratic relation. In the presence of pirmenol, time constants of onset of use-dependent conduction block became shorter as the stimulation frequency and the concentration of drug increased. These results showed a similar trend for $\dot{V}_{max}$ as well (Table 2).

Recovery from the use-dependent effect of pirmenol

To investigate and compare the rest recovery process from use-dependent inhibition of $\dot{V}_{max}$ and conduction velocity by pirmenol, a single extrastimulus was applied after the cessation of each train of 2 Hz stimulations. Figure 3 shows the results of one experiment for $\dot{V}_{max}$ and conduction. In the presence of 30 μM pirmenol the mean recovery time constant from the use-dependent block of $\dot{V}_{max}$ for 8 experiments was 15.73 s. The mean recovery time constant of conduction velocity for 4 experiments was 27.80 s for 30 μM pirmenol. (The recovery time constant for $\dot{V}_{max}$ for 4 experiments that corresponded to the conduction velocity measurements was 15.83 ± 2.14 s.)

The recovery rate for $\dot{V}_{max}$ was somewhat faster than that of the conduction velocity, but this was not significant ($P = 0.07$). Figure 4 represents the results of one such experiment.

Discussion

Pirmenol depressed the $\dot{V}_{max}$ by 6 and 13% at concentrations of 3 and 10 μM, respectively, but at the same time it increased the action potential duration of guinea-pig ventricular muscles. A previous study in canine Purkinje fibres showed that pirmenol had effects on $\dot{V}_{max}$ at concentrations greater than 1 μM and on action potential amplitude and duration when concentrations were greater than 10 μM (Reder *et al.*, 1980). Dukes *et al.* (1986) studied the depressant effects of pirmenol on $\dot{V}_{max}$ in the atrial and ventricular muscles as well as the Purkinje cells of rabbit heart at concentrations greater

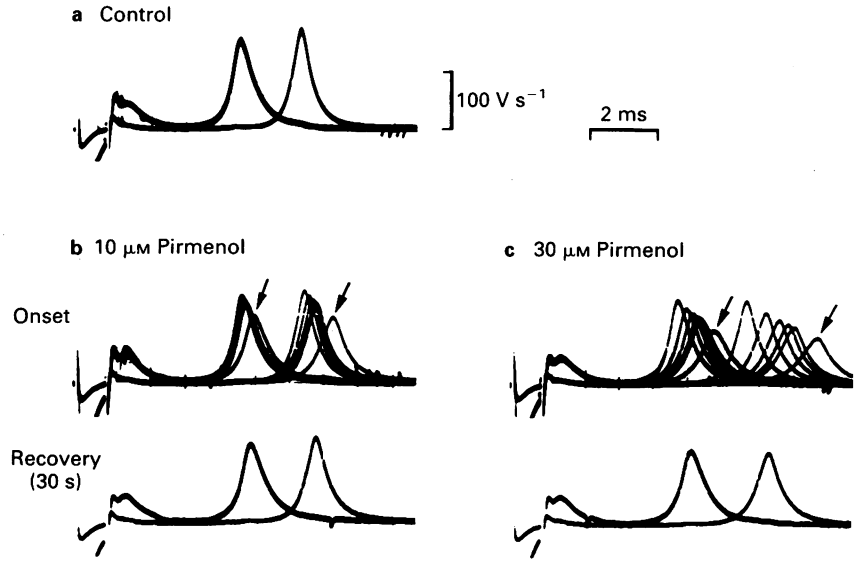


Figure 3 Recovery of $\dot{V}_{max}$ and conduction velocity from a pirmenol induced use-dependent block. (a) The first 5 $\dot{V}_{max}$ signals during a 2 Hz stimulation for 30 s in the control are superimposed. (b and c) The first 5 and the last (steady-state value indicated by arrows) $\dot{V}_{max}$ signals during a train of stimulation (Onset, upper traces), and $\dot{V}_{max}$ signals induced by one extrastimulation after a 30 s pause (Recovery, lower traces) were recorded in the presence of 10 (b), and 30 (c) μM pirmenol.

Table 2 Onset and recovery time constants of use-dependent block of $\dot{V}_{max}$ and conduction velocity induced by pirmenol (s)

	Onset				
	0.2 Hz	*1 Hz*	*2 Hz*	*3 Hz*	*Recovery*
$\dot{V}_{max}$					
10 μM		5.90 ± 1.99	3.25 ± 0.54 } ***	1.67 ± 0.38	
30 μM	13.44 ± 2.24 ***	3.03 ± 0.27	1.32 ± 0.15 } ****	1.05 ± 0.26 ***	15.73 ± 2.29
CV					
10 μM		3.00 ± 0.60	2.44 ± 0.45 } *	0.93 ± 0.23 *	
30 μM	40.53 ± 26.73	2.03 ± 0.31	1.28 ± 0.09 } *	0.93 ± 0.08 *	27.80 ± 8.74

Values are mean ± s.e.mean. For 0.2 Hz stimulation $n = 3$, $n = 7$–8 for $\dot{V}_{max}$ and $n = 4$ for conduction velocity during 1, 2 and 3 Hz stimulation and experiments for recovery. * $P < 0.05$, *** $P < 0.005$, **** $P < 0.001$. In the presence of 30 μM pirmenol the onset time constant for conduction velocity is faster than that for $\dot{V}_{max}$ only at 1 Hz stimulation ($P < 0.01$). Stimulation frequency-dependent differences were compared with values at 1 Hz.

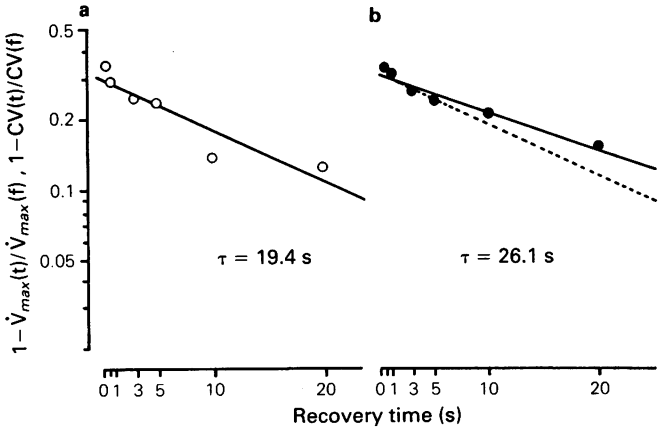


Figure 4 Recovery rates from the pirmenol-induced use-dependent block of $\dot{V}_{max}$ and conduction velocity. The recovery kinetics from the use-dependent block of $\dot{V}_{max}$ (a, ○) and conduction velocity (b, ●) were analysed against the coupling interval of single stimulus, after cessation of a 2 Hz train of stimuli in the presence of 30 μM pirmenol. Results of a typical experiment are shown. For comparison, in (b) the $\dot{V}_{max}$ slope (dashed line) was plotted against the conduction velocity line of regression.

than 1.5 μM. The difference in concentrations at which pirmenol exerts its effects may be due to variations in the cardiac tissues, species of animal and K^+ concentrations of Tyrode solution used, which are responsible for the resting potential. The dual effects of pirmenol on action potential duration observed in the present study show similarities with results from a previous study on the same preparation (Nakaya *et al.*, 1988), namely 3 and 10 μM pirmenol prolonged and 100 μM pirmenol shortened the action potential duration significantly. At clinically used concentrations, 7.3 to 9.1 μM for intravenous administration (Estes *et al.*, 1987; Nieminen *et al.*, 1987) and 4.7 to 7.7 for oral administration (Farnham, 1987; Estes *et al.*, 1987), pirmenol seems to show the ability to prolong action potentials.

The characteristics of the use-dependent inhibition of $\dot{V}_{max}$ induced by pirmenol are similar to those of slow kinetic drugs, such as disopyramide which was shown to have a recovery time constant of 12.0 to 37.8 s (Campbell, 1983a; Varro *et al.*, 1985), rather than of fast kinetic drugs, according to the classification proposed by Courtney (1980a). Previous studies indicate that the onset rate of the use-dependent block correlates directly with molecular weight, (Courtney, 1979; 1980a,b) and increases with increasing drug concentration (Courtney, 1980a; Gintant *et al.*, 1983; Grant *et al.*, 1984). The studies of

recovery of $\dot{V}_{max}$ from the block show a strong correlation between molecular weight and recovery times (Courtney, 1980a,b; Grant *et al.*, 1984). The relationship between the use-dependent kinetics and pK_a (the dissociation constant), or the log P (the log of the n-octanol : partition coefficient) has also been investigated (Courtney, 1979; 1980a,b; Grant *et al.*, 1984). Pirmenol (338.5) has a very similar molecular weight to disopyramide (339) and a somewhat larger molecular weight than quinidine (324). Values of p*K*a and log P for pirmenol are 4.0 and 0.52, respectively. The results of the present experiments were compatible with these observations. Hence, the slow recovery kinetics of pirmenol may be due mostly to its molecular size.

In the present study, the relationship between pirmenol-induced inhibition of $\dot{V}_{max}$ and conduction velocity were examined with respect to the degree of inhibition and the onset and recovery rate from the use-dependent block. The extents of both the resting and use-dependent block (%), induced by pirmenol, were dose- and stimulation frequency-dependent. The degrees of these blocks for $\dot{V}_{max}$ were much larger (nearly double) than those for the conduction velocity (Table 1). These variations between the degrees of block in $\dot{V}_{max}$ and in conduction velocity seem to be compatible with the quadratic relationship previously described by Buchanan *et al.* (1985).

As summarized in Table 2, the recovery rate of the conduction velocity from the use-dependent block induced by pirmenol was much slower than that of $\dot{V}_{max}$, whereas the onset rate of use-dependent block of the conduction velocity showed similar or slightly faster values than that of $\dot{V}_{max}$ for each stimulation frequency. A train of stimulation has been shown to increase intracellular Ca^{2+} activity (Lado *et al.*, 1982) which leads to cell decoupling, and increases intercellular (Spray *et al.*, 1982) and inside longitudinal resistance (Dahl & Isenberg, 1980). This may be responsible for discrepancies in the recovery time constants between $\dot{V}_{max}$ and conduction velocity observed in the present study. In summary, pirmenol has electrophysiological properties which suggest that it can be categorized as a class I antiarrhythmic drug and a slow kinetic drug such as disopyramide, according to the classification proposed by Courtney (1980a).

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Investigation of the selectivity of α, β-methylene ATP in inhibiting vascular responses of the rat *in vivo* and *in vitro*

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1 The proposal that α,β-methylene adenosine 5'-triphosphate (mATP) inhibits pressor responses in the pithed rat by selective desensitization of P_{2x}-purinoceptors was examined by comparing the selectivity of its inhibitory effect on vascular responses *in vitro* and *in vivo*.

2 In isolated ring preparations of rat femoral and tail artery, which had been denuded of endothelium, mATP markedly reduced the contractile response to exogenous ATP but had no effect on the response of the arteries to exogenous noradrenaline (NA).

3 In the pithed rat a substantial proportion of the pressor response to sympathetic nerve stimulation was resistant to α-adrenoceptor blockade, suggesting a non-adrenergic component to the sympathetic vasoconstriction.

4 In the pithed rat, repeated administration of desensitizing doses of mATP attenuated the pressor response to sympathetic nerve stimulation by approximately 80%, suggesting that a component of the sympathetic vasoconstriction is mediated by ATP acting on vascular P_{2x}-purinoceptors. However, the same mATP treatment also attenuated, to a similar degree, the pressor responses to intravenous NA, angiotensin II and vasopressin, indicating that the desensitization procedure was non-selective.

5 These results demonstrate that while mATP can be used to desensitize selectively P_{2x}-purinoceptors *in vitro*, its attenuation of the sympathetic nerve-mediated pressor response *in vivo* is non-selective.

Introduction

The stable analogue of adenosine 5'-triphosphate (ATP), α,β-methylene ATP, has been shown to reduce selectively responses of various isolated smooth muscle preparations to exogenously applied ATP *in vitro* by desensitizing the P_{2x}-purinoceptors (Kasakov & Burnstock, 1982; Meldrum & Burnstock, 1983). Thus, mATP has been used extensively *in vitro* to examine cotransmission by noradrenaline (NA) and ATP in a variety of sympathetically innervated tissues, including vascular smooth muscle (see for example Sneddon & Burnstock, 1984; Kennedy *et al.*, 1986; Machaly *et al.*, 1988). There is evidence that in some vessels, such as the rabbit saphenous artery, most of the sympathetic vasoconstriction is mediated by ATP acting on P_{2x}-purinoceptors (Burnstock & Warland, 1987).

The pressor response of the pithed rat to electrical stimulation of the preganglionic sympathetic nerves is biphasic, the first phase being due to direct vasoconstriction and the second phase due to the action of released adrenomedullary catecholamines on the vasculature (Gillespie *et al.*, 1970; Flavahan *et al.*, 1985). The initial 'neuronal' phase of this response has been shown to be resistant to a combination of α_1- and α_2-adrenoceptor blockade, whilst the secondary 'adrenal' phase was abolished by this treatment (Flavahan *et al.*, 1985). These findings are of particular interest in the light of *in vitro* investigations which suggest the involvement of a non-adrenergic neurotransmitter, possibly ATP, acting as a cotransmitter with NA, in vascular sympathetic nerves (Sneddon & Burnstock, 1984; Muramatsu *et al.*, 1984; Muramatsu, 1986; 1987; Burnstock & Warland, 1987; Nagao & Suzuki, 1988; Machaly *et al.*, 1988).

Until recently the *in vivo* use of mATP to examine the α-antagonist-resistant component of the nerve-mediated vasoconstriction for a possible purinergic involvement was complicated by several factors. Firstly, mATP ($\geq 25\,\mu g\,kg^{-1}$) stopped the heart. Secondly, mATP had no effect on the sympathetic nerve-mediated pressor response before α-adrenoceptor blockade. Thirdly, only partial antagonism of the α-blocker-resistant pressor response was observed after cumulative addition of mATP (0.005–0.1 mg kg^{-1}) (Flavahan *et al.*, 1985). More recently, it was found that a novel administration regime of mATP in the pithed rat avoided the cardiotoxic effects of the drug. This modified regime of administration of mATP reduced the sympathetic pressor response to spinal stimulation by 60% in the absence of α-adrenoceptor blockade, and completely abolished the residual response after α-adrenoceptor antagonism (Bulloch & McGrath, 1988). The previous inability of mATP to affect the pressor response before α-antagonism, or to abolish the residual response after α-antagonism completely (Flavahan *et al.*, 1985), was attributed to the transient nature of its action. Thus, the effects of mATP and α-adrenoceptor antagonists appeared to be additive, and dissect the sympathetic pressor response of the pithed rat into α-adrenergic (40%) and purinergic (60%) components. The use of mATP was demonstrated to be selective in that it had no effect on the pressor response to intravenously administered NA (Bulloch & McGrath, 1988).

In the present study, the selectivity of mATP was compared *in vitro* and *in vivo*. This was done by establishing the selectivity of mATP in isolated arteries from the rat, and by examining the selectivity of the novel mATP desensitization procedure (Bulloch & McGrath, 1988) in the pithed rat by use of sympathetic nerve stimulation and several pressor agents.

A preliminary account of this work has been presented to the British Pharmacological Society (Dalziel *et al.*, 1989).

Methods

In vitro *recording of vasoconstrictor responses*

Male Sprague-Dawley rats (250–300 g) were killed by stunning and exsanguination. The tail was amputated, and the covering skin layer removed. The caudal artery was excised from the ventral surface and carefully cleared of any connective tissue under Krebs solution. The femoral artery was ligated close to the abdominal aorta. These arteries were carefully removed and placed in bubbled Krebs solution (see below). Arterial rings (4 mm in length, 0.5–1.0 mm in diameter) were prepared from both arteries, the proximal end being used in the case of the tail artery.

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The artery rings were mounted in jacketed 10 ml organ baths using 2 parallel stainless steel wires, one of which was attached to a force-displacement transducer (Grass FTO3C). Each ring was allowed to equilibrate for 1 h at a resting tension of 1.0 g. Isometric contractions were recorded on a Grass 79D four channel pen recorder. Preliminary experiments indicated that insertion of the wires into the lumen in this manner removed endothelial responses such that no relaxant responses to acetylcholine (10 μM) were observed in arteries precontracted with NA (1 μM). All drug actions, therefore, were assumed to be due to a direct effect on the smooth muscle. The Krebs solution used throughout these experiments was of the following composition (mM): NaCl 118, KCl 5.4, NaH_2PO_4 1.16, $NaHCO_3$ 25, $MgSO_4$ 1.16, $CaCl_2$ 2.5, glucose 11.1. The solution was bubbled with 95% O_2/5% CO_2 and maintained at 36.5 ± 0.5°C.

Drug additions were made directly to the baths. Three reproducible control responses to NA (1 μM) and ATP (1 mM) were obtained in each tissue. Introduction of mATP (2.5 μM), which remained in contact with the tissue for the remainder of the experiment, produced a transient contraction; the tone of the preparations returned towards basal levels over a period of 10–15 min as the P_{2x}-purinoceptors became desensitized. The tissues were re-challenged with NA and ATP after exposure to the mATP, once the baseline tension had recovered.

In vivo *pressor responses in pithed rats*

Male rats (230–270 g) were prepared for artificial ventilation by tracheal cannulation and then pithed while under ether anaesthesia. The tracheal cannula was immediately attached to a small animal ventilator (Harvard), which drew its gaseous supply from a Douglas bag prefilled with 100% O_2. The ventilator was pre-adjusted to deliver 1 ml 100 g^{-1}. The preparation was prepared for electrical stimulation of the preganglionic sympathetic outflows by a modified version of the pithed rat preparation described by Gillespie *et al.* (1970). Electrical stimulation (5 Hz, 0.1 ms, 50 V for 5 s, with a Grass S44 stimulator via a Grass SIU5 stimulus isolation unit) of the preganglionic sympathetic neurones at the level of the 6th thoracic to the 8th thoracic vertebrae (T_6–T_8) produced a substantial pressor response while having little or no chronotropic effects. The left carotid artery was cannulated for monitoring of blood pressure. The cannula was connected to a Gould P23XL pressure transducer, the output from the pressure transducer being displayed on a Gould 2200S 2-channel pen recorder. The jugular veins were then exposed and cannulated for administration of drugs. The temperature of the animal was maintained at 37°C by a heating lamp controlled by a rectal thermistor. Arterial blood samples (0.1 ml) were taken via the carotid cannula and the Po_2 and Pco_2 of the arterial blood determined by an Instrumentation Laboratories (Model 213) blood gas analyser. The ventilation parameters were finely adjusted to achieve a Pco_2 value between 26 and 34 mmHg (the rats were invariably hyperoxic, Po_2 > 280 mmHg, when ventilated with 100% O_2).

Following surgery the preparation was left for 30 min to allow the blood pressure and Pco_2 to stabilise. Gallamine (10 mg kg^{-1} i.v.) was administered, immediately followed by an infusion of gallamine (10 mg kg^{-1} h^{-1}), to achieve neuromuscular blockade. This was to avoid skeletal muscular twitching during electrical stimulation of the spinal neuronal outflows. The rat was then allowed to stabilise for a further 30 min. A single dose of 1 mg kg^{-1} propranolol was administered 10 min before the commencement of the experiment, to erradicate β-adrenoceptor-mediated vasodilatation in response to circulating catecholamines (Flavahan *et al.*, 1985). This dose of propranolol was previously shown (by preliminary experiments) to abolish the depressor response to 1 μg kg^{-1} isoprenaline over the period of the experiment.

Three reproducible responses to pressor agents or electrical stimulation of the sympathetic outflows were obtained before administration of the mATP according to the method of Bulloch & McGrath (1988). The mATP desensitization procedure involved i.v. administration of 3 low doses (0.05 mg kg^{-1}), followed by five higher doses (0.5 mg kg^{-1}), all doses being separated by a period of 1 min. The time cycle of sympathetic nerve stimulation or agonist administration was recommenced 2 min following the last dose of mATP.

Drugs

Drugs were obtained from the following sources: gallamine triethiodide (Flaxedil) from May and Baker Ltd. Adenosine 5'-triphosphate, disodium salt (ATP); α,β-methylene ATP, lithium salt (mATP); angiotensin II, acetate salt; isoprenaline (isoproterenol hydrochloride); noradrenaline ((−)-arterenol hydrochloride, NA); propranolol hydrochloride and [Arg^8]-vasopressin, acetate salt from Sigma Chemicals Ltd. Doses of drugs were calculated as mg of the appropriate salt per kg body weight. In the case of vasopressin the doses given were expressed as iu per kg body weight.

