SJC

British Journal of Pharmacology

November 1990

Volume 101 Number 3 pages 499–768

Br. J. Pharmacol. (1990), 101, 501–503

SPECIAL REPORT

Selective chiral inhibitors of 5-lipoxygenase with anti-inflammatory activity

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The studies described here, using enantiomers of an optically-active methoxy alkyl thiazole ICI216800 (1-methoxy-6-(naphth-2-yl-methoxy)-1-(thiazol-2-yl)indane), provide unequivocal evidence for a specific, chiral interaction with 5-lipoxygenase. In accordance with their biochemical efficacy these compounds also demonstrate enantio-specific anti-inflammatory activity in a leukotriene-mediated model of inflammation. This is the first class of compounds for which 5-lipoxygenase inhibition and anti-inflammatory activity have been shown to be mediated via a specific chiral interaction.

Introduction Discovery of 5-lipoxygenase inhibitors which are orally active and selective compared to a related enzyme, cyclo-oxygenase, has proved difficult. Most of the previously described inhibitors have the potential to participate in redox reactions or to chelate iron and may have effects on other biological systems that involve iron or redox processes. Although we and others have identified orally-active and selective inhibitors based on these mechanisms (Tateson *et al.*, 1988; Carter *et al.*, 1989; Foster *et al.*, 1990) there has been little evidence for a specific interaction with 5-lipoxygenase. In particular there is no difference in biological potency between enantiomers of optically-active compounds (Salmon *et al.*, 1989).

The studies described here, using enantiomers of an optically-active methoxy alkyl thiazole, ICI216800, provide unequivocal evidence for a specific, chiral interaction with 5-lipoxygenase. Moreover, these compounds also demonstrate enantio-specific anti-inflammatory activity in a leukotriene-mediated model of inflammation.

Methods *Enantiomer synthesis and resolution* Each of the two enantiomers of ICI216800 [1-methoxy-6-(naphth-2-yl-methoxy)-1-(thiazol-2-yl)indane] were obtained pure (as confirmed by analytical high performance liquid chromatography (h.p.l.c.) and nuclear magnetic resonance spectroscopy) by preparative h.p.l.c. using a Pirkle ionic preparative chiral column (Pirkle & Finn, 1982). For the (+)-enantiomer the chiral phase of the column was prepared from Nucleosil 10 NH2 (10 μm aminopropyl) silica (Machery-Nagel GMBH, Duren, Germany) and S(+)-3,5-dinitrobenzoylphenylglycine and was eluted with hexane/dioxane (97:3). For the (−)-enantiomer a similar column was prepared but with R(−)-3,5-dinitrobenzoylphenylglycine as the chiral component to the phase.

Spectrophotometric analysis of 5-lipoxygenase Inhibition of cell-free 5-lipoxygenase activity was determined spectrophotometrically by modification of the procedure of Aharony & Stein (1986), using the high speed (150,000 *g*) supernatant from rat basophilic leukaemia (RBL-1) cells as the enzyme source.

Eicosanoid generation Eicosanoid generation in heparinised rat and human blood and protein-free mouse peritoneal macrophage cultures was determined as previously described (Foster *et al.*, 1990).

Arachidonic acid-induced inflammation in rabbit skin Anti-inflammatory efficacy of 5-lipoxygenase inhibitors was determined by a modification of the procedure previously described (Aked & Foster, 1987). Fur from the back of New Zealand White rabbits (3.0–4.0 kg: Ranch Rabbits, Crawley Down, Sussex) was removed with clippers 24 h before an intravenous injection of ^{125}I-labelled human serum albumin, in a 0.5% solution of Evans blue in physiological saline. Five min later inflammation was induced by the topical application of arachidonic acid alone (300 μg, in 10 μl acetone; 6 sites) or together with the appropriate concentrations of lipoxygenase inhibitor (3 sites). Sites treated with 10 μl of acetone were used to assess background ^{125}I-counts. Animals were killed 2 h later with a lethal dose of pentobarbitone sodium (Euthatal; May and Baker, Dagenham). The skin was removed and the injection sites were separated with a 15 mm diameter metal punch. ^{125}I-counts were determined in the skin sites and aliquots of plasma with an LKB ultra-gamma counter and the plasma volume at each inflammation site was calculated in μl after subtracting the mean background count.

Statistical analysis Differences between means were assessed either by Student's paired t test or by the use of a one-tailed Student's t test with $P < 0.05$ regarded as significant in both cases.

Materials Materials used were as previously described (Foster *et al.*, 1990 and references therein). REV5901 and all ICI compounds were synthesized in Chemistry Department 1, ICI Pharmaceuticals or at ICI Pharma, Centre de Recherches, Reims.

Results The optically-active compound ICI216800 (Table 1), is a potent and selective inhibitor of 5-lipoxygenase. In plasma-free peritoneal macrophage cultures, the compound produced potent inhibition of leukotriene C_4 (LTC_4) synthesis (mean $IC_{50} = 0.014\,\mu$M; $n = 2$) but did not inhibit synthesis of prostaglandin E_2 (PGE_2) at doses up to 30 μM which indicates a selectivity ratio (cyclo-oxygenase: lipoxygenase) of more than 2000. Inhibition of LTB_4 synthesis by ICI216800 in human blood ($IC_{50} = 0.69 \pm 0.18\,\mu$M; $n = 4$) was also selective with no significant inhibition of synthesis of the cyclo-oxygenase product, thromboxane B_2 (TxB_2), at doses up to 100 μM.

In order to investigate the chiral specificity of the inhibition, the enantiomers of ICI216800 were resolved and their effects compared to the parent compound (Table 1). The racemic mixture produced potent inhibition of 5-lipoxygenase in a cell-free enzyme assay and in human blood. Evaluation of the resolved enantiomers demonstrated that the lipoxygenase

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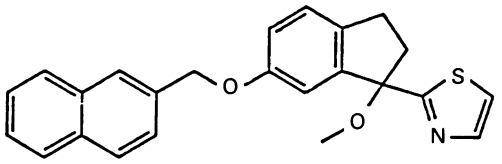
Table 1 Structure and chiral inhibition of 5-lipoxygenase by ICI216800



	IC_{50} (μM)		
	Racemate	*(+)-Enantiomer*	*(−)-Enantiomer*
LTB_4 synthesis in human blood	0.69 ± 0.18	0.54 ± 0.17	>40
Cell-free 5-lipoxygenase	0.77	0.13	>20

LTB_4 = leukotriene B_4.
Results in human blood are mean IC_{50} values ± s.d. ($n = 4$).
IC_{50} values for cell-free data are derived from mean values of duplicate determinations.

inhibitory activity resided predominantly in the (+)-enantiomer: (+)-ICI216800 was at least 70 times more potent than (−)-ICI216800 in both assays (Table 1).