Statistics

Significance of the results was tested by Student's *t* test for paired data, and differences were considered to be statistically significant when $P < 0.05$. All data points in the figures and tables are expressed as the mean ± s.e.

Results

Selectivity of mATP on isolated arteries in vitro

In the isolated tail artery, mATP reduced significantly the magnitude of the contractile response to ATP (1 mM) from 0.49 ± 0.10 g to 0.21 ± 0.07 g ($P < 0.01$, $n = 7$). mATP had no significant effect on the magnitude of the contractile response to NA (1 μM), which was 1.96 ± 0.21 g before and 1.98 ± 0.23 g after introduction of mATP ($n = 7$). Further experiments were performed to examine the effects of mATP on similar sized responses to NA and ATP in the same preparation. In four preparations the responses to NA (0.1 μM) were 0.38 ± 0.10 g before mATP and 0.43 ± 0.12 g after mATP. In contrast, responses to ATP (1 mM) were markedly depressed, being 0.33 ± 0.11 g before and 0.05 ± 0.03 g after mATP. Similarly, in the isolated femoral artery, mATP reduced the contractile response to ATP from 1.05 ± 0.21 g to 0.75 ± 0.21 g ($P < 0.01$, $n = 7$), whereas mATP had no significant effect on the magnitude of the contractile response to NA, which was 2.62 ± 0.41 g before and 2.63 ± 0.41 g after introduction of mATP ($n = 7$).

Pressor responses of the pithed rat to electrical stimulation of the preganglionic, sympathetic neuronal outflows; effect of α-adrenoceptor blockade

Figure 1a shows a typical pressor response of the pithed rat to electrical stimulation of the preganglionic sympathetic spinal nerves at the level of T_6–T_8. The response was biphasic being composed of an initial 'neuronal' and a secondary 'adrenal' component (Flavahan *et al.*, 1985). The neuronal component of the response was largely resistant to α-adrenoceptor blockade since administration of the α-antagonist, phentolamine (1 mg kg^{-1}) produced only a small attenuation of the neuronal component but almost abolished the adrenal component (Figure 1b). A higher dose of phentolamine (5 mg kg^{-1}) had no further inhibitory effect on the pressor response but did unmask a slight vasodilatation which was probably due to the action of endogenous adrenaline on vascular β-adrenoceptors. In all subsequent experiments rats were pretreated with propranolol (1 mg kg^{-1}), as described in the Methods section, to block vascular β-adrenoceptors.

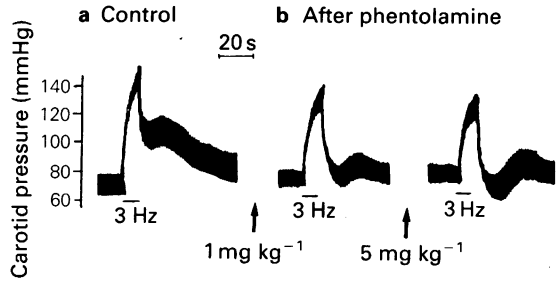


Figure 1 Effect of α-adrenoceptor antagonism on the pressor response to electrical stimulation of the sympathetic neuronal outflows in the pithed rat (3 Hz, 0.1 ms pulse duration, 50 V for 5 s). (a) Control pressor response to sympathetic nerve stimulation. (b) Phentolamine (1 mg kg^{-1}) slightly reduced the initial, neuronal component of the pressor response, and almost abolished the secondary adrenal phase. Subsequent injection of 5 mg kg^{-1} phentolamine had no further inhibitory effect of the neuronal component but converted the adrenal component to a depressor response.

Effect of mATP on pressor responses to sympathetic nerve stimulation

mATP produced a marked, transient, pressor response following each administration, successive doses producing a smaller pressor response (Figure 2). Treatment with mATP produced a marked attenuation of both the neuronal and adrenal components of the response to sympathetic nerve stimulation (5 Hz, 0.1 ms pulse duration, 50 V for 5 s at the level of T_6–T_8) which recovered in a time-dependent manner. Figure 3 represents a suummary of the data and shows in more detail the effect of mATP on each component of this response.

Effect of mATP on pressor responses to exogenous agonists

The effect of mATP on pressor responses to intravenously administered NA (0.1 μg kg^{-1}), angiotensin II (0.1 μg kg^{-1}) and vasopressin (7×10^{-5} i.u. kg^{-1}) is shown in Figure 4. Three reproducible responses were obtained to each of the pressor agents before administration of mATP, the last of the three being shown as the control response in Figure 4. In preliminary experiments, responses to NA were reproducible over the time course of the experiment, the change in mean arterial pressure (MAP) being 22 ± 4, 25 ± 4 and 24 ± 3 mmHg during three successive injections at 30 min intervals ($n = 8$). The mATP desensitization procedure markedly attenuated the pressor response to all 3 of the pressor agents. The observation that the responses to angiotensin II and vasopressin recovered towards the pre-mATP control level with a similar time course to the recovery seen to NA (see Figure 5) or sympathetic nerve stimulation (see Figure 3), indicates that the

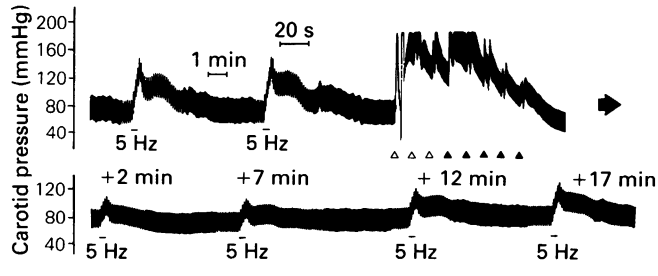


Figure 2 Effect of the α,β-methylene ATP (mATP) desensitization procedure on pressor responses of the pithed rat to sympathetic nerve stimulation (5 Hz, 0.1 ms pulse duration, 50 V for 5 s). Control responses were obtained at 5 min intervals before administration of the first dose of mATP. Doses of mATP were given at 1 min intervals (△, 0.05 mg kg^{-1} and ▲ 0.5 mg kg^{-1}). The 5 min time cycle of spinal stimulation was recommenced 2 min after the last dose of mATP was given, by which time the basal blood pressure had recovered to control levels.

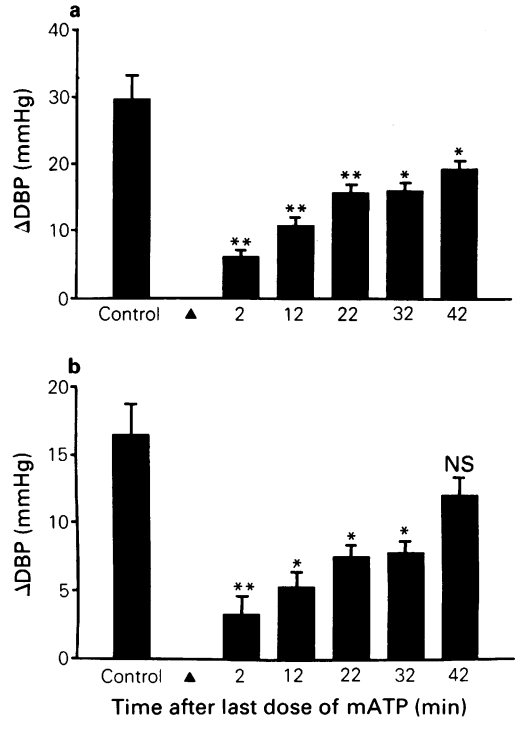


Figure 3 Summary of the effect of the α,β-methylene ATP (mATP) desensitization procedure (▲) on the neuronal phase (a) and adrenal phase (b) of the pressor response to sympathetic nerve stimulation (5 Hz, 0.1 ms pulse duration, 50 V for 5 s), expressed as the change in diastolic blood (ΔDBP). Pressor responses were obtained at 10 min intervals before and after the mATP desensitization procedure. The 10 min time cycle of stimulation was recommenced 2 min after the last dose of mATP was given. Both phases were depressed to a similar degree by the mATP, and both recovered towards control with a similar time course. Each column is the mean of results from 6 animals and vertical bars show s.e.mean. $*P < 0.05$, $**P < 0.01$, NS, no significant difference vs control.

attenuation of these responses was due to the action of mATP. Figure 5 shows, in greater detail the effect of mATP on the pressor response to NA (0.1 μg kg^{-1}). The response was attenuated by approximately 70% at 2 min after the last dose of mATP and subsequently returned towards control values in a time-dependent manner, until after 22 min the response was

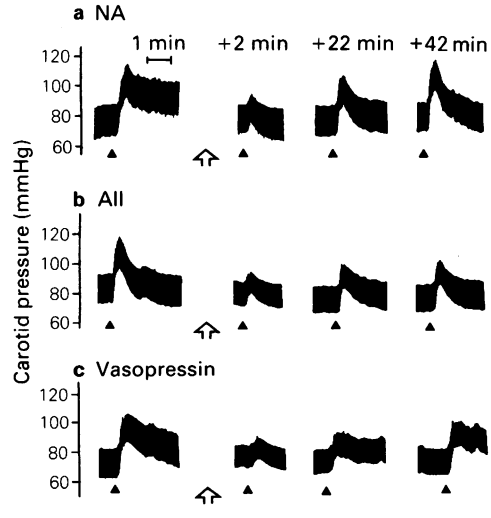


Figure 4 Effect of α,β-methylene ATP (mATP) desensitization (open arrow) on pressor responses to noradrenaline (NA, 0.1 μg kg^{-1}) (a), angiotensin II (AII, 0.1 μg kg^{-1}) (b) and vasopressin (7×10^{-5} iu kg^{-1}) (c). The mATP desensitization procedure reduced the pressor response to each agent to a similar degree.

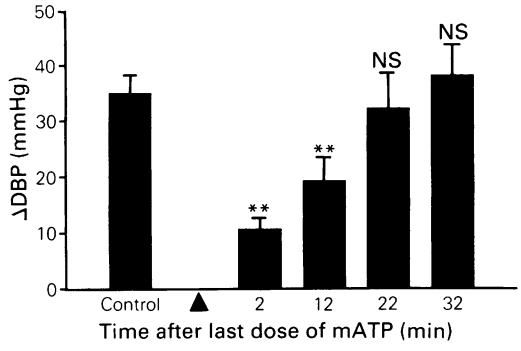


Figure 5 Summary of the effect of the α,β-methylene ATP (mATP) desensitization procedure on pressor responses of the pithed rat to noradrenaline (NA, 0.1 $\mu g kg^{-1}$), expressed as a change in diastolic blood pressure (ΔDBP). Responses to NA were obtained at 10 min intervals before and after the mATP desensitization procedure. The 10 min time cycle of NA administration was recommenced 2 min after the last dose of mATP was given. Each column represents the mean of the results from 6 animals and vertical bars show s.e.mean. $*P < 0.05$, $**P < 0.01$, NS, no significant difference vs control.

not significantly different from control. In some experiments the effect of mATP on sympathetic pressor responses and NA responses was examined in the same animal. In these experiments, the pressor response to both sympathetic nerve stimulation and NA were attenuated to a similar degree and recovered with a similar time course (not shown).

Effect of alterations of the pithed rat model on the selectivity of mATP

The lack of selectivity of mATP for reducing responses to nerve stimulation compared to noradrenaline was independent of the strain of rat employed, since the lack of selectivity was also observed when Wistar rats ($n = 2$) were used instead of the Sprague-Dawley strain in the rest of the experiments. With Wistar rats, the mean change in diastolic blood pressure produced by NA (0.1 $\mu g kg^{-1}$) before mATP was 27.5 mmHg, whilst that for the neuronal phase of the sympathetic nerve-mediated response was 55.0 mmHg. Two minutes after the last dose of mATP the mean change in diastolic blood pressure in response to NA was reduced to 6.5 mmHg. The neuronal phase of the response to sympathetic nerve stimulation 12 min after the last dose of NA was reduced to 20 mmHg.

In another experiment, the concentrations of mATP used in the desensitization procedure were scaled down by a factor of ten. This resulted in a smaller pressor response to mATP and a less marked attenuation of the sympathetic nerve-mediated response by mATP (25% attenuation). However, the attenuation of pressor responses to injected noradrenaline persisted with this alteration (75% attenuation).

To ensure that the hyperoxic state of the rats was not responsible for the lack of selectivity of the mATP desensitization procedure, an experiment was carried out where rats were ventilated with room air (21% oxygen), which produced an arterial Po_2 of approximately 80 mmHg. Under these conditions mATP was still non-selective, in that it reduced the pressor responses to administered NA (by 70%) as well as sympathetic nerve stimulation (by 60%).

Finally, in one experiment the effect of mATP on responses to NA in the absence of gallamine was examined. In the absence of gallamine mATP still produced an 80% reduction of the pressor response to NA.

Discussion

The results presented here, with the rat isolated tail and femoral arteries, confirm that *in vitro*, mATP produces a selective desensitization, in that contractile responses mediated by ATP acting on P_{2x}-purinoceptors were inhibited whereas those to NA were unaffected. The selectivivity of mATP has previously been established in the guinea-pig urinary bladder (Kasakov & Burnstock, 1982), chick rectum (Meldrum & Burnstock, 1985), rodent vas deferens (Sneddon & Westfall, 1984; Sneddon *et al.*, 1984; Sneddon & Burnstock, 1984a), dog mesenteric artery (Muramatsu, 1986; Machaly *et al.*, 1988), rabbit mesenteric artery (Kugelgen & Starke 1985; Ramme *et al.*, 1987) and rat tail artery (Sneddon & Burnstock, 1984b).

In agreement with previous studies the results of this investigation indicate that there is a component of the pressor response of the pithed rat to sympathetic nerve stimulation which is resistant to α-adrenoceptor blockade (Flavahan *et al.*, 1985; Bulloch & McGrath, 1988). The results also confirm the findings of Bulloch & McGrath (1988) that a novel regime of administration of mATP produces a substantial reduction of the sympathetic nerve-mediated pressor response in the absence of α-adrenoceptor blockade. However, the present results indicate that the *in vivo* use of mATP to antagonise nerve-mediated vasoconstrictor responses was non-selective as suggested by the following findings. Firstly, pressor responses to NA were substantially attenuated by the mATP desensitization procedure. Secondly, pressor responses to angiotensin II and vasopressin were also reduced to a similar degree to the NA response. Thirdly, the secondary, adrenal component of the biphasic pressor response to sympathetic nerve stimulation, which has been attributed to the action of endogenous catecholamines of adrenal origin acting on α-adrenoceptors (Flavahan *et al.*, 1985), was also markedly reduced by mATP. This would not be expected if the mATP was acting selectively to desensitize the postjunctional P_{2x}-purinoceptors. These results disagree with previous findings obtained with mATP in the pithed rat (Flavahan *et al.*, 1985; Bulloch & McGrath, 1988).

The possibility that the slight differences between the pithed rat model used here and that used by Bulloch & McGrath (1988) may have had some effect on the selectivity of the mATP desensitization procedure was also considered. Firstly, in these experiments the strain of rat used (Sprague-Dawley) was different from that used by Bulloch & McGrath (1988) (Wistar strain). However, when experiments were carried out using Wistar rats, it was found that the pressor responses to NA and sympathetic nerve stimulation were again reduced by mATP to a similar degree. Secondly, since it was observed that a given dose of mATP produced a markedly greater pressor response in our experiments, than those obtained by Bulloch & McGrath (1988), the effect of scaling down the doses used in the desensitization procedure by 10 fold was investigated. This was done to determine whether the large pressor responses to mATP were having a traumatic effect on the cardiovascular system of the rat, which may account for the non-selective depression of vascular responsiveness. Whilst the lower concentrations of mATP produced comparable pressor responses to those observed in the previous study, no increase in selectivity was observed. Thirdly, it was shown previously that the arterial Po_2 of the pithed rat may affect the pressor responses obtained to agonists (Grant *et al.*, 1985). Experiments were therefore carried out, with room air as the ventilation gas, to investigate the effect of reducing the arterial Po_2 from >280 mmHg to approximately 80 mmHg. Under these conditions mATP was still non-selective, in that it reduced pressor responses to intravenous NA as well as sympathetic nerve stimulation. Lastly, Bulloch & McGrath (1988) used a bolus dose of gallamine (10 $mg kg^{-1}$) to induce skeletal-neuromuscular blockade, and in the present investigations one bolus dose (10 $mg kg^{-1}$) followed by a maintenance infusion (10 $mg kg^{-1} h^{-1}$) was employed. It was thus possible that prolonged gallamine treatment produced some non-specific depression of vascular smooth muscle responsiveness. In the absence of gallamine, however, mATP still produced a substantial reduction of the pressor responses to NA. These

observations indicate that the differences in the pithed rat model used here were unlikely to account for the non-selective nature of the action of mATP *in vivo*.

The paradoxical inability of mATP to act selectively in the whole animal when it is selective in abolishing responses mediated by P_{2x}-receptors *in vitro* is interesting. In the isolated mesenteric vascular bed of the rat perfused with Krebs solution, mATP produced selective antagonism of the vasoconstrictor responses to a variety of P_2-purinoceptor agonists and had no antagonistic effect on the vasoconstrictor response to NA (Ralevic & Burnstock, 1988). However, in the autoperfused intestinal circulation of the cat, mATP was non-selective in that it attenuated, by approximately 60%, the vasoconstrictor effects of administered noradrenaline and methoxamine, as well as the vasoconstrictor response to sympathetic nerve stimulation (Taylor & Parsons, 1989). This suggests that some other factor may be involved in the whole animal as a consequence of intravenous administration of mATP, resulting in a transient non-specific depression of vascular smooth muscle function.

In conclusion, these results support previous findings that mATP selectively reduces responses to ATP *in vitro*. However, the desensitization procedure *in vivo* produced a non-selective attenuation of pressor responses to a variety of pressor agents, as well as to electrical stimulation of the preganglionic sympathetic outflows. It is therefore not possible to use mATP, *in vivo*, to draw conclusions about the role of ATP in cotransmission in the rat peripheral vascular system.

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Rapid tolerance to the hypotensive effects of glyceryl trinitrate in the rat: prevention by N-acetyl-L- but not N-acetyl-D-cysteine

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1 A new model of tolerance to the hypotensive effect of organic nitrates has been developed in the rat.

2 The fall in mean arterial pressure (MAP) in response to bolus doses of sodium nitroprusside (NP) (4 $\mu g\,kg^{-1}$) and glyceryl trinitrate (GTN) (10 $\mu g\,kg^{-1}$) was recorded both before and after a 60 min infusion of either 0.9% saline, NP (20 $\mu g\,kg^{-1}\,min^{-1}$) or GTN (40 $\mu g\,kg^{-1}\,min^{-1}$).

3 The hypotensive effects of NP or GTN were unchanged following saline infusion, but were reduced in both cases by approximately 40% following the infusion of NP.

4 Infusion of GTN for 60 min virtually abolished the hypotensive effect of a GTN bolus (i.e. nitrate tolerance), whilst the effect of a NP bolus was reduced only to a similar extent (30%) as after an infusion of NP. This latter effect is attributed to a degree of non-specific cross-tolerance between GTN and NP.

5 Co-treatment of a group of rats with N-acetyl-L-cysteine (L-NAC) prevented the development of nitrate tolerance, confirming the role of thiols in this phenomenon, whereas N-acetyl-D-cysteine (D-NAC) did not.

6 The stereospecificity in the effect of NAC in preventing this specific tolerance to GTN suggests that the interaction between GTN and NAC and/or cysteine involves an enzyme-dependent step.

7 NAC was unable to prevent the non-specific cross-tolerance to NP which followed infusion of GTN, suggesting that the mechanism does not directly involve NAC and/or cysteine.

Introduction

Organic nitrates (such as glyceryl trinitrate, GTN), nitric oxide (NO) and NO-containing nitrocompounds (such as sodium nitroprusside, NP) exert their vasodilator action through the activation of soluble guanylate cyclase (Ignarro *et al.*, 1984). There is evidence for the involvement of intracellular thiols at one or more sites within the biochemical pathway that culminates in the activation of this enzyme. Firstly, stimulation of partially purified soluble guanylate cyclase by NO or NO-containing nitrocompounds *in vitro* requires the presence of thiols, probably as precursors for the formation of S-nitrosothiol intermediates (Ignarro *et al.*, 1981). Secondly, thiols may be involved at a stage in the biotransformation of organic nitrates which precedes the formation of NO or S-nitrosothiol intermediates, though the exact mechanism remains a subject of some controversy (Needleman *et al.*, 1973; Ignarro *et al.*, 1981; Yeates *et al.*, 1985).

Tolerance to the beneficial effects of organic nitrates can develop within 24 h of continuous administration (Elkayam *et al.*, 1987). Needleman & Johnson (1973) demonstrated that the induction of tolerance to GTN *in vivo* in the rat was accompanied by depletion of tissue sulphydryl groups, and several clinical studies have shown prevention and reversal of nitrate tolerance by the cysteine precursor N-acetylcysteine (Packer *et al.*, 1987; May *et al.*, 1987). These data are compatible with the hypothesis that it is the unavailability of critical intracellular sulphydryl groups, possibly in the form of cysteine, which determines the development of nitrate tolerance. The results of other studies performed *in vitro*, however, suggest that more than one mechanism may be involved (Gruetter & Lemke, 1985; Abdollah *et al.*, 1987; Mulsch *et al.*, 1988; Henry *et al.*, 1989; Romanin & Kukowetz, 1989). In an attempt to elucidate further the mechanism by which N-acetyl-cysteine (NAC) prevents tolerance *in vivo*, we have developed a new model of tolerance to the hypotensive effects of intravenous GTN in the anaesthetized rat. We have used this model to study the interaction between GTN and the two enantiomers of NAC, and to compare the effects of GTN with those of an inorganic nitrate, NP.

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Methods

Preparation of animals

Male Sprague-Dawley rats (200–450 g) (obtained from Harlan Olac Ltd, Bicester, U.K.) were anaesthetized with sodium pentobarbitone (60 $mg\,kg^{-1}$ i.p. and 40 $mg\,kg^{-1}$, s.c.) and placed on a heated mat (37–39°C, Remploy). Further anaesthetic (sodium pentobarbitone 40 $mg\,kg^{-1}$, i.p.) was administered 20 min after the start of the 60 min infusion period. The trachea was cannulated and blood pressure recordings were made via a polyethylene cannula inserted into the right carotid artery, using a Gould pressure transducer (model P321D) and Grass polygraph recorder (model 79D). Intravenous drugs were administered via a polyethylene cannula inserted into the right jugular vein. Heparin (1 iu g^{-1}, i.v.) was administered immediately after cannulation, and the experiment was begun after a 10 min equilibration period.

Experimental protocol

Two bolus doses of NP (4 $\mu g\,kg^{-1}$) and then of GTN (10 $\mu g\,kg^{-1}$) were administered i.v. both before and again 5 min after a 60 min infusion of either 0.9% saline ($n = 4$), NP (20 $\mu g\,kg^{-1}\,min^{-1}$) ($n = 8$) or GTN (40 $\mu g\,kg^{-1}\,min^{-1}$) ($n = 8$). Two additional groups of animals received either L-NAC ($n = 8$) or D-NAC ($n = 8$) before and during a 60 min infusion of GTN (40 $\mu g\,kg^{-1}\,min^{-1}$) as follows: 200 $mg\,kg^{-1}$ in 1 ml 0.9% saline i.p. and 200 $mg\,kg^{-1}$ in 0.5 ml 0.9% saline administered i.v. before infusion, and 200 $mg\,kg^{-1}$ (in 0.9% saline) at 5 $ml\,kg^{-1}\,h^{-1}$ over the 60 min infusion period. A 'Y' connector system ensured that extracorporeal admixture of GTN and NAC was kept to a minimum. In all experiments the total infusion volume was 10 $ml\,kg^{-1}$ over 60 min.

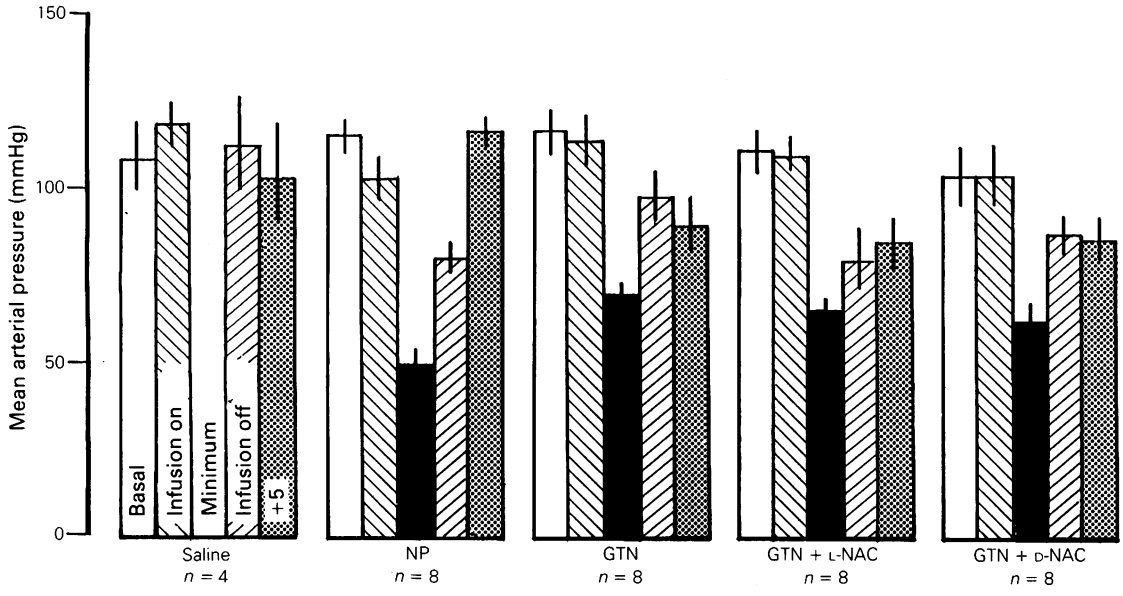


Figure 1 Mean arterial pressure (MAP) recorded at the onset of the experiment (Basal) and at the beginning (Infusion on), the end (Infusion off) and 5 min following (+5) a 60 min i.v. infusion of 0.9% saline, sodium nitroprusside (NP) ($20\,\mu g\,kg^{-1}\,min^{-1}$) or glyceryl trinitrate (GTN) ($40\,\mu g\,kg^{-1}\,min^{-1}$) in rats anaesthetized with sodium pentobarbitone ($60\,mg\,kg^{-1}$, i.p. and $40\,mg\,kg^{-1}$, s.c.). MAP fell rapidly during the infusion of NP and GTN, reached a minimum (Minimum, solid column) within 2 min of the onset, but then rose towards the pre-infusion level during the remainder of the infusion period. MAP did not decrease during infusion of 0.9% saline, so that no value for Minimum is shown for this group of animals. Two groups of animals were co-treated with either N-acetyl-L-cysteine (L-NAC) or N-acetyl-D-cysteine (D-NAC) (total dose $600\,mg\,kg^{-1}$) before and during the infusion of GTN (see text), but such treatment had no effect on basal MAP. Values are mean for numbers of animals indicated with s.e. shown by vertical lines.

Drugs

Glyceryl trinitrate (as a 0.5% solution in 30% v/v ethanol and 30% v/v propylene glycol) and sodium nitroprusside powder were obtained from David Bull Laboratories. N-Acetyl-L-cysteine and D-cystine were obtained from Sigma Chemical Co. N-Acetyl-D-cysteine was synthesized according to the method described by Sheffner *et al.* (1966). Essentially, D-cystine (2 g) was dissolved in aqueous sodium hydroxide (2 M, 8.7 ml) and cooled in an ice/salt bath. Six portions of acetic anhydride (0.7 ml) and sodium hydroxide solution (6.6 ml) were added and the solution allowed to stand for 0.5 h in the cold and a further 0.5 h at room temperature. Sodium ions were removed by passage through a Dowex column (H^+ form, 25 × 1 cm). Acetyl-D-cystine was reduced by zinc/acetic acid at 60°C for 3 h; zinc ions were removed on a Dowex (H^+) column and the resulting solution lyophilised. N-Acetyl-D-cysteine was recrystallised from hot water. The molecular rotation of the product was confirmed as that of the D-isomer by Miss S. Johnson, Department of Chemistry, Imperial College, London.

Analysis of data

Mean arterial pressure (MAP) was estimated from the systolic (SDP) and diastolic (DBP) blood pressure recordings using the formula MAP = DBP + ((SBP − DBP)/3). All results have been expressed as mean ± s.e.mean. Comparisons amongst groups were made using two-way analysis of variance, and individual comparisons using Student's *t* test for paired samples. The null hypothesis was rejected at $P < 0.05$.

Results

Basal MAP was similar in all groups of rats, including those co-treated with either L-NAC or D-NAC (Figure 1). Infusion of 0.9% saline for 1 h had no effect on MAP ($n = 4$). During infusion of GTN ($40\,\mu g\,kg^{-1}\,min^{-1}$), MAP fell rapidly to a minimum of 69.4 ± 3.5 mmHg, rose progressively over the remainder of the 60 min infusion period, but remained below the preinfusion MAP during the first 5 min after completion of the infusion. A similar pattern was observed in those animals co-treated with either L-NAC or D-NAC (Figure 1). Infusion of NP at a dose of $20\,\mu g\,kg^{-1}\,min^{-1}$ resulted in a somewhat greater fall in MAP, reaching a minimum of 49.4 ± 4.2 mmHg. MAP again rose progressively over the remainder of the infusion period, recovering rapidly during the immediate post-infusion period to reach pre-infusion MAP within 5 min (Figure 1).

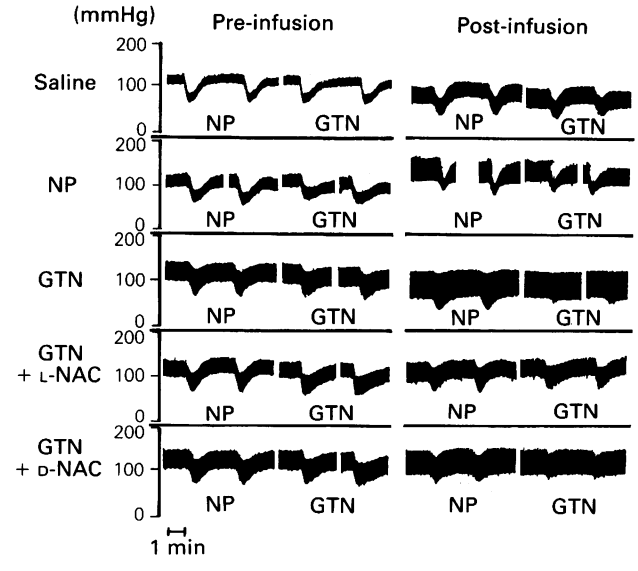


Figure 2 Representative individual arterial pressure recordings showing the hypotensive effect of duplicate intravenous bolus doses of sodium nitroprusside (NP) ($4\,\mu g\,kg^{-1}$) and glyceryl trinitrate (GTN) ($10\,\mu g\,kg^{-1}$) before (Pre-infusion) and 5 min after a 60 min infusion (Post-infusion) of 0.9% saline, NP ($20\,\mu g\,kg^{-1}\,min^{-1}$) or GTN ($40\,\mu g\,kg^{-1}\,min^{-1}$). GTN infusions were also administered to animals which had been co-treated with either N-acetyl-L-cysteine (L-NAC) or N-acetyl-D-cysteine (D-NAC) (total dose $600\,mg\,kg^{-1}$).

Table 1 Baseline and minimum (min.) mean arterial pressure (MAP) (±s.e.mean) in response to intravenous bolus doses of sodium nitroprusside (NP) (4 $\mu g\,kg^{-1}$) and glyceryl trinitrate (GTN) (10 $\mu g\,kg^{-1}$), both before and 5 min after the 60 min infusion period

	Pre-infusion				*Post-infusion*			
Infusion	*NP*		*GTN*		*NP*		*GTN*	
type	*Baseline*	*Min.*	*Baseline*	*Min.*	*Baseline*	*Min.*	*Baseline*	*Min.*
Saline	112.6	70.6	112.3	67.3	120.8	77.0	119.3	78.6
	(7.7)	(2.8)	(8.2)	(2.4)	(10.4)	(5.8)	(11.4)	(7.7)
NP	115.6	72.4	106.3	69.6	127.2	103.4	126.3	102.0
	(3.1)	(2.8)	(3.3)	(2.3)	(4.2)	(4.2)	(3.8)	(4.0)
GTN	120.6	76.5	119.7	82.0	110.6	80.5	116.1	106.6
	(5.8)	(3.8)	(4.8)	(3.2)	(3.8)	(4.3)	(4.0)	(4.3)
GTN +	113.3	75.3	109.2	76.8	96.5	73.1	100.0	79.8
L-NAC	(4.0)	(2.1)	(3.8)	(2.4)	(5.5)	(4.4)	(6.2)	(4.7)
GTN +	105.5	75.7	102.6	77.5	104.3	79.8	109.1	97.6
D-NAC	(5.1)	(4.4)	(4.4)	(4.0)	(2.8)	(2.8)	(2.9)	(3.4)

It was established during preliminary experiments that bolus injections of NP (4 $\mu g\,kg^{-1}$) and GTN (10 $\mu g\,kg^{-1}$) in untreated anaesthetized rats caused a similar, transient, fall in MAP, and these doses were used thereafter. Representative tracings from each group of animals are shown in Figure 2 and the pooled results in Table 1 and Figure 3. There was no significant difference between groups in the hypotensive effect of bolus doses of NP or GTN administered immediately after the equilibration period, including those animals which had been pretreated with either L-NAC or D-NAC. The fall in MAP induced by a bolus dose of NP was similar pre- and post-infusion of 0.9% saline for 60 min (42.0 ± 9.1 mmHg pre-, 43.8 ± 8.0 mmHg post-), but was significantly and similarly attenuated following infusion of NP (43.3 ± 2.5 mmHg pre-, 23.8 ± 2.5 mmHg post-, $P < 0.001$) and GTN (44.0 ± 4.6 mmHg pre-, 30.1 ± 3.7 mmHg post-, $P < 0.05$), most probably due to a degree of non-specific tolerance to NP. This reduction in the effect on MAP of a bolus dose of NP following a GTN infusion was unaffected by co-treatment with L-NAC (fall in MAP 38.0 ± 3.9 mmHg pre-, 23.4 ± 2.3 mmHg post-infusion of GTN, $P = 0.005$), although D-NAC seemed to have some protective effect (31.9 ± 3.5 mmHg pre-, 24.6 ± 2.8 mmHg post-, $P > 0.1$). However, this appeared to be due to the fact that the hypotensive response to the pre-infusion bolus dose of NP was impaired in these animals, the reason for which is not clear.

The hypotensive effect of a bolus dose of GTN was also unaffected by infusion of 0.9% saline for 60 min, and was attenuated following the infusion of NP (fall in MAP 36.7 ± 2.4 mmHg pre-, 24.3 ± 2.4 mmHg post-, $P < 0.005$) by a similar degree to that observed for the bolus dose of NP post-infusion of NP, again suggesting a degree of non-specific cross-tolerance between GTN and NP. Following infusion of GTN, however, the hypotensive effect of a bolus dose of GTN was markedly reduced (37.7 ± 4.1 mmHg pre-, 9.6 ± 1.1 mmHg post-, $P = 0.0001$), indicating an additional element of tolerance specific to GTN and not NP. The specific component of the tolerance to GTN could be completely prevented by co-treatment with L-NAC (fall in MAP 32.3 ± 2.8 mmHg pre-, 20.2 ± 2.7 mmHg post-) but not D-NAC (26.8 ± 2.5 mmHg pre-, 11.5 ± 1.9 mmHg post-), ($P = 0.001$ by ANOVA). This is perhaps most clearly illustrated by comparisons of the ratio of the fall in MAP induced by a bolus of GTN compared to that induced by NP (GTN : NP ratio) under different conditions (Figure 3).

Discussion

The mechanisms responsible for vascular tolerance to organic nitrates remain unclear. Previous work in rats has shown that tolerance induced *in vivo* is accompanied by depletion of tissue

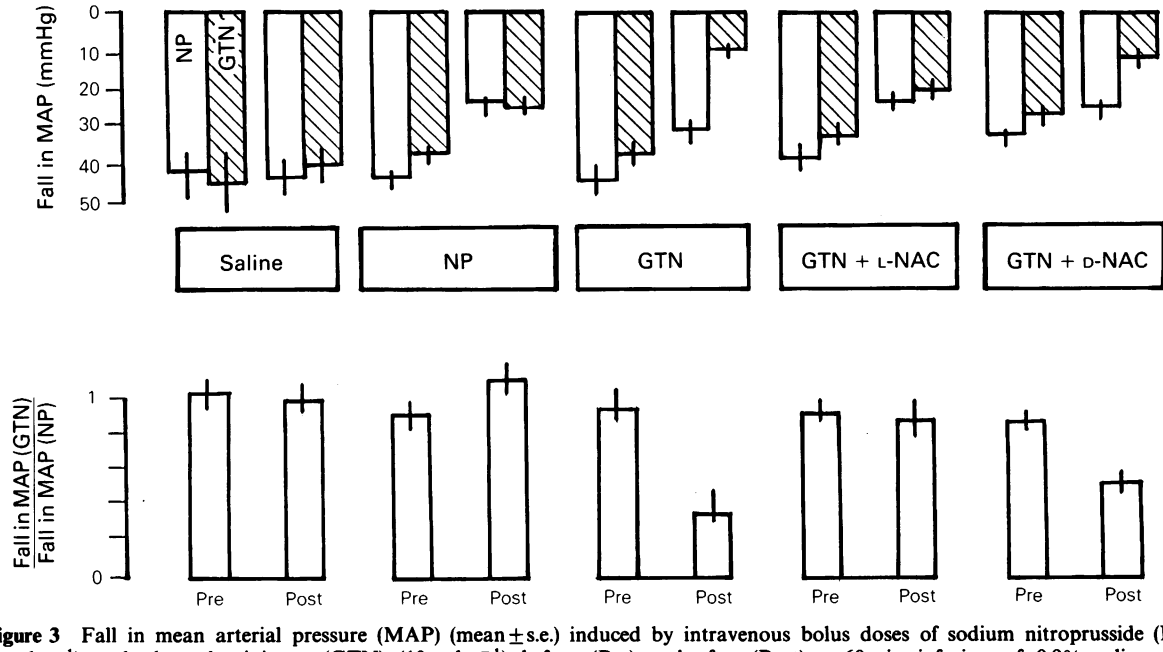


Figure 3 Fall in mean arterial pressure (MAP) (mean ± s.e.) induced by intravenous bolus doses of sodium nitroprusside (NP) (4 $\mu g\,kg^{-1}$) and glyceryl trinitrate (GTN) (10 $\mu g\,kg^{-1}$) before (Pre) and after (Post) a 60 min infusion of 0.9% saline, NP (20 $\mu g\,kg^{-1}\,min^{-1}$) or GTN (40 $\mu g\,kg^{-1}\,min^{-1}$). Shown in the bottom panel is the ratio of the fall in MAP induced by a bolus dose of GTN compared to that induced by a bolus dose of NP (GTN : NP ratio).

sulphydryl groups (Needleman & Johnson, 1973). More recently, Ignarro et al. (1981) showed that activation of partially purified soluble guanylate cyclase by organic nitrates, nitrites, nitroprusside and nitric oxide *in vitro* requires the addition of thiols; GTN differed from the other nitrocompounds tested in that cysteine was the only effective thiol donor. One interpretation of these data is that tolerance to GTN is secondary to a specific depletion of intracellular cysteine, rather than of the total thiol pool.

Intracellular cysteine may be replenished by L-NAC, which enters cells where it is de-acetylated to free L-cysteine. The intracellular concentration of L-cysteine is tightly controlled, largely by conversion to glutathione (Cooper, 1985) and levels above 150 μM cannot be achieved, even when isolated cells are incubated with high concentrations of L-NAC *in vitro* (Cotgreave et al., 1987). D-NAC will also readily enter cells, but it is not metabolised further (Sjodin et al., 1989). Assuming that D-NAC is distributed throughout total body water, then it can be estimated that the tissue concentration in our experiments would have been greater than one millimolar, thereby providing a much higher concentration of free cysteine-derived sulphydryl groups than the same dose of L-NAC. If the maintenance of an adequate concentration of free cysteine-derived sulphydryl groups for participation in a non-enzymatic reaction was the only requirement for the prevention of tolerance to GTN, then D-NAC should have been the more effective of the two enantiometers. In fact we found the exact opposite. Thus, our data are more compatible with an enzyme-dependent interaction between GTN and NAC and/or cysteine.