Figure 1 compares the anti-inflammatory activity of the enantiomers against arachidonic acid-induced inflammation in rabbit skin, which is mediated in part by LTB_4 (Aked & Foster, 1987). Topical administration of (+)-ICI216800 produced dose-dependent inhibition of plasma extravasation with ED_{30} ($n = 4$) of 15.2 nmol per site. In accord with the *in vitro* data described above, (−)-ICI216800 was inactive at doses up to at least 78 nmol per site.

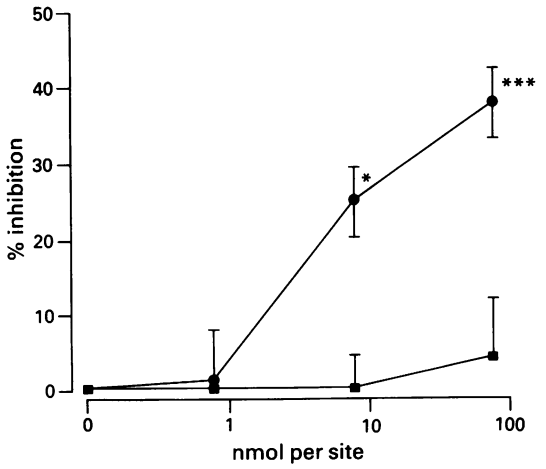


Figure 1 Topical anti-inflammatory activity of enantiomers of ICI216800: (●), (+)-ICI216800; (■), (−)-ICI216800. Values are the mean percentage inhibition of plasma extravasation in 4 rabbits; vertical bars show s.e.mean. The amount of plasma extravasation induced by arachidonic acid alone was 88.0 ± 10.5 μl. * $P < 0.05$; *** $P < 0.001$.

Discussion The identification of compounds that produce specific chiral inhibition of 5-lipoxygenase should allow more definitive pharmacological characterization of the biological roles of leukotrienes. Most previously described inhibitors of 5-lipoxygenase have the potential to participate in redox reactions or to chelate iron. Orally active compounds that selectively inhibit 5-lipoxygenase compared to cyclo-oxygenase have been derived from redox-based compounds (ICI207968: Foster *et al.*, 1990) and iron chelators (BWA4C: Tateson *et al.*, 1988; A-64077: Carter *et al.*, 1989). However little evidence for specific interaction with 5-lipoxygenase has been obtained with such compounds and the fact that enantiomers of optically active compounds of these classes are equipotent (Salmon *et al.*, 1989) suggests that they inhibit 5-lipoxygenase via a non-specific interaction with the enzyme. Thus, interpretation of pharmacological studies is complicated since similar effects on other redox or iron-dependent processes cannot be excluded.

The data presented here demonstrate that the optically-active compound ICI216800 is a potent, selective inhibitor of 5-lipoxygenase and the marked difference in inhibitory potency observed with the enantiomers demonstrates that inhibition involves a chiral interaction with the enzyme. Although we have not yet investigated the kinetics of inhibition, it is probable that methoxy alkyl thiazoles are like REV5901, which has been shown to interact at the active site of 5-lipoxygenase (Aharony *et al.*, 1986).

Proof of the importance of arachidonic acid metabolites as mediators of inflammation has depended largely on pharmacological evidence. Thus, the pro-inflammatory role of prostaglandins was strengthened with the demonstration that non-steroidal anti-inflammatory drugs (NSAIDs) inhibited prostaglandin biosynthesis. Studies in animal models of inflammation have demonstrated that the anti-inflammatory potency of NSAIDs correlates with their potency as cyclo-oxygenase inhibitors. In addition, with optically-active NSAIDs, anti-inflammatory activity and cyclo-oxygenase inhibition have been shown to reside in the same enantiomer (Ham *et al.*, 1972; Tomlinson *et al.*, 1972). Similarly we have previously shown, using arachidonic acid-induced oedema in rabbit skin, that the anti-inflammatory efficacy of a series of redox-based compounds, 2-substituted indazolinones, is directly related to their potency as lipoxygenase inhibitors (Foster *et al.*, 1990). The same is true for methoxy alkyl thiazoles (S.J. Foster, unpublished) and, in this study with enantiomers of ICI216800, anti-inflammatory activity is shown to reside solely in the enantiomer which inhibits 5-lipoxygenase.

In summary, ICI216800 and related compounds represent a novel series of 5-lipoxygenase inhibitors which are the first to show chiral interaction with the enzyme. Such chiral inhibitors will be valuable experimental compounds for clarifying the roles of leukotrienes in inflammation and other pathophysiological processes. In addition, such compounds provide a structural template for the development of compounds for clinical use.

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(Received July 2, 1990
Accepted July 27, 1990)

Br. J. Pharmacol. (1990), **101**, 505–512

Activation of μ- and δ-opioid receptors present on the same nerve terminals depresses transmitter release in the mouse hypogastric ganglion

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1 The inhibitory actions of μ- and δ-opioid receptor agonists on the strong, single fibre synaptic input to neurones contained in the mouse hypogastric ganglion have been examined.

2 The opioid agonists [D-Ala^2,NMePhe^4,Gly-ol^5]enkephalin (DAMGO, 10 nM–10 μM), morphine (10–30 μM), [D-Ser^2,Leu^5,Thr^6]enkephalin (DSLET, 3 nM–1 μM), [D-Pen^2,D-Pen^5]enkephalin (DPDPE, 10 nM–10 μM), all depressed the single fibre, all-or-nothing, nicotinic, excitatory synaptic potential (e.p.s.p.) recorded in mouse hypogastric ganglion neurones. U50488H (0.3–1 μM) was without effect.

3 The effect of DSLET, but not that of DAMGO, was reversed by the δ-opioid receptor-selective antagonist, ICI 174864 (0.3 μM). Naloxone (0.3 μM) antagonized the effect of both DSLET and DAMGO.

4 The site of action of the μ- and δ-receptor agonists was on the presynaptic terminals, since at the concentrations which depressed the e.p.s.p. these drugs did not affect the resting membrane potential or input resistance of the postganglionic neurone body, nor did they depress the postganglionic, nicotinic response to exogenously applied acetylcholine.