Whilst depletion of intracellular cysteine or other thiol compounds may represent the main cause of tolerance to organic nitrates, additional mechanisms of tolerance may exist, at least under experimental conditions. For example, some *in vitro* data suggest that tolerance may be induced to inorganic nitrocompounds (such as NP) as well as to GTN and other organic nitrates (Axelsson et al., 1982; Axelsson & Andersson, 1983; Keith et al., 1983; Molina et al., 1987). It has been suggested that the mechanism of this tolerance may involve desensitization of guanylate cyclase (Waldman et al., 1986; Schroder et al., 1988; Romanin & Kukowetz, 1989; Henry et al., 1989). However, any desensitization of guanylate cyclase observed in cell-free preparations *in vitro* may not accurately reflect the situation within intact cells (Mulsch et al., 1988). We found that infusions of either NP or GTN were associated with a moderate attenuation of the hypotensive effect of bolus doses of NP. Infusion of NP for 60 min caused a similar loss of potency of bolus doses of GTN. This second, nonspecific, cross-tolerance was not prevented by L-NAC, is therefore unlikely to be due to depletion of intracellular cysteine, and could be mediated at the level of guanylate cyclase. Further studies with guanylate cyclase-independent vasodilators are required to investigate this point further.

The original *in vivo* model of nitrate tolerance in the rat was developed by Needleman and involved repeated dosing over several days (Needleman, 1970). *In vitro* data suggested that tolerance to high concentrations of organic nitrates may occur within 1 h of exposure (Mulsch et al., 1988; Henry et al., 1989). We have been able to produce tolerance *in vivo* over a similarly short period by the use of a continuous intravenous infusion. The characteristics of the specific tolerance to GTN in our model, particularly its prevention by L-NAC, imply that the mechanisms involved are similar to those of clinical nitrate tolerance, and suggest that it is a suitable model for further *in vivo* studies.

The stereospecificity of the prevention of nitrate tolerance in this model by L-NAC suggests the involvement of an enzyme-dependent step in the interaction of GTN with NAC and/or cysteine. Additional work with other thiol donors is required to elucidate the interaction further.

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Synergistic internal carotid vasodilator effects of human α-calcitonin gene-related peptide and nimodipine in conscious rats

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1 In a first series of experiments, male Long Evans rats were chronically instrumented for the measurement of internal carotid blood flow and systemic arterial blood pressure; cardiovascular changes were assessed during and after 30 min infusions of human α-calcitonin gene-related peptide (CGRP) (0.06 and 0.6 nmol h^{-1}), or nimodipine (60 and 600 nmol h^{-1}) or human α-CGRP plus nimodipine. The effects of human α-CGRP or nimodipine on internal carotid vasoconstriction induced by endothelin-1 were also measured.

2 Human α-CGRP (0.06 nmol h^{-1}) caused a small (+15%), transient increase in internal carotid blood flow and a tachycardia (+33 beats min^{-1}), but no change in mean blood pressure. Nimodipine (60 nmol h^{-1}) caused a brief internal carotid hyperaemia (+16%) but no changes in blood pressure or heart rate. However, concurrent administration of human α-CGRP (0.06 nmol h^{-1}) and nimodipine (60 nmol h^{-1}) caused a sustained increase in internal carotid blood flow (+40%) unaccompanied by significant changes in heart rate or blood pressure.

3 Human α-CGRP at a dose of 0.6 nmol h^{-1} or nimodipine at a dose of 600 nmol h^{-1} caused substantial reductions in internal carotid vascular resistance (−43 and −40%, respectively); concurrent administration of these doses did not have an additive vasodilator effect.

4 Infusion of endothelin-1 (1.2 nmol h^{-1}) for 20 min caused incremental constriction of the internal carotid vascular bed; human α-CGRP infusion (0.6 and 6.0 nmol h^{-1}) begun 10 min after the onset of endothelin-1 infusion reversed this effect (dose-dependently); nimodipine (600 nmol h^{-1}) also caused a substantial attenuation of the effects of endothelin-1.

5 In a second series of experiments the haemodynamic effects of human α-CGRP and/or nimodipine were assessed in rats chronically instrumented for the measurement of renal, superior mesenteric and hindquarters blood flow together with systemic arterial blood pressure.

6 Administration of human α-CGRP (0.06 nmol h^{-1}) alone or in conjuction with nimodipine (60 nmol h^{-1}) had no significant effects on renal or superior mesenteric vascular resistances, although there was a slight hindquarters vasodilatation. Human α-CGRP at a dose of 0.6 nmol h^{-1} caused hypotension, tachycardia and reductions in renal and superior mesenteric blood flows, together with a marked (+31% maximum) hindquarters hyperaemia. Nimodipine at a dose of 600 nmol h^{-1} caused hypotension, tachycardia and a reduction (−34%) in renal blood flow; mesenteric blood flow was unchanged and there was an increase in hindquarters flow (+59%).

7 Concurrent administration of human α-CGRP (0.6 nmol h^{-1}) and nimodipine (600 nmol h^{-1}) did not have an additive hypotensive effect or an enhanced hindquarters hyperaemic effect, but was associated with a marked impairment of renal blood flow (−48%).

8 The present results indicate that concurrent administration of low doses of human α-CGRP and nimodipine might be particularly helpful in the acute treatment of patients with cerebral vasospasm and impaired renal perfusion, since this intervention improved internal carotid blood flow without compromising blood flow to the kidney.

Introduction

Rat and human α- and β-calcitonin gene-related peptide (CGRP) cause marked vasodilatation of the common carotid vascular bed *in vivo* in conscious rats (Gardiner *et al.*, 1989a,b). Although in man it appears that human α-CGRP may increase internal carotid blood flow (Bell *et al.*, 1989), this phenomenon has not been demonstrated in other species in the conscious state. The first aim of the present study was to assess the influence of human α-CGRP on internal carotid blood flow in conscious, unrestrained rats.

Currently, there is a great deal of interest in the clinical application of calcium channel antagonists, particularly nimodipine, in the treatment of cerebral vascular disorders (see Marin, 1988; Pickard *et al.*, 1989; Scriabine *et al.*, 1989; Langley & Sorkin, 1989). The second aim of the present study was to assess the effects of nimodipine alone on internal carotid blood flow, and to determine if human α-CGRP and nimodipine had synergistic effects on internal carotid haemodynamics. We also assessed the ability of human α-CGRP or nimodipine to reverse internal carotid vasoconstriction induced by an infusion of endothelin-1 (Yanagisawa *et al.*, 1988), since the latter peptide has been found to have potent effects on the cerebral vasculature (Asano *et al.*, 1989; Saito *et al.*, 1989).

The final aim of this study was to determine whether or not there were interactions between the effects of human α-CGRP and nimodipine on renal, superior mesenteric and hindquarters haemodynamics, that could prove detrimental under conditions in which human α-CGRP and nimodipine were being administered together. Some of the results of this work have been presented to the Physiological Society (Bennett *et al.*, 1989a).

Methods

All studies were carried out on male, Long Evans rats (350–450 g). Animals were anaesthetized (sodium methohexitone,

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60 mg kg^{-1} i.p., supplemented as required) and had one of the following procedures carried out: (a) implantation of a pulsed Doppler probe (Haywood *et al.*, 1981) around the left common carotid artery after the external carotid artery on the same side had been ligated. This arrangement permitted assessment of flow through the patent internal carotid artery. (b) Implantation of pulsed Doppler probes around the left renal and superior mesenteric arteries and the distal abdominal aorta below the level of the ileocaecal artery (to monitor flow to the hindquarters).

Following 7–14 days recovery animals were briefly re-anaesthetized (sodium methohexitone, 40 mg kg^{-1}) for the implantation of intravascular catheters for drug or peptide administrations (jugular vein) and the recording of instantaneous heart rate (HR) and blood pressures (BP) from the abdominal aorta (via the caudal artery). The experiments began on the next day and ran over 3 days.

The protocols involved continuous recordings from conscious unrestrained animals of mean BP, HR and Doppler shifts from the implanted probes. Percentage changes in regional blood flows and vascular resistances were calculated as described elsewhere (Gardiner *et al.*, 1989a,b).

The following protocols were run:

Animals with internal carotid probes

Effect of human α-CGRP1 (1) Human α-CGRP (0.06 or 0.6 nmol h^{-1}) was infused for 30 min. Measurements were made before, during, and for 30 min after infusion.

Effects of nimodipine vehicle (2) Nimodipine vehicle (see below) was infused for 30 min. Measurements were made before, during and for 30 min after infusion.

Effect of nimodipine (3) Nimodipine (60 or 600 nmol h^{-1}) was infused for 30 min. Measurements were made before, during and for 30 min after infusion.

Effects of human α-CGRP plus nimodipine (4) Human α-CGRP and nimodipine (0.06 and 60 nmol h^{-1}, respectively, or 0.6 and 600 nmol h^{-1}, respectively) were infused concurrently for 30 min. Measurements were made before, during and for 30 min after infusion.

Effects of human α-CGRP during endothelin-1 infusion (5) Endothelin-1 (1.2 nmol h^{-1}) was infused for 10 min before the onset of human α-CGRP infusion (0.6 or 6 nmol h^{-1}); the substances were infused concurrently for 10 min. A control experiment involving infusion of endothelin-1 alone (1.2 nmol h^{-1}) for 20 min was also carried out.

Effects of nimodipine during endothelin-1 infusion (6) Endothelin-1 was infused for 10 min before nimodipine infusion (600 nmol h^{-1}) was started. The substances were given concurrently for 10 min.

The experiments that involved administration of nimodipine vehicle, low and high doses of nimodipine, low and high doses of human α-CGRP, and low dose nimodipine and human α-CGRP together were run in one group of rats ($n = 8$). A second group ($n = 8$) of rats was used in the protocols that involved high dose nimodipine and human α-CGRP together, and nimodipine administration during endothelin-1 infusion. The effects of endothelin-1 alone and of human α-CGRP during endothelin-1 infusion were assessed in a third group of rats ($n = 8$).

Animals with renal, mesenteric and hindquarters probes

Protocols (1)–(4) above were run also in animals ($n = 8$) with renal, mesenteric and hindquarters probes.

Drugs and peptides

Human α-CGRP (Celltech Ltd) and endothelin-1 (Peptide Institute) were dissolved in isotonic saline containing 1% bovine serum albumin. Nimodipine (pure substance, Bayer, U.K.) was dissolved in a mixture of polyethylene glycol 400, sterile water and glycerine BP in the ratio 9.69 : 1.0 : 0.6 (by weight). Nimodipine solutions were made up in a dark room under filtered light (wave length > 500 nm) and all catheters and syringes were masked to avoid exposure of the solutions to ambient lighting; the infusion rate was 0.4 ml h^{-1}.

Statistics

All data were subjected to non-parametric analysis of variance (Friedman's test) with Wilcoxon's rank sum test, or the Mann-Whitney U test as appropriate.

Results

Animals with internal carotid probes

Effects of human α-CGRP Infusion of human α-CGRP at 0.06 nmol h^{-1} caused an increase in HR and a modest internal carotid hyperaemia, accompanied by a fall in vascular resistance (Figure 1).

Human α-CGRP at 0.6 nmol h^{-1} caused tachycardia, hypotension and marked hyperaemic vasodilatation in the internal carotid vascular bed (Figure 1).

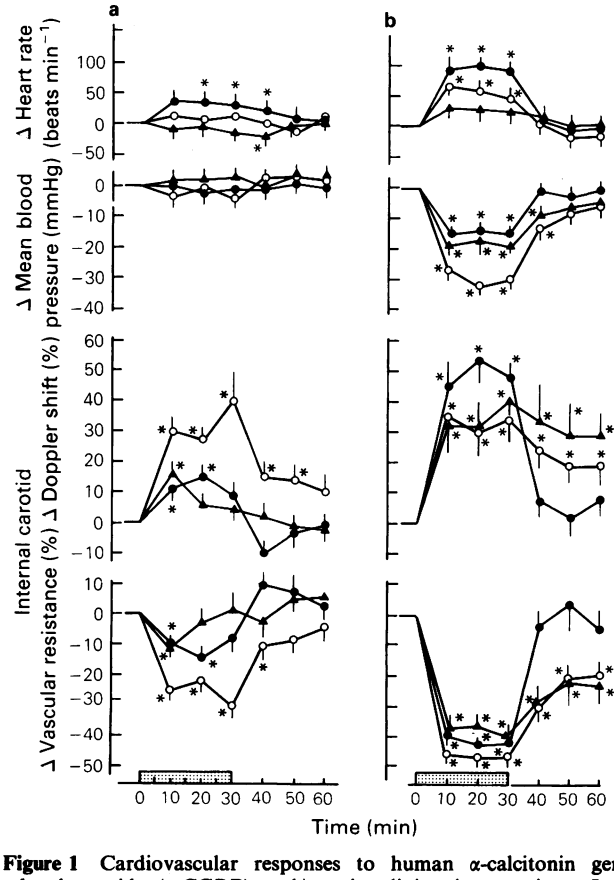


Figure 1 Cardiovascular responses to human α-calcitonin gene-related peptide (α-CGRP) and/or nimodipine in conscious, Long Evans rats ($n = 8$). (a) Responses to a 30 min infusion of human α-CGRP (●, 0.06 nmol h^{-1}), or nimodipine (▲, 60 nmol h^{-1}) or human α-CGRP (0.06 nmol h^{-1}) plus nimodipine (60 nmol h^{-1}) (○). (b) Responses to a 30 min infusion of human α-CGRP (●, 0.6 nmol h^{-1}), or nimodipine (▲, 600 nmol h^{-1}) or human α-CGRP (0.6 nmol h^{-1}) plus nimodipine (600 nmol h^{-1}) (○). Values are mean ($n = 8$); vertical lines show s.e.mean. * $P < 0.05$ versus baseline; statistics for inter-group comparisons are in the text.

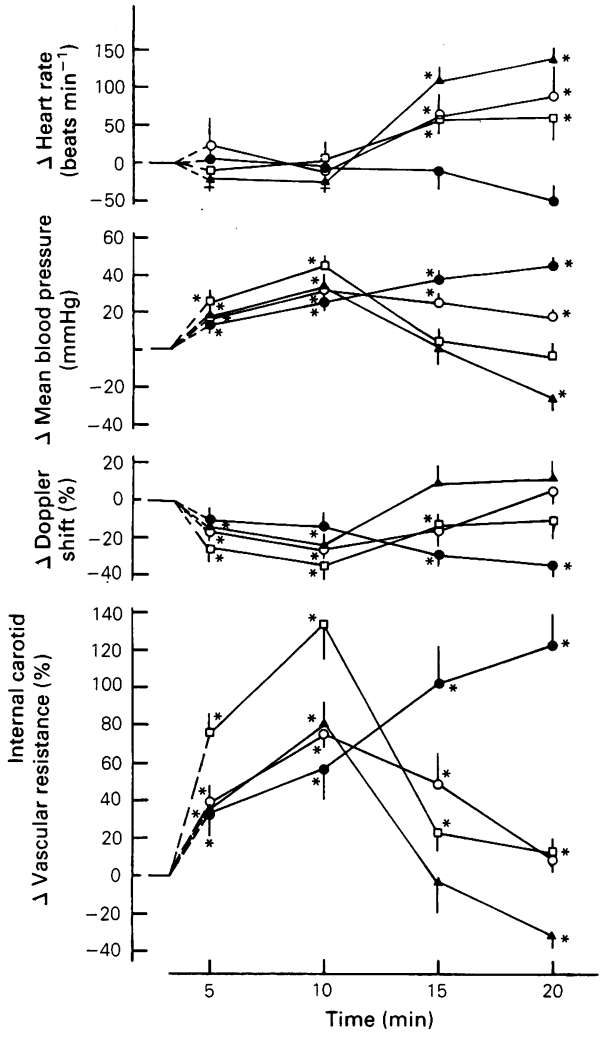


Figure 2 Cardiovascular responses to a 20 min infusion of endothelin-1 alone (1.2 nmol h^{-1}; ●) or a 20 min infusion of endothelin-1 together with human α-calcitonin gene-related peptide (α-CGRP) (○, 0.6 nmol h^{-1}; ▲, 6 nmol h^{-1}) or nimodipine (□, 600 nmol h^{-1}). Infusions of human α-CGRP or nimodipine were begun 10 min after the onset of endothelin-1 infusion and both substances were administered together for the following 10 min. * $P < 0.05$ versus baseline. At the 20 min time point there is a significant difference between the experiment involving endothelin-1 alone and the others for all variables. Values are mean ($n = 8$) and vertical lines indicate s.e.mean.

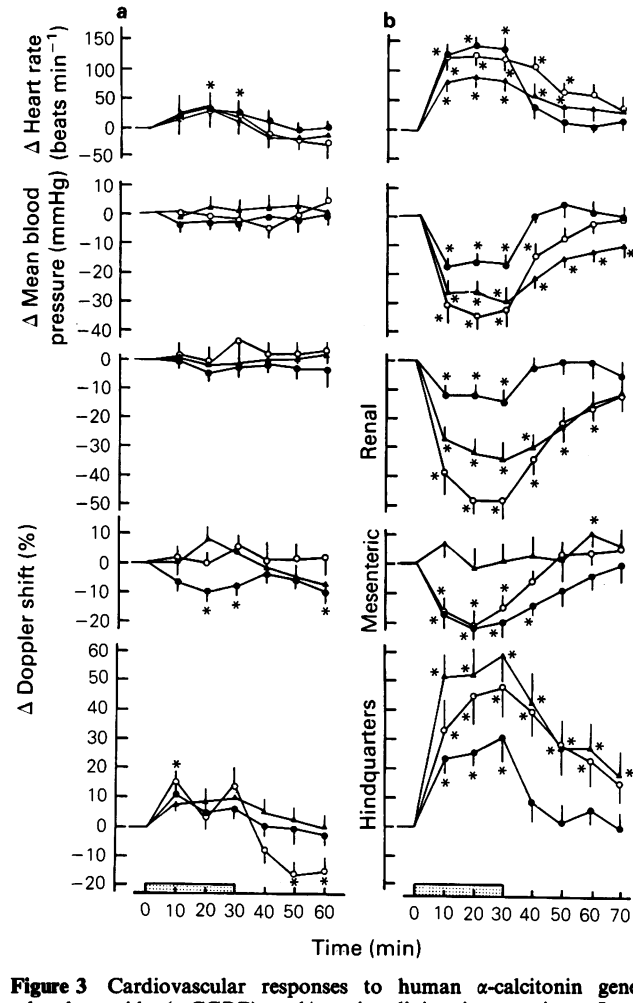


Figure 3 Cardiovascular responses to human α-calcitonin gene-related peptide (α-CGRP) and/or nimodipine in conscious, Long Evans rats ($n = 8$). (a) Responses to a 30 min infusion of human α-CGRP (●, 0.06 nmol h^{-1}), or nimodipine (▲, 60 nmol h^{-1}) or human α-CGRP (0.06 nmol h^{-1}) plus nimodipine (60 nmol h^{-1}) (○). (b) Responses to a 30 min infusion of human α-CGRP (●, 0.6 nmol h^{-1}), or nimodipine (▲, 600 nmol h^{-1}) or human α-CGRP (0.6 nmol h^{-1}) plus nimodipine (600 nmol h^{-1}) (○). Values are mean ($n = 8$) and vertical lines indicate s.e.mean. * $P < 0.05$ versus baseline; statistics for inter-group comparisons are in the text.

Effects of nimodipine vehicle Infusion of nimodipine vehicle was associated with a significant ($P < 0.05$) fall in HR (-23 ± 9 beats min^{-1} at 30 min) and a slight rise in mean BP ($+5 \pm 2$ mmHg at 30 min; $P < 0.05$), but no change in internal carotid haemodynamics.

Effects of nimodipine Nimodipine infused at 60 nmol h^{-1} caused a transient increase in internal carotid flow and fall in vascular resistance (Figure 1). The higher dose of nimodipine (600 nmol h^{-1}) caused hypotension and substantial hyperaemic vasodilatation in the internal carotid vascular bed. These effects persisted after the infusion was stopped, unlike those of human α-CGRP (Figure 1).

Effects of human α-CGRP plus nimodipine Infusion of human α-CGRP (0.06 nmol h^{-1}) together with nimodipine (60 nmol h^{-1}) caused significantly ($P < 0.05$) greater increases in internal carrotid flow and falls in vascular resistance than seen with either compound alone (Figure 1). However, there were no significant changes in mean BP or HR (Figure 1).

Concurrent administration of human α-CGRP (0.6 nmol h^{-1}) and nimodipine (600 nmol h^{-1}) did not enhance significantly the vasodilatation seen with either compound alone (Figure 1).