5 Quantal analysis further confirmed the presynaptic site of action; μ- and δ-opioid receptor agonists decreased the mean number of quanta released per stimulus but did not reduce the mean amplitude of the quantal unit.

6 It was concluded that μ- and δ-opioid receptors were located on the same presynaptic nerve terminals since, in the same neurones, μ- and δ-opioid receptor agonists depressed the same single fibre inputs.

7 The potassium channel blockers barium and quinine, at concentrations known to block opioid-activated somatic potassium conductances, reduced slightly but did not abolish the μ- and δ-opioid receptor-mediated inhibition of the e.p.s.p.

Introduction

Whilst the phenomenon of opioid inhibition of transmitter release from nerve terminals in the central and peripheral nervous systems has been well documented, the ionic mechanism(s) which may underly this inhibition still remains unclear. In contrast, at the level of the neuronal soma the changes in ionic conductances resulting from activation of opioid receptors have been elucidated. Opioids acting through μ- and δ-receptors have been observed to activate a potassium conductance and to inhibit a calcium conductance in peripheral (North & Tonini, 1977; Mihara & North, 1986) and central (Pepper & Henderson, 1980; Werz & Macdonald, 1983; North & Williams, 1985) neurones and through δ- and κ-receptors to inhibit a calcium conductance in peripheral and central neurones (Macdonald & Werz, 1986; Gross & Macdonald, 1987; North *et al.*, 1988; Bean, 1989) and through μ- and δ-receptors to inhibit a calcium conductance in neuronal cell lines (Tsunoo *et al.*, 1986; Hescheler *et al.*, 1987; McFadzean & Docherty, 1989; Seward *et al.*, 1989). The opioid activation of a potassium conductance and the inhibition of a calcium conductance appear to be mediated through a pertussis toxin-sensitive G protein (Aghajanian & Wang, 1986; Hescheler *et al.*, 1987; North *et al.*, 1987) but they are not secondary to inhibition of adenylate cyclase (North & Williams, 1985; McFadzean & Docherty, 1989).

In the present study, we have examined the action of μ-, δ- and κ-opioid receptor agonists on synaptic transmission through the mouse hypogastric ganglion. The cluster of neuronal somata comprising the hypogastric ganglion lie close to and innervate the vas deferens. This preparation has been chosen because the postganglionic cell bodies are ovoid in shape, lack a dendritic arborization and each is innervated by only a single preganglionic nerve fibre (Rogers *et al.*, 1990). Single inputs are commonly observed in autonomic ganglia in which the neurone somata lack any dendritic arborization (Tabatabai *et al.*, 1986; Snider, 1987; Purves *et al.*, 1988). The relatively simple anatomical arrangement in the mouse hypogastric ganglion has permitted us to determine whether different opioid receptor types exist on the same nerve terminal and to examine the mechanisms by which inhibition of transmitter release are produced. Some of the results contained in this paper have previously been communicated (Henderson & Rogers, 1987a,b).

Methods

Electrophysiological recording

The hypogastric ganglion was isolated from male mice (DBA I/1a; 20–25 g) as described previously (Rogers *et al.*, 1990). The preparation was pinned out in the base of a shallow, Sylgard-lined bath (volume 0.4 ml) and superfused at a rate of 2–3 ml min^{-1} with an oxygenated Krebs solution at 37°C.

Intracellular recordings were made from individual ganglion neurones by use of glass micropipettes filled with 3 M KCl (tip resistance 30–80 MΩ). The recording electrode was incorporated into the bridge circuit of a conventional preamplifier

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which permitted simultaneous current injection and voltage recording. Voltage and current records were captured on a digital storage oscilloscope and displayed on a pen recorder.

Preganglionic inputs to the hypogastric ganglion were stimulated by brief current pulses (0.1 ms duration; 0.017 Hz) applied through a fine-tipped (<10 μm) Krebs-filled micropipette placed on a fibre tract. The stimulus intensity was adjusted to ensure that the stimulus was sufficient to evoke synaptic potentials.

Quantal analysis

After a synaptic response was obtained, the Krebs solution superfusing the ganglion was changed to one containing low calcium (0.3 mM) and high magnesium (3 mM). When the low calcium/high magnesium Krebs had been applied for 10 min the rate of preganglionic nerve stimulation was increased to 1 Hz and a 2–4 min sample of responses recorded. Drugs were then applied and after 3 min of application, when the effect of the drug was maximal, another 2–4 min sample of responses was recorded. Responses were recorded on an FM tape recorder for subsequent play-back and analysis. Frequency-response amplitude histograms were constructed.

If a Poisson distribution for transmitter release was assumed when the probability of release had been lowered (see Discussion), the quantal content of excitatory synaptic potentials (e.p.s.ps) was calculated by inserting the number of failures observed into the Poisson relationship which then simplifies to

$$n_0/N = e^{-m}$$

where n_0 represents the number of failures, N represents the total number of stimuli in the train and m represents the mean number of quanta released per stimulus (Del Castillo & Katz, 1954). The mean amplitude of the response to a single quantal unit of transmitter release was obtained by dividing the mean amplitude of the synaptic responses by m.

Solutions and drugs

The composition of the modified Krebs solution superfusing the ganglion was (mM): NaCl 126, KCl 2.5, NaH_2PO_4 1.2, $MgCl_2$ 1.3, $CaCl_2$ 2.4, $NaHCO_3$ 26, glucose 10 and saturated with 95% O_2 plus 5% CO_2 at 37°C. Drugs were dissolved in the Krebs solution and, with the exception of acetylcholine, applied to the ganglion in known concentrations by addition to the superfusing solution. Acetylcholine (1 M) was made up daily from solid. It was dissolved in Krebs solution and applied by pressure ejection from a micropipette (tip diameter 5–10 μm) placed above the ganglion, close to the impaled neurone. Pressure pulses 2–6 psi (1 psi = 6.8 kPa), 50–800 ms duration were applied at 4 min intervals by a pneumatic pressure system. Pressure application of acetylcholine evoked reproducible, dose-dependent depolarizations of the postganglionic neurone.