Effects of human α-CGRP or nimodipine during endothelin-1 infusion Infusion of endothelin-1 alone (1.2 nmol h^{-1}) for 20 min caused an increase in mean BP associated with a reduction in internal carotid blood flow and a vasoconstriction (Figure 2). These effects were reversed by human α-CGRP in a dose-dependent fashion (Figure 2). Nimodipine (600 nmol h^{-1}) also reversed the effects of endothelin-1 on mean BP and internal carotid blood flow (Figure 2).

Animals with renal, mesenteric and hindquarters probes

Effects of human α-CGRP Infusion of human α-CGRP (0.06 nmol h^{-1}) caused tachycardia but no significant change in mean BP (Figure 3). There was a reduction in mesenteric flow only (Figure 3) and a transient fall in hindquarters vascular resistance (Figure 4). During infusion of human α-CGRP at 0.6 nmol h^{-1} there was tachycardia and hypotension accompanied by reductions in renal and mesenteric blood flows and a hindquarters hyperaemia (Figure 3). Only the fall in hindquarters resistance was significant during the infusion,

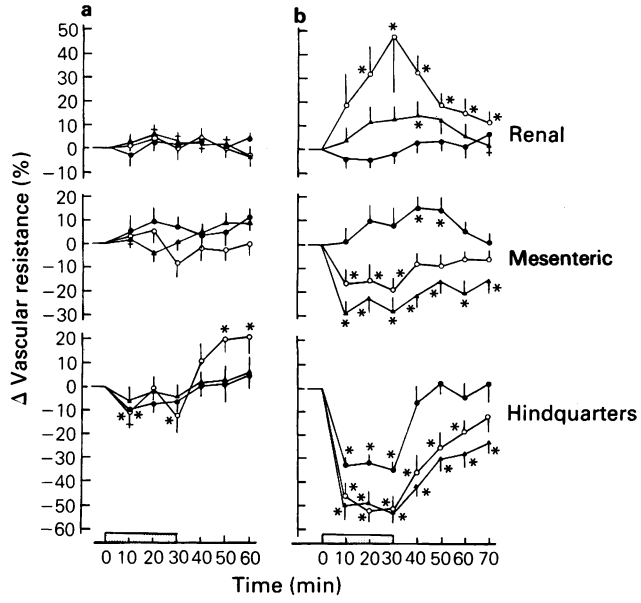


Figure 4 Changes in regional vascular resistances derived from the data in Figure 3. (a) Responses to a 30 min infusion of human α-calcitonin gene-related peptide (α-CGRP) (●, 0.06 nmol h^{-1}), or nimodipine (▲, 60 nmol h^{-1}) or human α-CGRP (0.06 nmol h^{-1}) plus nimodipine (60 nmol h^{-1}) (○). (b) Responses to a 30 min infusion of human α-CGRP (●, 0.6 nmol h^{-1}), or nimodipine (▲, 600 nmol h^{-1}) or human α-CGRP (0.6 nmol h^{-1}) plus nimodipine (600 nmol h^{-1}) (○). Values are mean ($n = 8$) and vertical lines indicate s.e.mean. * $P < 0.05$ versus baseline; statistics for inter-group comparisons are in the text.

although mesenteric vascular resistance rose significantly after the infusion (Figure 4).

Effects of nimodipine vehicle Infusion of nimodipine vehicle was associated with an overall bradycardia, but this was not significant at any specific time point. There were no changes in mean BP or regional blood flows (data not illustrated).

Effects of nimodipine Infusion of nimodipine at 60 nmol h^{-1} had no significant effects (Figures 3 and 4). However, at an infusion rate of 600 nmol h^{-1} nimodipine caused reductions in mean BP and renal flow accompanied by increases in HR and hindquarters flow, with no change in mesenteric flow (Figure 3). During and after infusion there were falls in mesenteric and hindquarters resistances (Figure 4), but renal vascular resistance rose significantly after infusion (Figure 4).

When assessed from areas under curves, all the cardiovascular effects of nimodipine (600 nmol h^{-1}) were significantly ($P < 0.05$) different from those of human α-CGRP (0.6 nmol h^{-1}).

Effects of human α-CGRP plus nimodipine During infusion of human α-CGRP (0.06 nmol h^{-1}) together with nimodipine (60 nmol h^{-1}) (Figure 3), there was no significant tachycardia or change in mesenteric haemodynamics, in contrast to the tachycardia and fall in mesenteric blood flow seen with human α-CGRP (0.06 nmol h^{-1}) alone (Figure 3). Hindquarters vasodilatation persisted throughout the infusion of human α-CGRP and nimodipine (Figure 4), in contrast to the transient effect seen with human α-CGRP alone or the lack of effect of nimodpine alone (Figure 4).

Concurrent infusion of human α-CGRP (0.6 nmol h^{-1}) and nimodipine (600 nmol h^{-1}) had significantly ($P < 0.05$) greater effects than human α-CGRP alone on all cardiovascular variables except HR and mesenteric flow (Figures 3 and 4). However, administration of human α-CGRP and nimodipine together did not enhance significantly the hypotension or mesenteric or hindquarters vasodilatations seen with nimodipine alone (Figures 3 and 4). Nonetheless, combined administration of human α-CGRP and nimodipine caused a significantly greater reduction in renal blood flow and enhanced renal vasoconstriction compared to those seen with nimodipine or human α-CGRP alone (Figures 3 and 4).

Discussion

The main findings of the present study were: (1) Low doses of human α-CGRP and nimodipine exerted synergistic internal carotid vasodilator effects without compromising other regional circulations or causing systemic hypotension. (2) Human α-CGRP or nimodipine reversed internal carotid vasoconstriction induced by infusion of endothelin-1. (3) Co-administration of high doses of human α-CGRP and nimodipine did not cause enhanced internal carotid vasodilatation compared to either compound alone, or compared to the effects of concurrent administration of low doses of human α-CGRP and nimodipine. However, although high doses of human α-CGRP and nimodipine given together did not cause inordinate hypotension, there was a marked impairment of renal blood flow.

Assuming our measurements of internal carotid Doppler shift signals give a good index of cerebral blood flow, the clear-cut internal carotid vasodilator effects of human α-CGRP and of nimodipine are interesting in the context of the clinical role these compounds might have in the treatment of cerebrovascular disorders. The findings with nimodipine are notable since several (e.g. Duncker *et al.*, 1986; Kanda & Flaim, 1986; Berger & Hakim, 1988; Young & Chien, 1989), but not all (e.g. Mohamed *et al.*, 1984), other studies have found this compound to have little influence on cerebral blood flow. This apparent absence of cerebral vasodilatation has raised the possibility that the beneficial effect of nimodipine on the outcome of subarachnoid haemorrhage (Pickard *et al.*, 1989) might be due to influences on metabolic processes (Berger & Hakim, 1988) and/or on cellular calcium fluxes (Lazarewicz *et al.*, 1989) following activation of excitotoxic amino acid-mediated mechanisms. If such a mechanism does contribute to the therapeutic influence of nimodipine (see Scriabine *et al.*, 1989; Langley & Sorkin, 1989), the present observation that low doses of human α-CGRP and nimodipine had synergistic effects in promoting internal carotid blood flow could result in more effective delivery of nimodipine to brain areas at risk. It is not known what effects human α-CGRP has on central neurotoxic events independently of its vascular action, or if such putative effects interact with those of nimodipine.

The present work indicates that co-administration of human α-CGRP and nimodipine in low doses was without detrimental effect on visceral circulations or on systemic arterial perfusion pressure, and caused as large an increase in internal carotid blood flow as was seen with higher doses of the two compounds together.

Human α-CGRP effectively reversed the internal carotid vasoconstriction induced by infusion of endothelin-1. This result is important since endothelin may be involved in cerebral vasospasm following subarachnoid haemorrhage (Asano *et al.*, 1989; Saito *et al.*, 1989). Elsewhere it has been shown that CGRP did not reverse the constrictor effects of endothelin in rabbit skin microvasculature (Brain *et al.*, 1988), thus it is feasible that the interaction of endothelin-1 and human α-CGRP differs in different vascular beds. Some indirect evidence for this comes from the finding that the lower dose of human α-CGRP reversed the internal carotid vasoconstrictor effects of endothelin-1 at a time when BP was still raised (Figure 2).

Although endothelin-1 was considered initially to be an endogenous ligand of the calcium channel (Yanagisawa *et al.*, 1988), subsequent observations have produced conflicting results concerning the influence of calcium channel antagonists on the cellular actions of endothelin-1, and it now seems likely that this is a 'tissue/species/dose' phenomenon (e.g.

Yanagisawa & Masaki, 1989; Secrest & Cohen, 1989; Bialecki *et al.*, 1989). In the present work infusion of nimodipine at 600 nmol h^{-1} reversed the internal carotid vasoconstrictor action of endothelin-1. However, this dose of nimodipine had substantial effects on internal carotid haemodynamics when given alone, so it does not follow that the reversal of the effects of endothelin-1 was due to calcium channel antagonism *per se*, rather than a functional antagonism as seen with human α-CGRP. Nonetheless, from a clinical point of view the overall effect rather than the mechanism of action may be more important. It remains to be determined if concurrent administration of the lower doses of human α-CGRP and nimodipine can reverse the vasoconstrictor effects of endothelin-1 or cerebral vasospasm due to pathophysiological mechanisms.

Although concurrent administration of high doses of human α-CGRP and nimodipine did not cause a greater hypotension than was seen with nimodipine alone, there was a significantly greater renal vasoconstriction and increased impairment of renal flow compared to the effects seen with human α-CGRP or nimodipine administered separately. The most likely explanation of this difference is that synergistic activation of the renin-angiotensin system by human α-CGRP (Kurtz *et al.*, 1988; Brasilis *et al.*, 1988; Bennett *et al.*, 1989b) and calcium channel antagonism (Hof & Hof, 1988), overrode any renal vasodilator effects of these compounds (Hof & Hof, 1988).

Concurrent administration of human α-CGRP and nimodipine together tended to cause less tachycardia than human α-CGRP alone, but since nimodipine vehicle had a bradycardic effect (as noted also by Kanda & Flaim, 1986), the apparent suppression of the tachycardic effects of human α-CGRP may not have been due to a specific interaction with nimodipine.

In summary, if the present findings can be extrapolated to the clinical situation, they indicate that concurrent administration of human α-CGRP and nimodipine at low doses might be helpful in the acute treatment of patients with cerebral vasospasm. Furthermore, the concurrent administration of low doses of human α-CGRP and nimodipine might be particularly useful in promoting cerebral blood flow in patients with cerebral vasospasm who also have compromised renal function, since this intervention did not impair renal perfusion, whereas administration of nimodipine alone in a dose sufficient to increase internal carotid blood flow reduced renal blood flow substantially.

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Evidence for noradrenergic-purinergic cotransmission in the hepatic artery of the rabbit

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1 Transmural electrical field stimulation produced a transient contraction of the isolated hepatic artery of the rabbit that was frequency-dependent up to 64 Hz. A contraction was rarely evoked at a stimulation frequency of less than 8 Hz and never at 4 Hz or less. All contractions were abolished in the presence of tetrodotoxin.

2 Neurogenic contractions were partially inhibited by prazosin. After desensitization of the P_{2X}-purinoceptor with α,β-methylene ATP, contractions in response to electrical stimulation were significantly reduced at all frequencies tested (4–64 Hz). In most cases, contractions were abolished by a combination of both drugs.

3 In vessels treated with 6-hydroxydopamine, no nerve-mediated contractile responses were observed.

4 In arteries from reserpine-treated rabbits, nerve stimulation evoked contractions that were resistant to prazosin. After desentization of the P_{2X}-purinoceptor with α,β-methylene ATP, no neurogenic contractile response remained.

5 The tissue contracted to exogenously applied noradrenaline and α,β-methylene ATP. There was an increase in sensitivity to noradrenaline in 6-hydroxydopamine-treated vessels compared with control vessels, but no difference in potency to α,β-methylene ATP was detected. The potency of noradrenaline and α,β-methylene ATP was not significantly affected by reserpine treatment.

6 In control tissues, fluorescence histochemistry demonstrated the presence of noradrenergic nerve fibres. Noradrenaline-containing nerves were not observed in 6-hydroxydopamine-treated or reserpine-treated vessels.

7 It is concluded that noradrenaline and ATP are cotransmitters in the sympathetic nerves supplying the hepatic artery of the rabbit. In contrast to other vessels, the hepatic artery requires a relatively high frequency of stimulation to evoke contractions and the purinergic component is not frequency-dependent. The significance of this finding in terms of the physiological demands of blood flow to the liver are discussed.

Introduction

Noradrenaline has been known for a long time to be the classical neurotransmitter released from perivascular nerves, producing its action via α_1-adrenoceptors. However, in certain arteries, a response to sympathetic nerve stimulation remains after adrenoceptor blockade (Holman & Surprenant, 1980; Muramatsu *et al.*, 1981; Suzuki & Kou, 1983). It has been proposed for several blood vessels that the response, which is resistant to α_1-adrenoceptor antagonists, may be a result of the purine adenosine 5'-triphosphate (ATP) being coreleased with noradrenaline producing its action via P_{2X}-purinoceptors (Sneddon & Burnstock, 1984; Kügelgen & Starke, 1985; Cheung & Fujioka, 1986; Kennedy *et al.*, 1986; Muramatsu, 1986; Burnstock & Warland, 1987).

The hepatic artery is a blood vessel that is richly innervated by sympathetic nerve fibres (see Lautt, 1983). Stimulation of the hepatic nerve induces a frequency-related vasoconstriction which results in a decrease in blood flow to the liver (Green *et al.*, 1959; Greenway *et al.*, 1967). However, nerve stimulation of the isolated hepatic artery of the dog has been shown to elicit a contraction that is resistant to α_1-adrenoceptor blockade (Varga *et al.*, 1984).

In the present study, the putative role of ATP as a cotransmitter with noradrenaline in the hepatic artery of the rabbit was investigated. The method of transmural electrical field stimulation was used to evoke neurogenic sympathetic responses, and the effects of prazosin and desensitization of the P_{2X}-purinoceptor with α,β-methylene ATP in control, 6-hydroxydopamine-treated and reserpine-treated vessels were examined.

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Methods

Male New Zealand white rabbits (2.3–3.3 kg) were killed by an overdose of pentobarbitone sodium (Sagatal) injected via the ear vein and exsanguinated. The proper hepatic artery which runs from the gastroduodenal artery to the porta hepatis was excised and cleaned of excess connective tissue and fat. Ring segments 4 mm in length were mounted horizontally in a 10 ml organ bath by inserting a tungsten wire through the lumen of the vessel ring and anchoring it to a stationary support. Another wire similarly inserted, was connected to a Grass FT03C force-displacement transducer and responses were recorded on a Grass ink-writing polygraph. The preparation was placed under a resting tension of 0.75–1.0 g and allowed to equilibrate for 1.5–2 h in Bülbring-modified Krebs solution of the following composition (mM): NaCl 133, KCl 4.7, $NaHPO_4$ 1.35, $NaHCO_3$ 16.3, $MgSO_4$ 0.61, glucose 7.8 and $CaCl_2$ 2.52, pH 7.2 (Bülbring, 1953). The solution was maintained at 37°C and aerated with 95% O_2 and 5% CO_2.

Transmural electrical stimulation was delivered to the tissue by means of two platinum wire electrodes placed parallel to, and on each side of the vessel, by a Grass S11 stimulator. A voltage of 60–70 V and a pulse width of 0.1–0.3 ms were used in experiments and the neurogenic origin of the response was confirmed by the abolition of all contractions with tetrodotoxin (3 μM).

The preparation was stimulated over a frequency range of 4–64 Hz for 1 s at 4 min intervals. This was repeated either

20 min after the application of prazosin (1 μM) or after desensitization of the P_{2X}-purinoceptor with α,β-methylene ATP (1 μM) and finally in the presence of both drugs. Desensitization was achieved by repeated exposure of the vessel to α,β-methylene ATP until no further contractile response was seen. If any responses remained after treatment of the tissue with both agents, stimulations were repeated in the presence of tetrodotoxin (3 μM).

Noradrenaline was added directly to the bath in a cumulative manner to establish a concentration-response curve. Since α,β-methylene ATP desensitizes its own receptors, a non-cumulative concentration-response curve was established by adding the purine analogue to the bath as single additions at 30 min intervals, with repeated washing between each addition.

These procedures were repeated in 6-hydroxydopamine-treated and control vessels and in reserpine-treated and control vessels.

6-Hydroxydopamine treatment

Segments of isolated artery were incubated in oxygenated Krebs solution containing 6-hydroxydopamine (4 μM) and ascorbic acid (0.1%) at 37°C for 4 h (Warland & Burnstock, 1987). For control experiments, segments of artery were incubated in Krebs solution containing ascorbic acid for the same period of time.

Preparations were then mounted and allowed to equilibrate for 1.5–2 h in normal Krebs solution before the beginning of the experiment.

Reserpine treatment

Rabbits received an intraperitoneal injection of reserpine 5 mg kg^{-1} 48 h and 3 mg kg^{-1} 24 h before killing. The drug was dissolved in a minimum amount of Tween 80 and made up to a 3 ml volume with Krebs solution.

Control animals were treated in a similar fashion with Tween 80 in 3 ml of Krebs.

Catecholamine fluorescence histochemistry

Vessels were cleaned of excess fat and connective tissue and then placed in a phosphate buffer solution containing glyoxylic acid (2% w/v) at pH 7.2 according to the method of Furness & Costa (1975). The segments were slit open longitudinally, stretched out on microscope slides, adventitial side uppermost, and air-dried. The stretched preparations were incubated at 100°C for 4 min, mounted in liquid paraffin and viewed under a Zeiss microscope fitted with an epi-fluorescence condenser 11RS.

This protocol was repeated in 6-hydroxydopamine-treated, reserpine-treated and control vessels.

Drugs and chemicals

α,β-Methylene adenosine 5'-triphosphate (lithium salt) (α,β-methylene ATP), (−)-noradrenaline bitartrate, reserpine, 6-hydroxydopamine hydrobromide, tetrodotoxin, polyethylenesorbitan monoleate (Tween 80) and glyoxylic acid monohydrate were obtained from Sigma Chemical Company, pentobarbitone sodium (Sagatal) was supplied by May and Baker Ltd, prazosin was obtained from Pfizer Ltd, and 0.1 M sodium dihydrogen phosphate from BDH.

Statistical analysis

Data are given as a mean ± s.e.mean. Results were analysed by Student's t test (paired or unpaired data as appropriate) and a probability of less than or equal to 0.05 was considered significant.

Results

Transmural electrical field stimulation evoked transient contractions of the hepatic artery of the rabbit that were frequency-dependent up to 64 Hz. A stimulation frequency of 4 Hz or less did not evoke a contraction (Figure 1) and in most cases, a frequency of less than 8 Hz rarely evoked a contraction. All neurogenic contractions were abolished after treatment of the vessel with tetrodotoxin (3 μM) and were partially inhibited by prazosin (Figures 1a and 2a). α,β-Methylene ATP (1 μM) itself elicited a large, transient contraction that fell

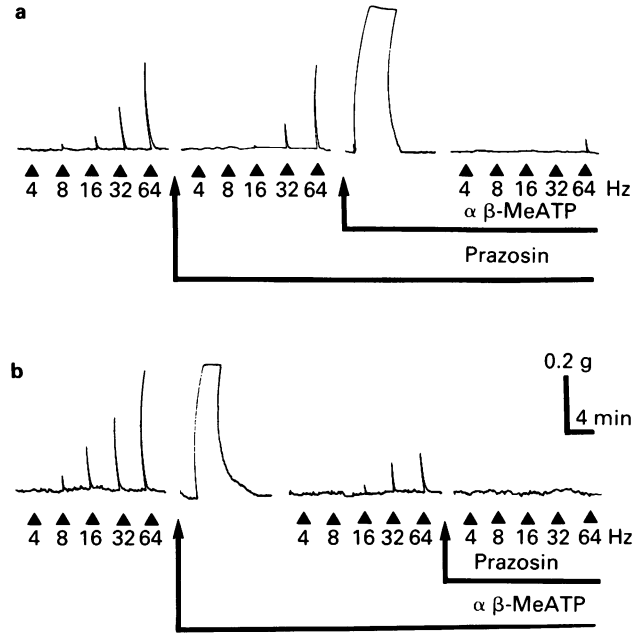


Figure 1 Contractions of the rabbit isolated hepatic artery to neurogenic transmural nerve stimulation for 1 s (4–64 Hz, 0.1–0.3 ms pulse width, 60–70 V). Nerve stimulations were repeated in the presence of 1 μM prazosin added before (a) or after (b) desensitization of the P_{2X}-purinoceptor with 1 μM α,β-methylene ATP (α,β-MeATP). α,β-MeATP produced a transient contraction which exceeded the recording range.