The drugs used were: acetylcholine chloride (Sigma), Allyl$_2$,Tyr,Aib,Aib,Phe,Leu-OH (ICI 174864; Cambridge Research Biochemicals (CRB)), atropine methylbromide (Sigma), [D-Ala^2,NMePhe^4,Gly-ol^5]enkephalin (DAMGO; CRB), [D-Pen^2,D-Pen^5]enkephalin (DPDPE; CRB), [D-Ser^2,Leu^5,Thr^6]enkephalin (DSLET; CRB), hexamethonium bromide (Sigma), mecamylamine hydrochloride (Sigma), morphine sulphate (Macarthy), naloxone hydrochloride (Endo), quinine hydrobromide (Sigma), tetrodotoxin (TTX; Sigma), *trans*-3,4-dichloro-N-methyl-(2-(1-pyrrolidinyl)cyclohexyl) benzene acetamine sulphonate hydrate (U50488H; Upjohn). Aib is aminoisobutyric acid and Pen is β,β dimethyl cysteine.

Where appropriate, results are presented as the mean ±s.e.mean. Statistical analysis was performed by Student's *t* test for paired data. Results were considered to be significantly different when *P* was less than 0.05.

Results

Inhibition by opioids of the evoked synaptic response

Mouse hypogastric ganglion neurones had passive and active membrane properties and synaptic responses similar to those previously described (Rogers *et al.*, 1990). Focal stimulation of preganglionic fibre tracts evoked an excitatory postsynaptic potential (e.p.s.p.) which in most neurones was suprathreshold for action potential initiation (see Figures 1 and 2). As the stimulus intensity was increased from zero, e.p.s.p./action potential complexes were evoked in an all-or-nothing manner (see also Figure 10 of Rogers *et al.*, 1990). This occurs because each neurone receives only a single, preganglionic strong input. Synaptic responses were blocked by TTX (1 μM) or by raising magnesium concentration of the superfusing solution to 20 mM. E.p.s.ps were nicotinic in nature since they were either markedly reduced or abolished by the ganglionic nicotinic antagonists hexamethonium (0.1–1 mM) or mecamylamine (10–100 μM) or abolished following desensitization of nicotinic receptors by prolonged (15 min) exposure to a high concentration (100 μM) of nicotine. As the degree of synaptic blockade increased e.p.s.ps eventually became subthreshold for action potential initiation.

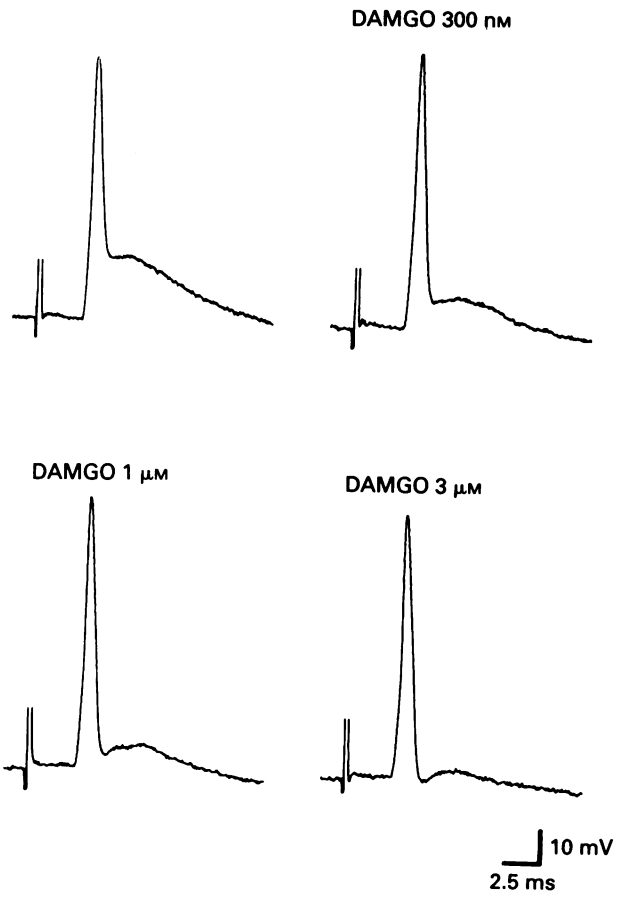

Figure 1 Depression by [D-Ala^2,NMePhe^4,Gly-ol^5]enkephalin (DAMGO) of the evoked synaptic response recorded in a single mouse hypogastric ganglion neurone. Stimulation of the preganglionic fibre with single pulses (0.017 Hz) evoked an e.p.s.p. which was suprathreshold for action potential initiation (top left-hand trace). For clarity the stimulus artefact has been attenuated. In this neurone the action potential arose from the resting membrane potential without an inflexion on the rising phase and the e.p.s.p. was apparent as an after-depolarization following the action potential. In the presence of increasing concentrations of DAMGO (each concentration was applied for 6 min with a 9 min wash between drug applications) the after-depolarization was reduced but the evoked synaptic response remained suprathreshold for action potential initiation.

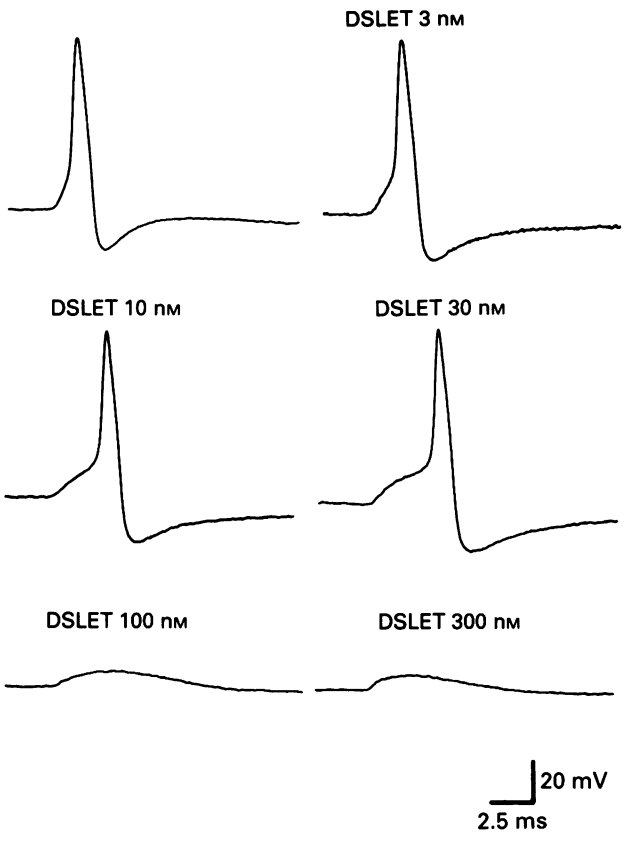

Figure 2 Depression by [D-Ser^2,Leu^5,Thr^6]enkephalin (DSLET) of the evoked synaptic response recorded in a single mouse hypogastric ganglion neurone. In this neurone preganglionic fibre stimulation (0.017 Hz) evoked an e.p.s.p. which was suprathreshold for action potential initiation (top left hand trace). For clarity the stimulus artefact has been removed. In the presence of increasing concentrations of DSLET (each applied for 5 min with a 10 min wash between drug applications) the e.p.s.p. was depressed. In the presence of 3–30 nM DSLET the depression is apparent as an increase in the time taken for the e.p.s.p. to reach threshold for action potential initiation but finally in the presence of 100–300 nM DSLET the e.p.s.p. is depressed to such an extent that it is rendered subthreshold for action potential initiation.