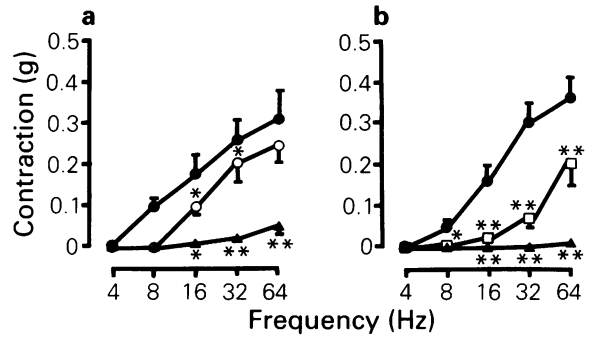


Figure 2 Contractions of the rabbit hepatic artery to neurogenic transmural nerve stimulation for 1 s (4–64 Hz, 0.1–0.3 ms pulse width, 60–70 V). (a) Neurogenic contractions produced in the absence of any drug (control) (●) are compared with those obtained in the presence of 1 μM prazosin (○) and subsequently following desensitization of the P_{2X}-purinoceptor with α,β-methylene ATP (1 μM) (▲). (b) Neurogenic contractions produced in the absence of any drug (control) (●) compared with those obtained after desensitization of the P_{2X}-purinoceptor with α,β-methylene ATP (1 μM) (□) and subsequently following the addition of prazosin (1 μM) (▲) ($n = 6$). Each value is the mean with s.e.mean shown by vertical lines. Significant differences (*$P < 0.05$; **$P < 0.01$) between control and experimental contractions were calculated by paired t tests.

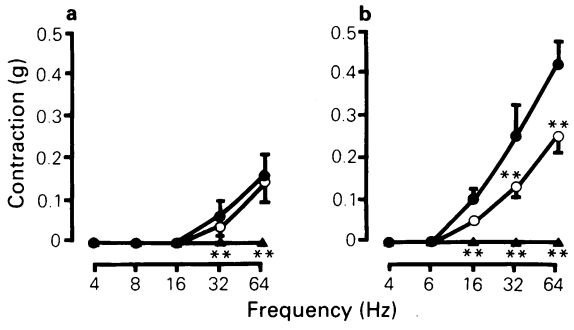


Figure 3 Effects of prazosin and α,β-methylene ATP on the neurogenic contractile responses of isolated hepatic artery from reserpine-treated rabbits (a, $n = 5$) and from control (vehicle-injected) rabbits (b, $n = 5$). Responses to 1 s periods of stimulation (4–64 Hz, 0.1–0.3 ms pulse width, 60–70 V) were measured in the absence of prazosin and α,β-methylene ATP (●) (a and b), in the presence of prazosin (1 μM) (○) (a and b) and in the presence of both prazosin (1 μM) and α,β-methylene ATP (1 μM) (▲) (a and b). Symbols represent the mean response and vertical lines denote the s.e.mean. Significant differences (**$P < 0.01$) were calculated by paired t tests.

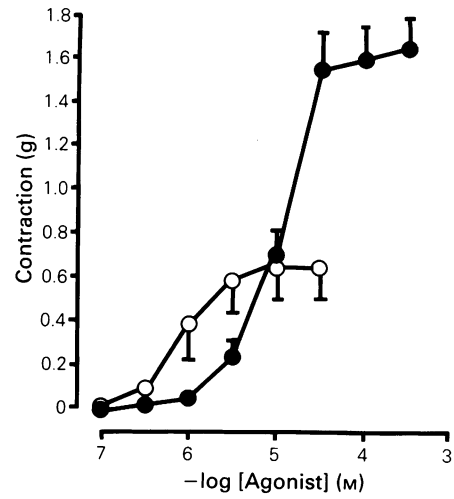


Figure 4 Concentration-response curves for noradrenaline (●) ($n = 10$) and α,β-methylene ATP (○) ($n = 7$) in the isolated hepatic artery of the rabbit. Each point represents the mean response and vertical lines denote the s.e.mean.

back to baseline after about 10 min. After complete desensitization of the P_{2X}-purinoceptor with α,β-methylene ATP, prazosin-resistant contractions were usually abolished. In preparations treated with α,β-methylene ATP before the addition of prazosin, the neurogenic response was markedly reduced. Subsequent addition of prazosin abolished any remaining responses in most cases (Figures 1b and 2b).

No nerve-mediated contractions were observed in vessels treated with 6-hydroxydopamine at any of the frequencies tested. Control vessels, incubated in ascorbic acid without 6-hydroxydopamine, demonstrated frequency-dependent contractile responses that were not significantly different in size when compared with those elicited in freshly prepared vessels ($n = 6$), and which were inhibited by prazosin and α,β-methylene ATP in a similar manner.

Hepatic arteries obtained from reserpine-treated rabbits demonstrated a contractile response to transmural electrical field stimulation at frequencies of 32 and 64 Hz and occasionally at 16 Hz. Addition of prazosin did not significantly affect the magnitude of these contractions ($n = 5$) (Figure 3a). Desensitization of the P_{2X}-purinoceptor with α,β-methylene ATP completely abolished the reserpine-resistant contraction even in the absence of prazosin. Neurogenic contractions in control vessels (from vehicle-treated rabbits) were not significantly different from those nerve-mediated responses observed in untreated vessels (Figure 3b) ($n = 5$).

Responses to drugs

Noradrenaline produced a concentration-dependent contraction of the rabbit hepatic artery (Figure 4). The maximal response was 1.6 ± 0.18 g ($n = 10$) and the EC_{50} was 10.0 ± 2.07 μM ($n = 10$). Desensitization of the P_{2X}-purinoceptor with α,β-methylene ATP (1 μM) did not affect the response to noradrenaline (maximal contraction was 1.65 ± 0.19 g; EC_{50} was 12.7 ± 1.57 μM; $n = 5$). There was a significant increase in sensitivity to noradrenaline in those vessels treated with 6-hydroxydopamine compared with their controls but no differences in the size of the maximal contraction (Table 1).

The stable analogue of ATP, namely α,β-methylene ATP, evoked a contraction that was concentration-dependent and transient in nature and could be reproduced only after 20–30 min of repeated washing (Figure 4). The maximal contraction (0.65 ± 0.18 g, $n = 6$) was less than that obtained with noradrenaline. However, this vessel appears to be more sensitive to the purine analogue, since the EC_{50} value for α,β-methylene ATP (1.6 ± 0.42 μM) was less than that for noradrenaline. α,β-Methylene ATP-induced contractions were not significantly affected by 6-hydroxydopamine treatment or reserpine treatment (Table 1).

Table 1 Effects of noradrenaline and α,β-methylene ATP (α,β-MeATP) on the isolated hepatic artery of the rabbit in 6-hydroxydopamine (6-OHDA)-treated and reserpine-treated tissue and their control tissue

	Maximal contraction (g)	EC_{50}
Noradrenaline treatment		
6-OHDA	1.47 ± 0.25 (6)	4.2 ± 0.87 (6)*
Control (6-OHDA)	1.47 ± 0.19 (6)	22.9 ± 8.4 (5)
Reserpine	1.74 ± 0.22 (9)	12.7 ± 2.2 (6)
Control (reserpine)	1.96 ± 0.19 (8)	10.7 ± 1.7 (8)
α,β-MeATP treatment		
6-OHDA	0.66 ± 0.14 (6)	9.7 ± 0.42 (6)
Control (6-OHDA)	0.69 ± 0.19 (7)	8.4 ± 0.27 (7)
Reserpine	0.56 ± 0.09 (7)	1.07 ± 0.85 (7)
Control (reserpine)	0.68 ± 0.17 (5)	1.07 ± 0.38 (5)

All values are given as mean ± s.e.mean with the number of observations n in parentheses.
* Significant difference between values ($P < 0.05$).

Catecholamine fluorescence histochemistry

All control tissues for 6-hydroxydopamine-treated and reserpine-treated vessels showed positive noradrenergic nerves with the glyoxylic acid treatment ($n = 6$ and 9 respectively).

There was no positive fluorescence in 6-hydroxydopamine- or reserpine-treated vessels ($n = 6$ and 9).

Discussion

The results presented provide evidence for the role of ATP as a cotransmitter with noradrenaline in the hepatic artery of the rabbit. The neurogenic contractile response induced by electrical transmural nerve stimulation was reduced, but not abolished by prazosin, a specific α_1-adrenoceptor antagonist (Cavero & Roach, 1980). However, desensitization of the P_{2X}-purinoceptor with α,β-methylene ATP substantially reduced the nerve-mediated response and abolished the

prazosin-resistant contraction in most cases. This suggests that contractions produced by stimulation of sympathetic perivascular nerves are largely purinergic in nature. The corelease of noradrenaline and ATP from sympathetic nerves supplying other blood vessels, such as the ear artery and the saphenous artery of the rabbit, has been demonstrated previously (Kügelgen & Starke, 1985; Sneddon & Burnstock, 1985; Kennedy *et al.*, 1986; Muramatsu, 1986; Burnstock & Warland, 1987; Muramatsu & Kigoshi, 1987). These vessels respond well to low frequencies of nerve stimulation and the relative contribution of the purinergic component is inversely related to the frequency of stimulation. It was suggested by Kennedy *et al.* (1986) that, since exogenous ATP desensitizes its own receptors, increased periods of stimulation could lead to a progressively smaller action of endogenously released ATP, whereas the action of noradrenaline could progressively increase. In contrast, the hepatic artery of the rabbit required a relatively high frequency of stimulation before a contraction was evoked and the purinergic component did not appear to be frequency-dependent. Studies in cats and dogs demonstrated that hepatic nerve stimulation causes an abrupt reduction in hepatic arterial blood flow (Greenway & Stark, 1971). However, the vasoconstriction is not maintained and blood flow recovers fully despite continuous stimulation. This response could be due to an accumulation of metabolic dilator factors that exert a powerful inhibitory effect on the arterioles. However, the existence of such metabolic dilator factors in the liver is obscure. The liver obtains its blood supply from two main sources, the hepatic artery and the portal vein. It has long been recognised that a reduced portal flow will produce an elevated hepatic arterial flow (Burton-Opitz, 1911). This relationship is now referred to as the 'hepatic arterial buffer response' (Lautt, 1981). The mechanism controlling the reaction remains speculative but it was suggested that a dilator substance is produced within the smooth muscle cells of the hepatic arterial resistance vessels; and further that the dilator substance may remain locally to produce dilatation of the artery or it may be washed away from the arterial vessel by the portal stream (Lautt, 1977). However, hepatic arterial hyperaemia following a serious loss of portal inflow is unable to provide complete haemodynamic compensation (Mathie & Blumgart, 1983). The hepatic artery is the sole means by which the blood flow to the liver is controlled and maintenance of a constant flow to the liver is of importance, since the hepatic clearance of many blood-borne drugs and hormones is blood flow limited. If ATP is the major neurotransmitter in the hepatic artery, increased sympathetic activity could lead to a self-limiting vasoconstriction as a result of desensitization. Therefore, it could be that a combination of this mechanism and the 'hepatic arterial buffer response' is required to prevent significant changes in total liver flow.

In the rabbit hepatic artery, treatment with 6-hydroxydopamine abolished all neurogenic contractions, whilst those in the control vessels remained intact and did not differ significantly in size when compared with those from freshly prepared vessels. The preparation contracted well to noradrenaline and supersensitivity developed in those vessels treated with 6-hydroxydopamine. The neurotoxic action of 6-hydroxydopamine is selective for sympathetic nerves (Theonen & Tranzer, 1968; Bennett *et al.*, 1970; Jonsson, 1980). The supersensitivity to noradrenaline observed in those vessels treated with 6-hydroxydopamine may be a result of the development of postsynaptic supersensitivity to the catecholamine (Westfall, 1981). Alternatively, it could be that, as a result of neurodegeneration, there is a loss in the catecholamine reuptake mechanism which leads to a potentiation of the action of noradrenaline. In this study, α,β-methylene ATP was selected for use as an agonist for the P_{2X}-purinoceptor rather than ATP, because it is more potent than ATP and because it can also be used to desensitize the P_{2X}-purinoceptor selectively (see Kasakov & Burnstock, 1983; Burnstock & Kennedy, 1985). In the rabbit hepatic artery, α,β-methylene ATP elicited a contraction that was not affected by treatment with 6-hydroxydopamine.

The complete abolition of nerve-mediated responses by 6-hydroxydopamine provides evidence that in the rabbit hepatic artery, noradrenaline and ATP are coreleased from sympathetic nerves and that both contribute to evoke a contraction in the vessel.

A contraction in response to electrical stimulation was also observed in those hepatic arteries from reserpine-treated rabbits. Reserpine depletes noradrenaline from sympathetic stores (Langer & Pinto, 1976; Suzuki *et al.*, 1984) without affecting the release of ATP or the prazosin-resistant component in several vessels and tissues (Cheung, 1982; Sneddon & Burnstock, 1984; Suzuki *et al.*, 1984; Kügelgen & Starke, 1985; Muramatsu, 1987; Warland & Burnstock, 1987). In this study, reserpine-treated vessels showed no positive catecholamine fluorescence following glyoxylic acid treatment, thus demonstrating the depletion of noradrenaline from sympathetic fibres innervating the artery. Prazosin did not affect the neurogenic contractile response remaining after reserpine treatment. However, α,β-methylene ATP abolished all reserpine-resistant neurogenic contractions. The responsiveness of the vessel to exogenously applied noradrenaline and α,β-methylene ATP was not affected by treatment of the rabbit with reserpine.

These results present evidence that in the rabbit hepatic artery, there appears to be a large purinergic component contributing to the production of a contraction on sympathetic nerve stimulation and that ATP is a cotransmitter with noradrenaline in the nerves innervating this vessel. Any residual responses after treatment with prazosin and α,β-methylene ATP were probably due to incomplete block by the two drugs, since the prazosin-resistant neurogenic contractions of reserpinised vessels could be completely inhibited after desensitization with α,β-methylene ATP. However, there is evidence to suggest that reserpine lowers the content of some neuropeptides that coexist with noradrenaline in peripheral sympathetic neurones (Gilbert *et al.*, 1981; Lundberg *et al.*, 1985; Allen *et al.*, 1986; Stjärne *et al.*, 1986; Nagata *et al.*, 1987) and so the possibility of a third neurotransmitter should not be ruled out.

The occurrence of noradrenergic-purinergic responses where α,β-methylene ATP has been shown to block selectively the ATP response has been well documented in many smooth muscle preparations, including the guinea-pig vas deferens (Meldrum & Burnstock, 1983), the mesenteric artery (Kügelgen & Starke, 1985; Muramatsu, 1986), the rat tail artery (Sneddon & Burnstock, 1985), the cat nictitating membrane (Duval *et al.*, 1985), the rabbit ear artery (Kennedy *et al.*, 1986), the dog cerebral artery (Muramatsu & Kigoshi, 1987) and the rabbit saphenous artery (Burnstock & Warland, 1987). It would appear for the hepatic artery of the rabbit also, that the sympathetic contraction consists of a noradrenergic and a purinergic component and that the purinergic response is mediated by ATP acting through postjunctional P_{2X}-purinoceptors.

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Effects of 5-HT_3 receptor antagonists on 5-HT and nicotinic depolarizations in guinea-pig submucosal neurones

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1 Intracellular recordings were made from neurones of the guinea-pig submucosal plexus. The effects of several 5-hydroxytryptamine$_3$ (5-HT_3) receptor antagonists on depolarizations produced by ionophoretic application of 5-HT and acetylcholine, as well as on fast excitatory postsynaptic potentials (fast e.p.s.ps) produced by nerve stimulation were examined.

2 ICS 205-930, GR 38032F, MDL 72222, cocaine and curare all inhibited the fast e.p.s.p. as well as the depolarizations in response to 5-HT and acetylcholine (ACh) ionophoresis in a dose-dependent fashion.

3 IC_{50} values for ICS 205-930, GR 38032F, MDL 72222, cocaine and curare in inhibiting the 5-HT mediated depolarizations were 12 nM, 100 nM, 3 μM, 3 μM and 20 μM, respectively.

4 IC_{50} values for ICS 205-930, GR 38032F, MDL 72222, cocaine and curare in inhibiting the nicotinic depolarizations were 4 μM, 12 μM, 11 μM, 6 μM and 17 μM, respectively. Similar IC_{50} values were obtained for inhibition of the fast e.p.s.ps by these antagonists.

5 The nicotinic receptor blocker, hexamethonium, inhibited the nicotinic depolarization and the fast e.p.s.p. with IC_{50} values of 10 μM. Hexamethonium (10 μM–5 mM) did not alter the depolarization induced by 5-HT.

6 These results demonstrate that the pharmacological profile of 5-HT_3 receptors present on submucosal neurones is identical to that of 5-HT_3 receptors on myenteric neurones and, thus, provide evidence that the enteric neuronal 5-HT_3 receptor forms a receptor subtype distinct from that characterized in other parts of the autonomic nervous system.

Introduction

5-Hydroxytryptamine (5-HT) has multiple actions on both pre- and post-synaptic membranes in autonomic neurones; this multiplicity of action is due largely to the presence of multiple 5-HT receptor subtypes (Bradley *et al.*, 1986; Peroutka, 1988). The cellular mechanism responsible for the 5-HT_3 response in autonomic ganglia is the best characterized of all 5-HT receptor responses: activation of 5-HT_3 receptors produces a rapidly desensitizing, 'nicotinic-like' depolarization resulting from an increased conductance to monovalent cations (Higashi & Nishi, 1982). More recently, patch-clamp studies from excised membranes have shown directly that the ganglionic 5-HT_3 receptor is a ligand-gated ion channel distinct from the nicotinic ion channel (Derkach *et al.*, 1989).

The pharmacology of 5-HT_3 receptors also has developed rapidly since the introduction of MDL 72222 (Fozard, 1984) and now several substances in addition to this one, particularly ICS 205-930, GR 38032F and zacopride, are known to be highly selective 5-HT_3 receptor antagonists (see Richardson & Engel, 1986; Peroutka, 1988). This selectivity pertains to the capacity of these antagonists to recognize 5-HT_3 receptors over other 5-HT subtypes and amine receptors such as dopamine and noradrenaline (Peroutka, 1988). Indeed, the development of these 5-HT_3 receptor antagonists has enabled these responses to be studied in isolation from other 5-HT responses and has been the major stimulus for the growing number of studies on the action of 5-HT in the autonomic nervous system (Mawe *et al.*, 1986; Surprenant & Crist, 1988; Wallis & Dun, 1988; Elliott & Wallis, 1988; Galligan *et al.*, 1988). 5-HT_3 antagonists have been shown to be selective for 5-HT_3 receptors over muscarinic acetylcholine receptors (Fozard, 1984; Richardson *et al.*, 1985; Surprenant & Crist, 1988) but the extent to which this selectivity pertains to the nicotinic acetylcholine receptor has received relatively little attention. This is an important question to examine in view of the similarities in the cellular mechanisms of 5-HT_3 and nicotinic responses as well as the well known action of curare in blocking both types of receptor response (Neild, 1978; Skok & Selyanko, 1979; Higashi & Nishi, 1982). Therefore, we undertook this study in order to examine the actions of a number of 5-HT_3 receptor antagonists on the 5-HT_3 and the nicotinic depolarizations in submucosal neurones and on the nicotinic excitatory synaptic potential produced by nerve stimulation of submucosal fibres. These studies have allowed us to determine the potency of action of these antagonists on both the 5-HT_3 and the nicotinic responses, as well as their relative selectivity of action at these two distinct receptors in submucosal neurones.

Methods

Experiments were carried out on submucosal plexus preparations obtained from the small intestine of guinea-pigs (175–250 g). Methods of dissection, intracellular recording and stimulation have been described previously (Surprenant & Crist, 1988; Surprenant & North, 1988). The preparation was continuously superfused with a physiological saline solution containing (mM): NaCl 126, NaH_2PO_4 1.2, $MgCl_2$ 1.2, $CaCl_2$ 2.5, KCl 5, $NaHCO_3$ 25, glucose 11; gassed with 95% O_2 and 5% CO_2.