Illustrated in Figure 1 is the type of e.p.s.p./action potential complex observed in 75% (113 of 150) of hypogastric ganglion neurones. The strong synaptic input evoked, in the postsynaptic neurone, an action potential that rose from resting membrane potential without an inflexion on its rising phase. The e.p.s.p. and the after-hyperpolarization following the action potential are apparent as the after-depolarization following the action potential. Synaptic input of this type could not be rendered subthreshold for action potential initiation by injection of hyperpolarizing current into the neurone through the recording electrode. In 19% (29 of 150) of neurones a less intense synaptic response was observed (Figure 2). In these neurones synaptic activation evoked a depolarizing potential which, on reaching threshold, gave rise to an action potential and the e.p.s.p. was partly occluded by the after-hyperpolarization following the action potential. In a small proportion (5%) of neurones subthreshold, yet still all-or-nothing, e.p.s.ps were observed. When evoked at a constant stimulus intensity these e.p.s.ps fluctuated in amplitude, presumably due to fluctuations in the quantal release of acetylcholine. The latency of the synaptic responses varied from 2 to 12 ms in different neurones and was not correlated with the type of synaptic response.

Both DAMGO (10 nM–10 μM; $n = 42$) and DSLET (3 nM–1 μM; $n = 49$) depressed the e.p.s.p. The depression was concentration-dependent (Figures 1, 2 and 6), reached a maximum within 3 min of applying the drug and readily reversed on washout. The depression of the e.p.s.p. by DAMGO and DSLET was observed in all neurones tested and was maintained for the duration of drug application up to 6 min. Figure 1 shows the effect of DAMGO on an e.p.s.p./action potential complex in which the synaptic response was more intense and the e.p.s.p. is apparent as the depolarization following the action potential. DAMGO did not render the synaptic response subthreshold but markedly reduced the depolarization following the action potential. Figure 2 shows the effect of DSLET (3–30 nM) on a neurone exhibiting a less intense synaptic response. DSLET increased the time taken for the e.p.s.p. to reach threshold for action potential initiation. At higher concentrations of DSLET (100–300 nM) the e.p.s.p. was reduced to such an extent that it was subthreshold for action potential initiation. In the presence of these concentrations of DSLET, however, injection of depolarizing current into the neurone through the recording electrode still evoked action potentials (not illustrated). Similar effects of both μ- and δ-selective agonists were seen on these two types of synaptic response. In addition, when e.p.s.ps were rendered subthreshold for action potential initiation by application of hexamethonium (1 mM; see Figure 6), or in the small percentage of neurones that showed a subthreshold e.p.s.p., DAMGO or DSLET depressed the amplitude of the e.p.s.p. in a graded manner. In general, DAMGO was slightly less potent than DSLET in that the percentage depression of subthreshold and suprathreshold e.p.s.ps was 62 ± 1 ($n = 11$) and 48 ± 5 ($n = 23$) respectively for DAMGO (300 nM) and 63 ± 5 ($n = 10$) and 49 ± 4 ($n = 30$) respectively for DSLET (100 nM).

To determine the nature of the opioid receptor subtypes present in the mouse hypogastric ganglion we have examined the effects of selective agonists and antagonists. E.p.s.ps were depressed in a concentration-dependent manner by the δ-receptor agonist, DPDPE (10 nM–1 μM; $n = 3$) and by morphine (10–30 μM; $n = 3$), but not by the κ-receptor agonist U50488H (0.3–1 μM; $n = 5$) applied for 15 min. The inhibition produced by DSLET was reversed by addition of the opioid antagonist naloxone (10–100 nM; $n = 4$) or the δ-selective antagonist ICI 174864 (300 nM; $n = 5$) (see Figure 3), whereas the inhibition produced by DAMGO was reversed by naloxone (100 nM; $n = 3$) but not by ICI 174864 (300 nM; $n = 5$) (see Figure 3).

Presynaptic locus of action of opioids

(i) *Lack of effect of opioids on neuronal membrane potential and input resistance* When applied for periods up to 15 min, over a wide range of concentrations, the opioid agonists DSLET (0.01–1 μM), DPDPE (0.01–1 μM), DAMGO (0.01–10 μM), morphine (10–30 μM) and U50488H (0.3–1 μM) did not cause a consistent, concentration-dependent or naloxone-reversible change in the resting membrane potential or input resistance of the ganglion neurone. The lack of effect of opioids directly on the somata of the postsynaptic neurones indicates that the inhibition of the synaptic responses observed was not secondary to an increase in the conductance of the postsynaptic membrane.

(ii) *Lack of effect of opioids on the response of ganglion neurones to exogenously applied acetylcholine* In the presence of atropine (1 μM), local application of acetylcholine from a pressure pipette located close to the impaled neurone evoked a membrane depolarization which was graded with the amount of drug applied and abolished by the ganglion blocking agent hexamethonium (300 μM). Neither DSLET (1 μM) nor DAMGO (1 μM), when applied for 5–10 min had any consistent effect on the nicotinic response to acetylcholine (Figure 4). The mean decrease in the amplitude of the response to acetylcholine was $1 \pm 4\%$ ($n = 8$) in the presence of DSLET and $5 \pm 6\%$ ($n = 5$) in the presence of DAMGO.