Acetylcholine and 5-HT were applied by ionophoresis from the same microelectrodes as were used for intracellular recording; the concentration of agonist in the pipette was 100 μM. Stimulus parameters were set so as to produce responses whose durations were, as far as was possible, comparable to the fast e.p.s.p. (50–200 ms); duration of the ionophoretic pulses ranged from 0.5 ms to 15 ms. Small amounts of backing current (< 5 nA) were applied if signs of leakage existed. In some experiments dopamine (1 M solution) was applied by ionophoresis. Ionophoretic pulses were delivered at a frequency of 0.05 Hz throughout the course of the experiment. Fast excitatory postsynaptic potentials (e.p.s.ps) were recorded in response to single nerve stimuli delivered at a frequency of 0.3 Hz.

Antagonist concentration-inhibition curves were constructed by applying antagonists in cumulative doses, at least 5 min superfusion was allowed between measurements; preliminary experiments in which single concentrations of

antagonists were used indicated that this length of time was sufficient for antagonists to achieve their maximum effect. Intracellular recordings were usually maintained for sufficient time (typically 3–4 h) to examine several antagonists on a single cell. All results are expressed as means ± s.e.means; tests of significance were carried out by Student's *t* test.

Drugs

The following drugs were used: (from Sigma) atropine, hexamethonium, 5-hydroxytryptamine creatinine sulphate (5-HT), cocaine hydrochloride, dopamine, acetylcholine, curare and physostigmine; (from Glaxo UK) 1αH,3α,5αH-tropan-3-yl-3,5-dichlorobenzoate methanesulphonate (MDL 72222) and 1,2,3,9-tetrahydro-9-methyl-3-[(2-methyl-1H-imidazol-1-yl)-methyl]-4H-carbazol-4-one hydrochloride (GR 32032F; odansetron); (from Reckitt & Colman) idazoxan; (from Sandoz) (3α-tropanyl) H-indole-3-carboxylic acid ester (ICS 205-930).

Results

Depolarizations evoked by 5-HT, ACh and nerve stimulation

Our aim was to examine the actions of antagonists on responses which were as similar to those produced by neurally released transmitter as we could obtain (Figure 1). Resting membrane potentials of submucosal neurones studied ranged from −50 mV to −63 mV ($n = 43$); all responses were recorded at a membrane potential of −90 mV (by applying steady hyperpolarizing current through the intracellular microelectrode). In this study the fast e.p.s.p. produced by single nerve stimuli averaged 21 ± 1.1 mV amplitude with half-duration of 57 ± 4 ms ($n = 18$); average amplitude and half-duration of the 5-HT potential was 26 ± 1 mV and 132 ± 21 ms ($n = 14$); that for the ACh potential was 33 ± 2 mV and 180 ± 30 ms ($n = 17$). All neurones responded to 5-HT and ACh with these fast depolarizations ($n = 43$); the amplitudes of the 5-HT and ACh potentials were constant (±5%) for periods of hours when evoked by ionophoretic pulses delivered at frequencies of less than once per 15–20 s (see Methods). Some cells also exhibited a slow depolarization whose onset followed the decay of the fast depolarization (e.g. Figure 2c). This slow 5-HT-mediated depolarization has been shown previously to be insensitive to blockade by ICS 205-930 and curare (Surprenant & Crist, 1988). Neurones which exhibited this slow, non 5-HT$_3$ response, were not used for analysis of the actions of 5-HT$_3$ antagonists.

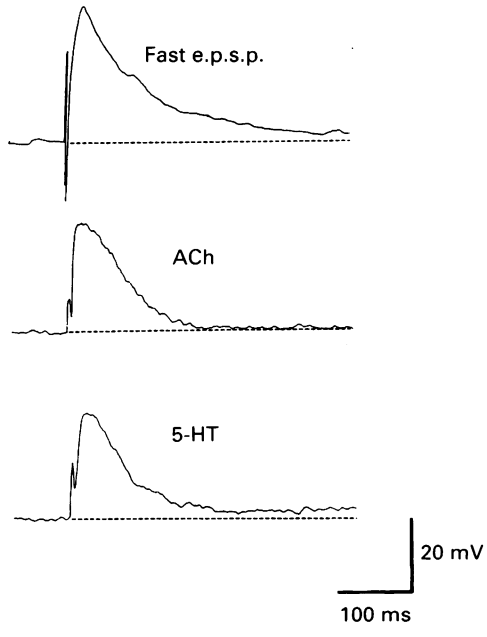


Figure 1 Examples of depolarizations evoked by nerve stimulation (fast excitatory postsynaptic potential (e.p.s.p.)), and ionophoretic pulses of acetylcholine (ACh) and 5-hydroxytrypamine (5-HT) in submucosal neurones; the actions of 5-HT$_3$ antagonists on these short duration responses were examined in this study. All responses recorded at a membrane potential of −90 mV.

Inhibition of responses by ICS 205-930 and GR 38032F

ICS 205-930 and GR 38032F inhibited both the 5-HT and ACh potentials as well as the fast e.p.s.p. in a dose-dependent and reversible fashion in all neurones examined (Figures 2 and 3). Figure 3 summarizes the results obtained from all experiments in which the actions of ICS 205-930 and GR 38032F on the 5-HT and ACh potential were examined. The concentrations of ICS 205-930 and GR 38032F which resulted in 50% inhibition (IC_{50}) of the 5-HT potential were 12 nM and 100 nM respectively. Tenfold higher concentrations were required to inhibit the ACh potentials (Figure 3; Table 1) with IC_{50} values for ICS 205-930 and GR 38032F inhibition of the nicotinic response being 4 μM and 12 μM respectively. The threshold concentration for inhibition of the fast e.p.s.p. by ICS 205-930 was 600 nM ($n = 4$).

Inhibition of responses by curare and hexamethonium

Curare produced equivalent inhibitions of the ACh potential, the 5-HT potential and the fast e.p.s.p., with IC_{50} values for

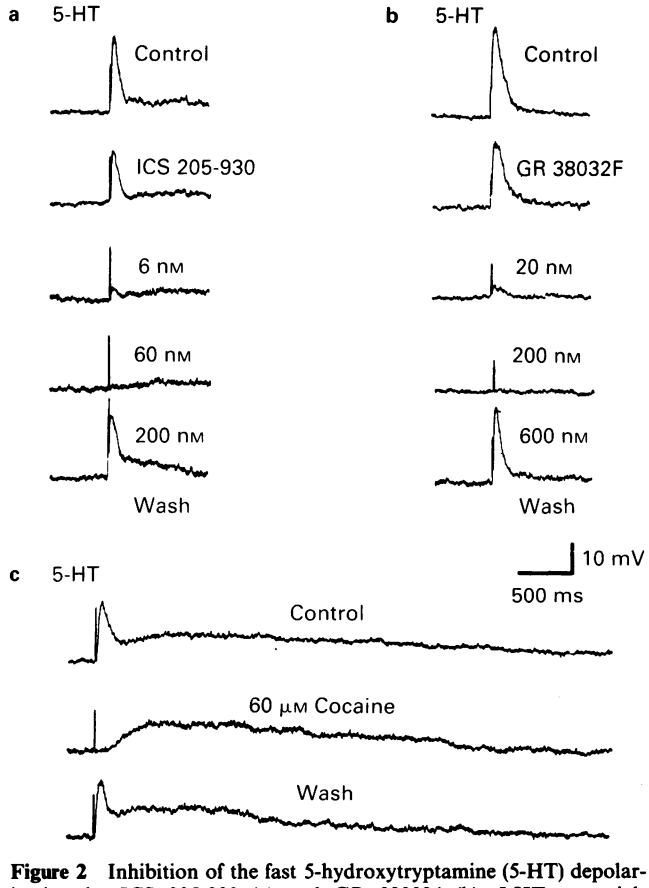


Figure 2 Inhibition of the fast 5-hydroxytryptamine (5-HT) depolarization by ICS 205-930 (a) and GR 380324 (b). 5-HT potentials evoked by repetitive ionophoretic pulses (frequency 0.05 Hz) in control solution and in the presence of increasing concentrations of ICS 205-930 and GR 38032F; each potential was obtained 5 min after the introduction of a new concentration of antagonist, by which time the maximum effect of the antagonist had been reached. All recordings in (a) and (b) are from the same cell. (c) Example of both fast and slow depolarizations recorded from a proportion of neurones in response to 5-HT ionophoresis. High concentrations of cocaine (60 μM) blocked the fast but not the slow depolarization.

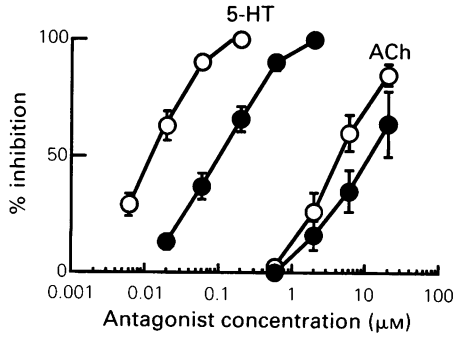


Figure 3 Inhibition of 5-hydroxytryptamine (5-HT, two leftmost curves) and acetylcholine (ACh, two rightmost curves) potentials by ICS 205-930 (○) and GR 38032F (●) in guinea-pig submucosal neurones. Each point is the mean from 4–8 experiments; vertical lines indicate s.e.mean.

all three responses being approximately 20 μM (Figure 4a; Table 1). Hexamethonium inhibited the ACh potential and the fast e.p.s.p. to the same degree (IC_{50} of 5 μM, Figure 4b), but had no effect on the 5-HT potential at concentrations of 500 μM ($n = 3$) or 5 mM ($n = 2$).

Inhibition of responses by MDL 72222 and cocaine

Cocaine and its close structural analogue, MDL 72222 (Fozard, 1984), inhibited both 5-HT and ACh potentials; the IC_{50} values for cocaine and MDL 72222 in inhibiting the 5-HT potential were the same (Figure 4c,d; Table 1). MDL 72222 was slightly, but significantly, more potent in blocking the 5-HT potential than the ACh potential (Figure 4d; Table 1). We also noted that neither cocaine (Figure 2c) nor MDL 72222 had any effect on the slow depolarization evoked in some cells by 5-HT ($n = 4$).

Voltage-dependence of inhibition by antagonists

In order to determine whether any of the antagonists might exhibit a voltage-dependence to their inhibition, we measured the inhibition produced by the IC_{50} concentration of each antagonist on the 5-HT and ACh potentials evoked at −60 mV, −90 mV and −110 mV. It was usually not possible to hold the membrane potential more depolarized than

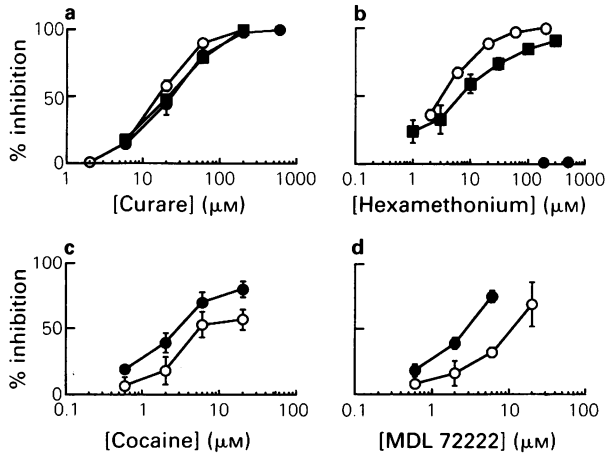


Figure 4 Inhibition of 5-hydroxytryptamine (5-HT) potentials (●), acetylcholine (ACh) potentials (○) and fast e.p.s.ps (■) by curare (a), hexamethonium (b), cocaine (c) and MDL 72222 (d) in guinea-pig submucosal neurones. Each point is the mean of 4–9 experiments (except in the case of hexamethonium on the 5-HT potential in which case $n = 2$); vertical lines show s.e.mean.

Table 1 Summary of IC_{50} values obtained for 5-hydroxytryptamine$_3$ (5-HT$_3$) antagonists examined in this study

Antagonist	*IC_{50} values* 5-HT	ACh	*Relative selectivity*
ICS 205-930	12 nM	4 μM	333
GR 38032F	100 nM	12 μM	120
MDL 72222	3 μM	11 μM	4
Cocaine	3 μM	6 μM	2
Curare	20 μM	17 μM	1

5-HT: acetylcholine (ACh) selectivity ratios are indicated for each antagonist.

approximately −55 mV because of the presence of a non-inactivating calcium current and calcium-activated potassium conductance at these potentials (Akasu & Tokimasa, 1989). ICS 205-930, GR 38032F and curare inhibited the 5-HT response to a similar degree at each membrane potential examined (Figure 5a). Both curare and hexamethonium produced a significantly greater inhibition of the ACh potential at −110 mV than at −60 mV (Figure 5b). However, this voltage-dependence of blockade of the nicotinic response was very weak (see Figure 5b) and thus appears similar to the nicotinic responses recorded in frog autonomic ganglia, where curare and hexamethonium inhibit nicotinic synaptic potentials in a voltage-independent manner (Lipscombe & Rang, 1988).

Dopamine activation of 5-HT$_3$ receptors

Depolarizations evoked by dopamine in mouse neuroblastoma cells have been shown to be mediated by 5-HT$_3$ receptors (Neijt *et al.*, 1986). In submucosal neurones, dopamine produces a membrane hyperpolarization by activating α_2-adrenoceptors (Hirst & Silinsky, 1974; Surprenant & North, 1988). We examined whether dopamine could also activate 5-HT$_3$ receptors on submucosal neurones by applying ionophoretic pulses of dopamine onto these neurones after

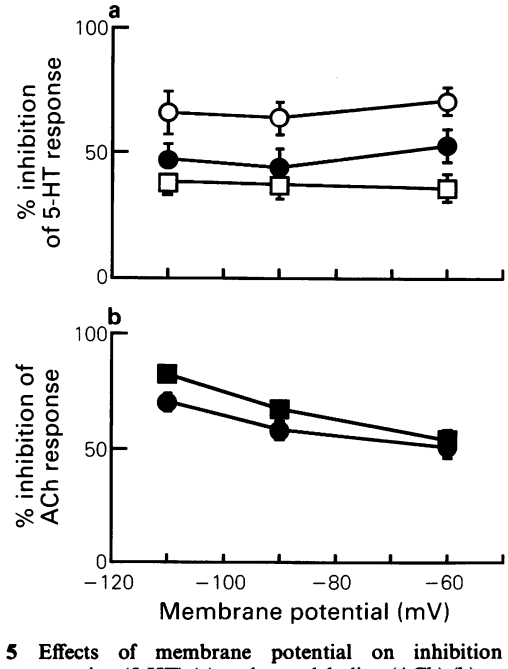


Figure 5 Effects of membrane potential on inhibition of 5-hydroxytryptamine (5-HT) (a) and acetylcholine (ACh) (b) potentials. (a) The membrane potential did not alter the inhibition of the 5-HT response produced by ICS 205-930 (10 nM, ○), GR 38032F (100 nM, □) or curare (20 μM, ●); $n = 4$–8 for each point. (b) The inhibition of the ACh potential by hexamethonium (■) and curare (●) was significantly greater when the membrane potential was held at −110 mV than when it was held at −60 mV; $n = 4$–8 for each point.

blockade of α_2-adrenoceptors by idazoxan (2 μM). In the presence of idazoxan, long duration (100–350 ms) ionophoretic pulses depolarized the membrane by 5–15 mV; the dopamine-mediated depolarization was abolished by 200 nM ICS 205-930 ($n = 3$) but not by the dopamine receptor antagonist haloperidol (2 μM).

Discussion

We have examined several agents which are known to inhibit 5-HT$_3$-mediated excitatory responses in autonomic ganglia and have determined their potency in inhibiting the 5-HT$_3$ depolarization in submucosal neurones (Table 1). We have also examined the ability of these antagonists to inhibit the nicotinic response in the same neurones and have demonstrated that they all block nicotinic responses with a distinct potency profile (Table 1). In submucosal neurones ICS 205-930 is over 300 fold selective in blocking 5-HT$_3$ over nicotinic responses, GR 38032F is greater than 100 fold selective for 5-HT$_3$ over nicotinic receptors while MDL 72222, cocaine and curare are approximately equipotent in blocking both 5-HT$_3$ and nicotinic receptors.

The IC_{50} values we obtained for ICS 205-930 (10 nM), GR 38032F (100 nM) and MDL 72222 (3 μM) in blocking the postsynaptic 5-HT potential on submucosal neurones are the same as dissociation equilibrium constants (K_D values) determined from more indirect assays in which the contractile responses of the whole ileum to 5-HT have been measured (Fozard, 1984; Richardson *et al.*, 1985; Richardson & Engel, 1986). The type of assay used in 'whole ileum' experiments primarily reflects 5-HT-induced release of acetylcholine from myenteric neurones (Fozard & Mobarok 1978; Furness & Costa, 1987); in other words, it is an indirect assay of 5-HT$_3$ receptors present on myenteric neurones. Our results demonstrate that 5-HT$_3$ receptors on submucosal neurones and on myenteric neurones are identical.

Results from the present study provide further evidence supporting the existence of subtypes of 5-HT$_3$ receptors. The pharmacological profile of 5-HT$_3$ receptors present on autonomic sympathetic neurones, such as the rabbit superior cervical ganglion, and sensory neurones (e.g. rabbit nodose ganglion) is clearly distinct from that of enteric neurones. K_D values for ICS 205-930, GR 38032F and MDL 72222 at sympathetic and sensory neurone 5-HT$_3$ sites are in the range of 40 pM, 5 nM and 10 nM, respectively; these values are ten to one hundred fold lower than obtained in this and other studies for 5-HT$_3$ receptors on enteric neurones (Fozard, 1984; Richardson & Engel, 1986; Butler *et al.*, 1988). Thus, while there is currently debate concerning whether the 5-HT$_3$ receptor present on sympathetic ganglia is pharmacologically distinct from that present on parasympathetic vagal fibres and sensory neurones (Richardson & Engel, 1986; Peroutka, 1988), our results on submucosal neurones combined with those on whole ileum (i.e. myenteric neurones) leave little doubt that the 5-HT$_3$ receptor on enteric neurones is a pharmacologically distinct receptor subtype.

Additional support for the existence of at least two 5-HT$_3$ receptor subtypes has come from recent electrophysiological studies of 5-HT$_3$-induced whole cell and single channel currents in neuroblastoma cells and submucosal neurones (Neijt *et al.*, 1988; Lambert *et al.*, 1989; Derkach *et al.*, 1989). The ionic mechanism and properties of desensitization underlying the 5-HT$_3$ responses in both tissues are identical. However, unitary conductances, measured either directly by single-channel recordings (submucosal neurones) or indirectly by noise analysis of 5-HT inward currents (neuroblastoma cells), demonstrate that the 5-HT$_3$ receptor in submucosal neurones is a 15 pS cationic channel while that in neuroblastoma appears to be a very small, subpicoseiman, cationic channel. The pharmacological profile of the 5-HT$_3$ receptor present on neuroblastoma cells partially resembles that of sensory neurones (Neijt *et al.*, 1986; 1988); thus it will be important to determine unitary conductance values for 5-HT$_3$ responses in other mammalian autonomic neurones and, particularly, to examine the actions of antagonists at the single channel level.

Few comparative data are available concerning the relative potencies of 5-HT$_3$ antagonists in a single tissue. One recent study, in which the properties of ICS 205-930, GR 38032F and zacopride at 5-HT$_3$ receptors in the guinea-pig ileum were compared (Cohen *et al.*, 1989), yielded K_D values for ICS 205-930 and GR 38032F which were identical to those obtained in the present study on submucosal neurones. Relative potencies of 5-HT$_3$ antagonists in blocking nicotinic responses have not been obtained previously, although micromolar concentrations of MDL 72222 are known to block nicotinic responses in the guine-pig ileum (Fozard, 1984). Our results demonstrating the relative selectivity between 5-HT and nicotinic responses should be of considerable practical use in investigations of these 5-HT$_3$ antagonists on *in vivo* and *in vitro* gastrointestinal functions.

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Characterization of cholecystokinin octapeptide-stimulated endogenous dopamine release from rat nucleus accumbens *in vitro*

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1 The effect of cholecystokinin sulphated octapeptide (CCK8S) on endogenous dopamine release was examined in rat striatal and nucleus accumbens slices, by high performance liquid chromatography (h.p.l.c.) with electrochemical detection.

2 CCK8S was shown to increase dopamine release from slices of nucleus accumbens but not striatum in a dose-dependent manner between 0.1 and 10 μM.

3 Pentagastrin was without effect on dopamine release at doses up to 10 μM.

4 The dopamine release produced in the presence of CCK8S was abolished by preincubation of the slice with 1.0 or 10.0 nM L-364,718 (the CCK_A-selective antagonist) while 1 μM L-365,260 (the CCK_B-selective antagonist) had no action.