(iii) *Quantal analysis of the action of opioids* When the ganglion was bathed with the low calcium/high magnesium Krebs

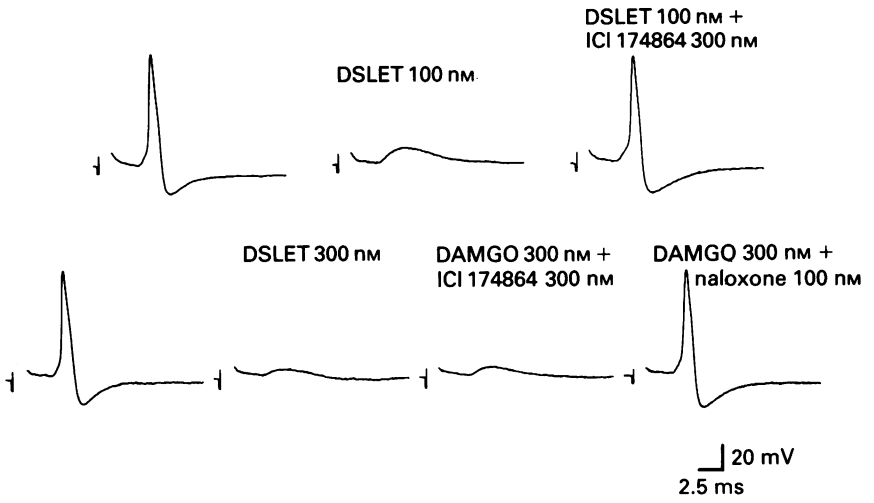

Figure 3 [D-Ser^2,Leu^5,Thr^6]enkephalin (DSLET) and [D-Ala^2,NMePhe^4,Gly-ol^5]enkephalin (DAMGO) depress the same synaptic response in a single mouse hypogastric ganglion neurone. In this neurone stimulation of the preganglionic fibre with single pulses (0.017 Hz) evoked an e.p.s.p. which was suprathreshold for action potential initiation (top left-hand trace). For clarity the stimulus artefact has been attenuated. DSLET (100 nM) applied for 5 min depressed the e.p.s.p. such that it became subthreshold for action potential initiation. The effect of DSLET was blocked by ICI 174864 (300 nM) administered for 5 min before and then during the application of DSLET. DAMGO (300 nM) also depressed the e.p.s.p., rendering it subthreshold. The depression produced by DAMGO was not antagonized by ICI 174864 (300 nM) but was antagonized by naloxone (100 nM) administered for 5 min before and then during the application of DAMGO

solution, e.p.s.ps were reduced in amplitude and became subthreshold for action potential initiation. Although e.p.s.ps were still evoked in an all-or-nothing manner, at a constant intensity of stimulation fluctuations in amplitude were apparent and some stimuli failed to evoke an e.p.s.p. We have analysed these data assuming that the distribution of e.p.s.p. amplitudes in the mouse hypogastric ganglion conforms to a Poisson distribution (see Discussion). Figure 5 illustrates graphically the data obtained from a single neurone, whilst the data from all such experiments are given in Table 1. Both the mean number of quanta released per stimulus and the mean amplitude of a single quantal unit varied between neurones but was consistent within individual neurones.

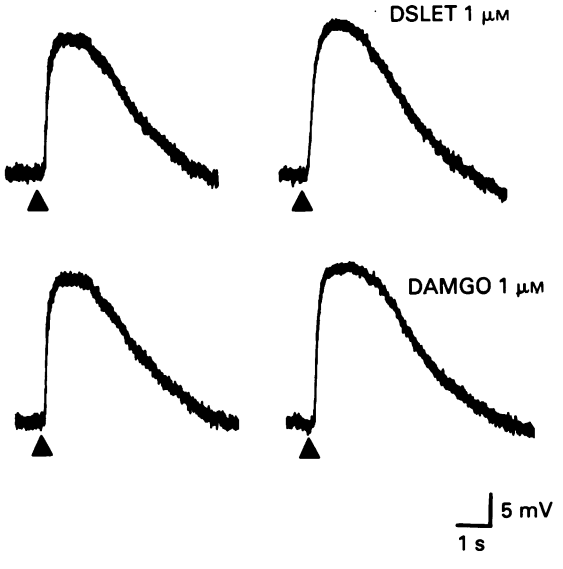

Figure 4 Lack of effect of [D-Ser^2,Leu^5,Thr^6]enkephalin (DSLET) and [D-Ala^2,NMePhe^4,Gly-ol^5]enkephalin (DAMGO) on the nicotinic depolarization evoked by pressure application of acetylcholine to a single mouse hypogastric ganglion neurone. Acetylcholine was applied by pressure application (pulses of 50 ms, 4 psi were applied at the ▲) at 4 min intervals from a micropipette positioned above the impaled neurone. DSLET (1 μM; upper right-hand trace) or DAMGO (1 μM; lower right-hand trace) was added to the Krebs solution bathing the neurone for 8 min before the responses illustrated were evoked. Throughout the experiment the Krebs solution contained atropine (1 μM).

When examined between 3 and 5 min after the start of application, DSLET (10–100 nM) increased the proportion of failures observed and reduced the mean number of quanta released per stimulus in a concentration-dependent manner. However, the mean amplitude of the quantal unit was virtually unchanged. Likewise, between 3 and 5 min after the start of application, DAMGO (10–300 nM) increased the number of failures and caused a reduction in the mean number of quanta released per stimulus in a concentration-dependent manner without reducing the mean amplitude of the quantal unit. These results confirm that the μ- and δ-opioid agonists act presynaptically to reduce the release of acetylcholine rather than postsynaptically to reduce the response to acetylcholine.

μ- and δ-receptors are present on the same preganglionic nerve terminals

The ovoid nature of mouse hypogastric ganglion neurones and the all-or-nothing nature of the e.p.s.ps recorded are consistent with the e.p.s.p. arising from stimulation of only a single preganglionic nerve fibre (Rogers *et al.*, 1990). In 34 neurones it was found that DSLET (100 nM) and DAMGO (300 nM) both depressed the e.p.s.p. in the same neurone. However, this was not due to DSLET and DAMGO activating the same opioid receptors since the response to DSLET was antagonized by ICI 174864 (300 nM), whereas the response to DAMGO was unaffected by this drug, but was antagonized by naloxone (100 nM) (Figures 3 and 6). Furthermore, the depression of the e.p.s.p. by a maximal concentration of DSLET or DAMGO was not increased when the agonists were applied simultaneously (data not shown). If the μ- or δ-receptors were present on separate fibres one would expect the effects of the agonists to be additive. Therefore, these results demonstrate that DSLET and DAMGO depressed the e.p.s.p. by activating different opioid receptor types (δ- and μ- respectively) present on the same preganglionic nerve terminal.