5 These results suggest that the CCK8S-evoked release of dopamine from the nucleus accumbens is mediated by a CCK_A-receptor.

Introduction

In 1980 Hokfelt and his co-workers using immunohistochemistry combined with retrograde tracing showed that cholecystokinin (CCK) was present in a subpopulation of dopaminergic neurones originating in the ventral tegmental area which project into the caudal medial nucleus accumbens (Hokfelt *et al.*, 1980). In contrast to this it was found that CCK immunoreactivity and dopamine did not appear to be co-localised in the caudate nucleus. The physiological significance of these results has recently been the subject of considerable investigation. Autoradiographic studies with iodinated CCK or tritiated pentagastrin suggest that the nucleus accumbens and striatum both contain CCK receptors in high concentration (Gaudreau *et al.*, 1987; Pelaprat *et al.*, 1988).

Evidence from electrophysiological experiments using extracellular recordings and iontophoresis of CCK sulphated octapeptide (CCK8S) shows CCK8S excites neurones in the nucleus accumbens (White & Wang, 1984). Intravenous injections of CCK8S were also found to excite these neurones (Skirboll *et al.*, 1981). Injections of CCK into the nucleus accumbens potentiate dopamine-induced increases in locomotor activity (Crawley *et al.*, 1985).

In release experiments the results were more confusing. In the nucleus accumbens, tritiated dopamine release has been shown to be stimulated by CCK8S (Vickroy & Bianchi, 1989) but endogenous potassium-evoked dopamine release was inhibited by CCK8S (Voigt *et al.*, 1986). In the striatum some workers have shown CCK to stimulate dopamine release (Starr, 1982), while others have found CCK to have no effect (Vickroy & Bianchi, 1989).

Two CCK receptor subtypes have been identified. The CCK_A receptor was originally characterized in the pancreas and gastrointestinal tract (Sankaran *et al.*, 1981). This receptor has a high affinity for CCK8S and CCK-33 but a low affinity for pentagastrin, CCK unsulphated octapeptide (CCK8US) and CCK tetrapeptide (CCK4). The compound L364,718 has been shown to be a potent antagonist at this receptor with a K_i of 0.1 nM (Chang *et al.*, 1986a). The CCK_B receptor has a high affinity for CCK, CCK8S, CCK8US, CCK4 and pentagastrin. Recently a CCK_B receptor specific antagonist has been described (Lotti & Chang, 1989). Autoradiographic studies indicate that the majority of CCK receptors in the rat brain are of the CCK_B type; however, CCK_A receptors are present in a number of brain nuclei (Moran *et al.*, 1986, Hill *et al.*, 1987).

The availability of potent antagonists for the CCK_A and CCK_B receptors has enabled us to re-examine the effects of CCK8S on endogenous dopamine release from slices of nucleus accumbens or striatum and to determine the type of receptor involved.

Methods

Male Wistar rats (150–200 g) were killed by cervical dislocation and their brains carefully removed. This involved removal of the overlying skull with a pair of sharp scissors followed by peeling away the dura. The brain was scooped out with a small spatula (taking care not to 'bruise' the surface) directly into a beaker of artificial cerebrospinal fluid (artificial CSF) at room temperature (20°C) gassed with 95% O_2, 5% CO_2. The artificial CSF was of the following composition, (concentrations in mM): NaCl 124, KCl 5, NaH_2PO_4 1.25, $MgSO_4$ 2, $CaCl_2$ 2.4, $NaHCO_3$ 26 and glucose 10.

Horizontal sections of the nucleus accumbens and coronal sections of the striatum (between 2.7 mm rostral to and 0.4 caudal to Bregma, as shown in Paxinos & Watson, 1986) were cut with a vibrating sectioning system (Vibratome). To cut these sections, the cortex was left intact and the pia removed from the ventral surface of the brain with watch maker's forceps. The block of tissue was then glued with cyanoacrylate adhesive on a glass slide. For horizontal sections of the nucleus accumbens, the dorsal surface of the brain was uppermost with the rostral end facing towards the vibrating blade. The brain and glass slide was held on the stage of the Vibratome filled with artificial CSF at 18–22°C. The 350 μm thick slices of accumbens were then cut. For coronal sections of the striatum a similar procedure was followed except that the rostral end of the brain was uppermost and the ventral surface was facing the vibrating blade. A tissue punch was used to punch out equal sized pieces of striatum or accumbens from either side of the brain slice.

The slices were transferred to a purpose-built chamber where they were perfused with the artificial CSF (0.5 ml min^{-1}) at 37°C. Each chamber contained the pieces of striatum or nucleus accumbens obtained from one slice (the wet weight of tissue per chamber was 13 $\pm$ 0.5 mg for the striatum and 8.7 $\pm$ 0.3 mg for the nucleus accumbens). After perfusing for 1 h, 2 min fractions were collected into tubes containing 50 μl of 2 M $HClO_4$. An aliquot of the sample was removed and to this was added dihydroxybenzylamine

(DHBA) as an internal standard. Depolarization was produced by perfusing the slices with artificial CSF containing a submaximal dose of KCl, 35 mM (see results section), for 2 min. The potassium stimulation was carried out after 4 min to give S1 and at 44 min to give S2. Basal release was measured in the two fractions prior to the pulses of high potassium. The peptide agonists were added at 38 min and the antagonists at 14 min. Samples were stored frozen at −20°C. At the end of the experiment the slices were removed and placed in an Eppendorf tube containing 1 ml of Milli Q water, the tubes were then sonicated and a 50 μl aliquot removed for protein estimation (Lowry et al., 1951).

Dopamine released into the medium was analysed by high performance liquid chromatography (h.p.l.c.) with electrochemical detection. The h.p.l.c. used was a Model 6410B Severn Analytical pump, an automatic sample injector (Wisp 710B, Waters), a 3 μM ODS column (Catecholamine HR-80, ESA) which was protected by a guard column. Effluent from the column passed through a model 5011 Analytical cell of an ESA Coulochem Model 5100A detector. A guard cell (Model 5020, ESA), set at 0.45 V, was placed between the pump and sample injector. The analytical cell contained two electrodes in series. The first electrode was set at 0.1 V and the second electrode at 0.35 V. The mobile phase was obtained from ESA (Cat-a-phase) and was modified by the addition of 4% methanol; it was delivered at a rate of 1 ml min^{-1}. A series of catecholamine standards was run in order to identify the peaks in the samples. The total run time for the samples was 10 min. The limit of detection for dopamine was 5–10 pg in both standards and samples with a signal to noise ratio of 3:1.

Data were pooled and compared by Student's t test. All means and standard errors were obtained from between 4 and 13 unpaired data points each representing individual animals.

CCK and pentagastrin were obtained from Peninsula laboratories. The purity of these peptides was checked by h.p.l.c. L-364,718 (3S(−)-N-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1H-1,4-benzodiazepin-3-yl)-1H-indole-2-carboxamide) and L-365,260 (3R(+)-N-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1H-1,4-benzodiazepin-3-yl)-N'-(3-methylphenyl)urea) were synthesized in Medicinal Chemistry, Parke-Davis, Cambridge, U.K.

Results

Potassium-stimulated release of dopamine

In the striatum, the basal dopamine release before S1 was 0.32 ± 0.07 pmol mg^{-1} protein (n = 5). Basal release from the nucleus accumbens was too low to be detected. Increasing concentrations of potassium in the artificial CSF resulted in a dose-dependent increase in dopamine release from the striatum and the nucleus accumbens (Figure 1). Potassium, 35 mM, was chosen as the stimulus for subsequent experiments as this submaximal concentration would allow for an increase or decrease in release following drug treatment.

Dopamine release following a 2 min pulse of 35 mM potassium was 23.4 ± 8 pmol mg^{-1} protein (n = 5) from the striatum and 2.9 ± 0.2 pmol mg^{-1} protein (n = 5) from the nucleus accumbens. Absolute amounts of dopamine in the perfusates were 16 pmol ml^{-1} from the striatum and 1 pmol ml^{-1} from the nucleus accumbens. As the area of the nucleus accumbens in the horizontal slices is so small it was necessary to cut around the corpus callosum in order to maximize the amounts of nucleus accumbens tissue in the slice. It should be realised that the values for the release (in pmol min^{-1} mg^{-1} protein) from the nucleus accumbens are artificially low due to protein estimations being values of the total protein in the slices, which included corpus callosum.

The potassium-stimulated release from both areas was found to be entirely calcium-dependent. The control S2/S1 ratio was 0.95 ± 0.1 (n = 8) in the striatum and 0.82 ± 0.05 (n = 8) in the nucleus accumbens.

Action of neuropeptides

CCK8S (0.1–10 μM) produced clear, dose-dependent increases in potassium-stimulated dopamine release in the nucleus accumbens (Figure 2 and Table 1). Pentagastrin had no action at any of the doses tested. The sodium channel blocker tet-

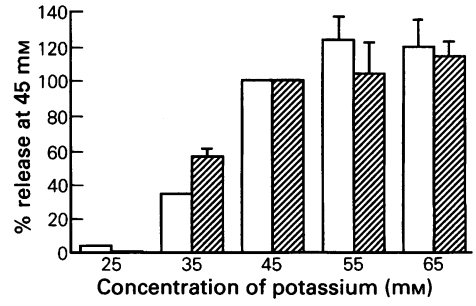


Figure 1 Histograms showing dose-dependent increases in dopamine release as a result of stimulation with increasing concentrations of potassium in the perfusing medium. The level of NaCl in the perfusing medium was correspondingly lowered to maintain osmolarity. The open columns show data from the nucleus accumbens, the hatched columns show data from the striatum. The results are expressed as a percentage of the release with 45 mM potassium. Each column represents the mean of 6 experiments. Standard errors of the means (vertical bars) are illustrated where they were greater than 3%.

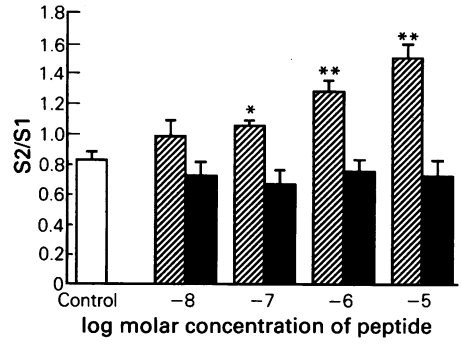


Figure 2 Histogram showing the dose-dependent effect of 10 to 10,000 nM cholecystokinin sulphated octapeptide (CCK8S) on potassium-stimulated dopamine release from nucleus accumbens slices (hatched columns). Each column represents an n of 4 to 13 from separate animals. The release stimulated by 1000 and 10,000 nM CCK8S was significantly different from the control 35 mM potassium-stimulated release (represented by the open column) where no peptides were present. There was no response to pentagastrin (solid columns) over a concentration-range from 10 to 10,000 nM. * $P < 0.05$, ** $P < 0.01$ comparing CCK8S data with control data. Perfusing of artificial CSF containing the peptides started 6 min before S2 and continued throughout the remainder of the experiment.

Table 1 Effects of cholecystokinin sulphated octapeptide (CCK8S) on 35 mM K^+-evoked dopamine release from the striatum and nucleus accumbens: S2/S1 ratios

	Nucleus accumbens	*Striatum*
Control	0.82 ± 0.05 (8)	0.95 ± 0.1 (8)
CCK 10.0 μM	1.52 ± 0.08 (4)**	
CCK 1.0 μM	1.26 ± 0.07 (13)**	1.01 ± 0.2 (4)
CCK 0.1 μM	1.04 ± 0.03 (4)*	1.04 ± 0.1 (4)
CCK 0.01 μM	0.97 ± 0.1 (5)	0.98 ± 0.28 (4)
CCK 0.001 μM	0.98 ± 0.5 (4)	0.88 ± 0.26 (4)
Control + TTX	0.65 ± 0.08 (4)	
CCK8S + TTX	1.28 ± 0.20 (4)	

TTX, tetrodotoxin.
* $P < 0.05$; ** $P < 0.01$; n, numbers in parentheses

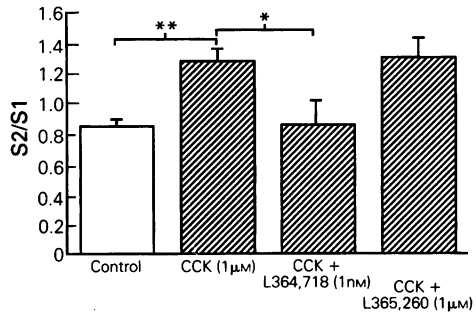


Figure 3 Histograms showing the response to 1000 nM cholecystokinin sulphated octapeptide (CCK8S) was reduced to control levels in the presence of 10 nM L-364,718 but not 1 μM L365,260 (** $P < 0.01$ comparing CCK8S with control data, * $P < 0.05$ comparing CCK8S in the presence of L-364,718 with control CCK8S data). Each column represents the mean and vertical bars the standard errors from 4 to 13 animals. Perfusion of artificial CSF containing the antagonists started 30 min before S2 and continued throughout the remainder of the experiment. The CCK was added to the perfusing medium 6 min before S2.

rodotoxin (1 μM) had no significant effect on the potassium-stimulated release and did not affect the CCK potentiation of this release (Table 1).

In contrast to the results obtained from the nucleus accumbens, CCK8S had no significant effect on basal or potassium-stimulated dopamine release from the striatum (Table 1). To ensure that the experimental conditions would allow us to detect a modulation of dopamine release from the striatum by neuropeptides, we tested the effects of neurotensin on the dopamine release from this area. It is now well documented that neurotensin increases dopamine release from striatal slices (DeQuidt & Emson, 1983, Hetier *et al.*, 1988). Neurotensin (0.01–1 M) in our system caused a dose-dependent increase in potassium-stimulated dopamine release from the striatum. Neurotensin 1 μM caused a 93% increase in the release of 35 mM potassium-stimulated dopamine release.

Classification of the cholecystokinin receptor in the nucleus accumbens

The CCK8S-stimulated dopamine release in the nucleus accumbens was abolished by 1 and 10 nM L-364,718, while 1 μM L-365,260 was without effect on the response to CCK8S (Figure 3). The blocking action of L-364,718 was dose-dependent (Figure 4).

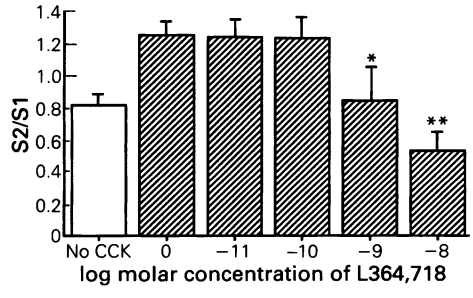


Figure 4 Dose-dependent blocking action of L-364,718 on the cholecystokinin sulphated octapeptide (CCK8S)-stimulated dopamine release from the nucleus accumbens (* $P < 0.05$, ** $P < 0.01$ compared with control CCK8S). Each column represents the mean and vertical bars the standard errors from 4 to 5 animals. Varying concentrations of the antagonist were perfused in the aCSF for 30 min before S2, 1 μM CCK was added to the perfusing medium 6 min before S2. Both CCK and the antagonist were then present for the remainder of the experiment. The open column represents the release from controls without CCK or antagonist.

Discussion

CCK8S produced a dose-dependent release of dopamine from the nucleus accumbens over the concentration range 0.1 to 10 μM. This suggests that the CCK binding sites in the nucleus accumbens observed in autoradiography studies (Pelaprat *et al.*, 1988) may be linked to effectors in this area.

Electrophysiological and biochemical studies suggest that pentagastrin is a selective agonist at CCK_B sites (Boden & Hill, 1988; Chang *et al.*, 1986a,b). Although high levels of pentagastrin binding sites have been found in the nucleus accumbens (Gaudreau *et al.*, 1987), the observation that pentagastrin was without effect on dopamine release suggests that in the nucleus accumbens the CCK8S-stimulated dopamine release was not mediated via these sites but by a CCK_A receptor population.

The use of selective CCK receptor antagonists confirmed that the CCK stimulation of endogenous dopamine release was mediated via CCK_A receptors. We found that the CCK_B selective antagonist L365,260 (Lotti & Chang, 1989) was without effect on the CCK stimulation of dopamine release while low concentrations of the CCK_A selective antagonist L-364,718 (Chang *et al.*, 1986a) potently inhibited this effect.

The sodium channel blocker, tetrodotoxin, had no effect on potassium-stimulated dopamine release or on the CCK8S facilitation of this release. These results suggest that CCK8S has a direct action on presynaptic dopamine terminals in the nucleus accumbens.

Thus our results are in agreement with previous studies measuring tritiated dopamine release, where a potentiation of dopamine release by CCK was found to be blocked by the CCK_A antagonist, A-65186 (Vickroy *et al.*, 1988). However, our findings that CCK8S caused an increase in potassium-stimulated dopamine release do not agree with previous experiments on endogenous dopamine release (Voigt *et al.*, 1986) where CCK8S was shown to decrease potassium-stimulated release. A possible explanation for the difference in the results is the different methodologies used in these studies. Voigt and co-workers used slices cut with a McIlwain tissue chopper and had a static incubation system whereas we used Vibratome cut slices and a superfusion system in our experiments. Evidence for CCK increasing dopamine release from the nucleus accumbens has also been found *in vivo* by use of microdialysis. Ruggeri *et al.* (1987) demonstrated that CCK8, CCK33 but not CCK4 could increase the release of dopamine from rostral parts of the nucleus accumbens. The fact that CCK4 had no effect is to be expected if the action of CCK is mediated by a CCK_A receptor.

In *in vivo* electrophysiological studies in the nucleus accumbens, lorglumide blocked the excitatory effects of CCK8S and both unsulphated CCK and CCK-4 were without effect (Wang *et al.*, 1988) suggesting CCK_A receptor-mediated excitations. However, if CCK8S were releasing dopamine it would be expected to inhibit rather than excite neurones. Microinjection of CCK into the nucleus accumbens of the rat has been shown to cause a potentiation of the effects of dopamine and apomorphine on two dopaminergically mediated behaviours, stereotypy and hyperlocomotion (Crawley, 1985); again this is to be expected if CCK were increasing dopamine release. In contrast to these results however Van Ree *et al.* (1983) found CCK8S, unsulphated CCK8 and ceruletide to antagonize hyperlocomotion induced by low doses of apomorphine.

Although there are now several lines of evidence from both release and electrophysiological experiments that CCK_A receptors mediate a number of CCK effects in the nucleus accumbens, binding and autoradiography have failed to show the presence of CCK_A receptors in this area. Displacing [^{125}I]-CCK8 binding with CCK_A receptor-specific antagonists has revealed the presence of CCK_A receptors in a number of regions of the CNS including the interpeduncular nucleus, area postrema and the nucleus tractus solitarius (Hill *et al.*, 1987); however, there was no evidence for a displace-

ment of binding from the nucleus accumbens. It is possible that a small population of CCK_A receptors do exist in the rat nucleus accumbens, but because the number is low compared to the CCK_B receptors in this region, binding and autoradiography experiments have not been sensitive enough to detect them. In contrast to the rat, in the primate nucleus accumbens, CCK_A receptors can be clearly visualised by autoradiography (Hill and Woodruff, personal communication).

We were unable to find any effect of CCK8S on basal or potassium-stimulated dopamine release from the striatum. To demonstrate that our system could detect a modulation of dopamine release from the striatum by a neuropeptide, we showed that dopamine release was increased by neurotensin as observed in previous studies. In the striatum, CCK receptors have been shown to be primarily located on neurones which have their cell bodies in the striatum and not on the dopamine nerve terminals, suggesting that any effect of CCK on dopamine in the striatum may be indirect (Beresford *et al.*, 1987). Unilateral intrastriatal injections of CCK8S cause contralateral rotation (Worms *et al.*, 1986), suggesting that there is a positive interaction with dopamine systems in the striatum. Furthermore, biochemical studies have demonstrated that CCK8S enhances cyclic AMP efflux from slices by stimulating dopamine release (O'Shaughnessy *et al.*, 1987). In other more direct release experiments CCK has been shown to increase (Starr, 1982), decrease (Markstein & Hokfelt, 1984) or have no effect (Vickroy & Bianchi, 1989) on dopamine release from the striatum. Differences between the effects of CCK on dopamine release from the nucleus accumbens and the striatum are perhaps to be expected in view of the fact that CCK is co-localised with dopamine in the nucleus accumbens but not in the striatum.

In conclusion our results demonstrate that CCK8S increases the potassium-stimulated release of dopamine from the nucleus accumbens but not the striatum. This effect appears to be mediated via a CCK_A type of receptor.

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