Figure 6b illustrates the relative potencies of DSLET, DPDPE and DAMGO at depressing the e.p.s.p. recorded in a single neurone. In this neurone the e.p.s.p. had been rendered subthreshold for action potential initiation by the addition of hexamethonium (1 mM) to the bathing medium. There was almost no difference in the potencies of the three agonists. In

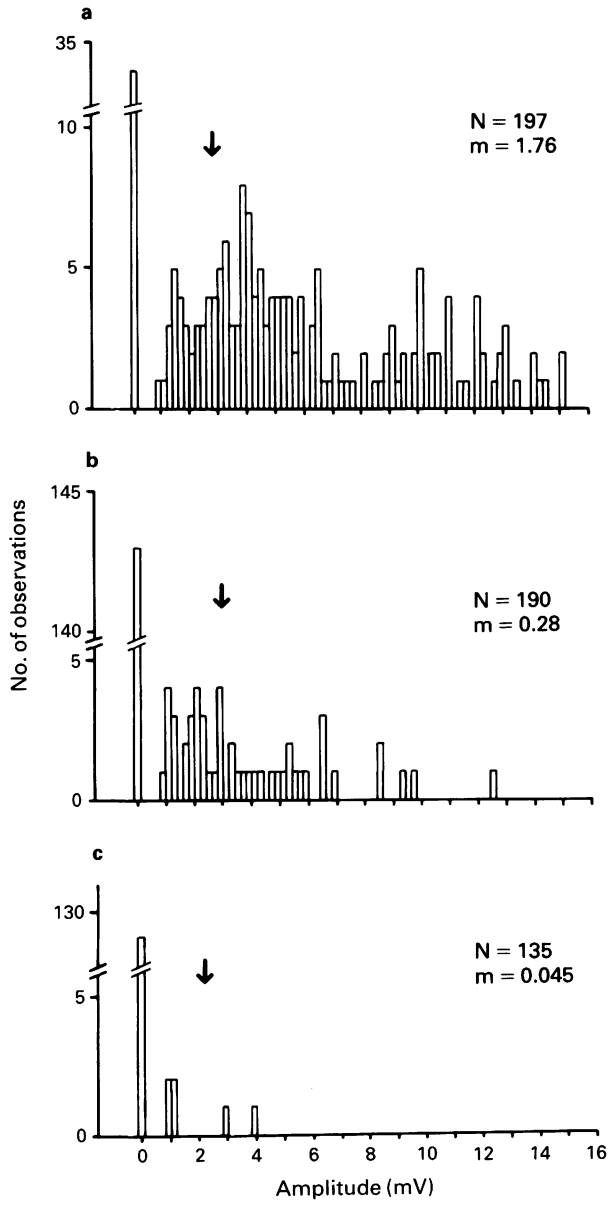

Figure 5 Quantal analysis of the inhibitory action of [D-Ser^2,Leu^5, Thr^6]enkephalin (DSLET) on synaptic transmission in a single mouse hypogastric ganglion neurone. When the ganglion was superfused with a Krebs solution containing 0.3 mM calcium and 3 mM magnesium preganglionic fibre stimulation (1 Hz) evoked e.p.s.ps which were subthreshold for action potential initiation. (a) Shows an amplitude-frequency distribution for evoked e.p.s.ps obtained after 10 min in the low calcium/high magnesium solution. Thereafter DSLET (10 nM) was applied; the amplitude-frequency distribution shown in (b) was obtained after 3 min exposure to this concentration of DSLET. The concentration of DSLET was then increased to 100 nM and the amplitude-frequency distribution shown in (c) was obtained after 3 min exposure to this concentration of DSLET. The abscissa scales show the amplitude of responses and the ordinate scales the frequency of occurrence. The arrow indicates the mean amplitude of the quantal unit calculated as described in the Methods. N is the number of stimuli and m the mean number of quanta released per stimulus. The neurone illustrated here is listed as neurone 3 in Table 1.

this experiment the IC_{50} values (concentrations required to depress the e.p.s.p. by 50%) were DAMGO 38 nM, DSLET 46 nM and DPDPE 59 nM.

Effect of potassium channel blocking agents on opioid inhibition of the e.p.s.p.

Although electrophysiological studies have revealed the ionic conductance changes resulting from activation of μ-, δ- and κ-receptors on the somata of various types of neurones, very

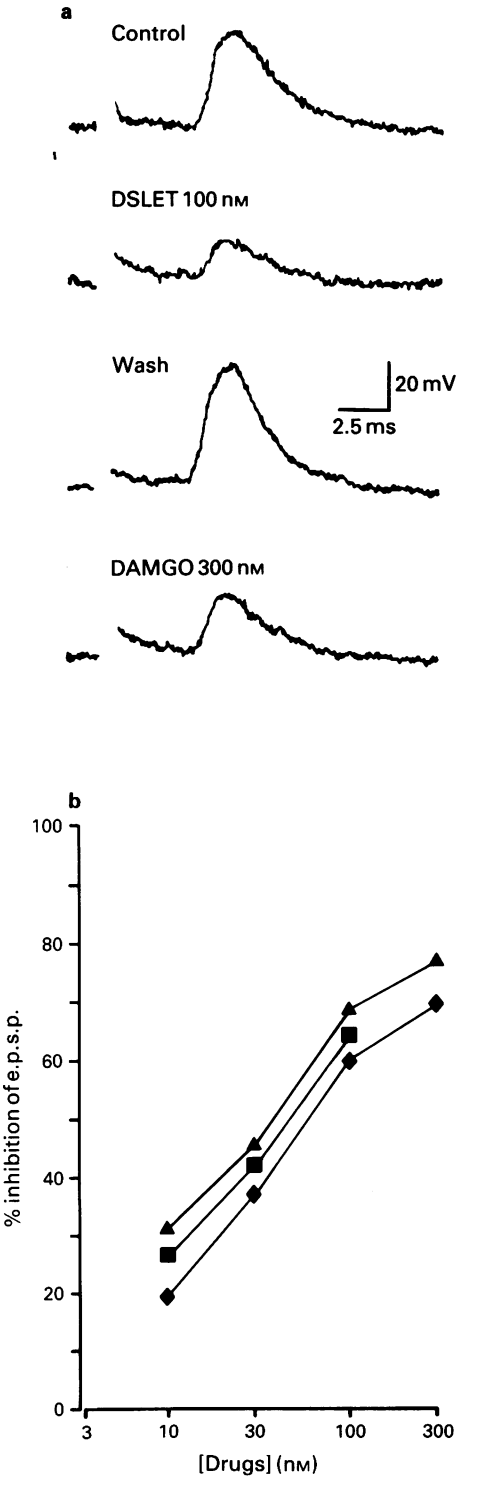

Figure 6 Concentration-dependent depression by [D-Ser^2,Leu^5, Thr^6]enkephalin (DSLET), [D-Pen^2,D-Pen^5]enkephalin (DPDPE) and [D-Ala^2,NMePhe^4,Gly-ol^5]enkephalin (DAMGO) of subthreshold synaptic potentials recorded in mouse hypogastric ganglion neurones. In the neurones illustrated in (a) and (b), a combination of membrane hyperpolarization by 5–10 mV from rest and addition of hexamethonium (1 mM) rendered the e.p.s.p. evoked by stimulation of the preganglionic fibre with single pulses (0.017 Hz) subthreshold. (a) E.p.s.ps evoked in a single neurone in the absence of opioid or after 5 min exposure to DSLET or DAMGO. In (b) a range of concentrations of DSLET (■), DPDPE (◆) and DAMGO (▲) were each applied to a single neurone for 5 min. The preparation was washed for 10 min between each application of drug. Results are expressed as the percentage depression of the e.p.s.p. against the logarithm of the agonist concentration. The depression produced in this neurone by DSLET (100 nM) or DPDPE (300 nM) but not by DAMGO (300 nM) was reversed by ICI 174864 (300 nM), whereas the depression produced by DAMGO (300 nM) was reversed by naloxone (100 nM).

Table 1 Quantal analysis of the action of opioids to inhibit synaptic transmission in the mouse hypogastric ganglion

A	*Neurone number*		*Control*	*DSLET 10 nM*	*DSLET 100 nM*
	1	$\bar{X}$	1.57 ± 0.21	0.20 ± 0.08	
		m	0.34	0.04	
		q.u.	4.61 ± 0.62	4.82 ± 1.86	
	2	$\bar{X}$	3.13 ± 0.36	0.86 ± 0.20	
		m	0.53	0.13	
		q.u.	5.91 ± 0.68	6.61 ± 1.54	
	3	$\bar{X}$	5.00 ± 0.30	0.96 ± 0.15	0.06 ± 0.08
		m	1.76	0.28	0.05
		q.u.	2.84 ± 0.16	3.10 ± 0.48	2.20 ± 1.25
	4	$\bar{X}$	8.27 ± 0.64	4.53 ± 0.39	2.96 ± 0.29
		m	2.72	1.17	0.74
		q.u.	3.94 ± 0.23	3.87 ± 0.34	4.00 ± 0.39
	5	$\bar{X}$	0.58 ± 0.10	0.20 ± 0.06	
		m	0.33	0.13	
		q.u.	1.76 ± 0.30	1.54 ± 0.46	
	6	$\bar{X}$	5.80 ± 0.70	0.78 ± 0.19	
		m	1.44	0.30	
		q.u.	3.99 ± 0.48	2.58 ± 0.60	

B	*Neurone number*		*Control*	*DAMGO 10 nM*	*DAMGO 30 nM*	*DAMGO 100 nM*	*DAMGO 300 nM*
	3	$\bar{X}$	1.60 ± 0.18	0.81 ± 0.14	0.38 ± 0.09		
		m	0.58	0.28	0.13		
		q.u.	2.76 ± 0.19	2.89 ± 0.48	2.92 ± 0.69		
	7	$\bar{X}$	2.77 ± 0.22		1.63 ± 0.20	0.45 ± 0.11	0.27 ± 0.08
		m	0.69		0.37	0.15	0.07
		q.u.	4.01 ± 0.32		4.41 ± 0.54	3.00 ± 0.73	3.70 ± 1.10
	8	$\bar{X}$	2.90 ± 0.35	0.51 ± 0.10			
		m	1.07	0.22			
		q.u.	2.71 ± 0.33	2.32 ± 0.45			

The results are from quantal analysis performed on 6 neurones to which [D-Ser^2,Leu^5,Thr^6]enkephalin (DSLET) was applied (A), and 3 neurones to which [D-Ala^2,NMePhe^4,Gly-ol^5]enkephalin (DAMGO) was applied (B). When the ganglion was superfused with a Krebs solution containing 0.3 mM calcium and 3 mM magnesium preganglionic fibre stimulation (1 Hz) elicited e.p.s.ps which were subthreshold for action potential initiation. Control data were obtained after 10 min in the low calcium/high magnesium solution. Thereafter, either DSLET or DAMGO, at the concentrations indicated, was applied for 3 min before data were collected. In some neurones increasing concentrations of each opioid were added cumulatively, each concentration being applied for 3 min before data collection. DSLET and DAMGO were applied to neurone 3 with a 15 min wash between the application of the different agonists. $\bar{X}$ is the mean response to a single stimulus over 100 to 200 stimuli: m is the mean number of quanta released per stimulus and was calculated as described in the Methods. The mean amplitude of the quantal unit (q.u.) was obtained by dividing $\bar{X}$ by m.

little is known of the ionic mechanism(s) by which opioids inhibit transmitter release. Several potassium channel blockers have been shown to reduce or abolish the somatic, outward potassium current resulting from μ-receptor activation on the somata of locus coeruleus neurones (Cherubini *et al.*, 1985; Cherubini & North, 1985; North & Williams, 1985). In the present study, the effects of two potassium channel blocking agents, barium and quinine, on the opioid depression of the e.p.s.p. were examined to gain insight into whether a potassium conductance was involved in the μ- or δ-opioid inhibition of the e.p.s.p.

Application of barium (1 mM), but not quinine (100 μM), depolarized neurones by 10–20 mV and increased input resistance. Therefore in experiments with barium the membrane potential was manually clamped back to the pre-barium level by injection of hyperpolarizing, direct current. In the presence of barium (1 mM; $n = 6$) the e.p.s.p. increased in amplitude from 14.3 ± 3.0 mV to 22.4 ± 4.5 mV, whilst in the presence of quinine (100 μM, $n = 3$) it decreased from 15.8 ± 3.8 mV to 7.8 ± 1.7 mV. The depression of the e.p.s.p. by DSLET and DAMGO was reduced but not abolished in the presence of either potassium channel blocking agent (Table 2). Barium appeared to reduce the effect of DSLET less than that of DAMGO, but the difference was not statistically significant.

Discussion

Neurones lying within the mouse hypogastric ganglion have a simple morphology, being ovoid in shape and giving rise to a single axonal process (Rogers *et al.*, 1990). They appear to lack completely dendritic processes. In several species, including the mouse, ganglion neurones which lack dendritic processes receive only a single synaptic input (Tabatabai *et al.*, 1986; Snider, 1987; Purves *et al.*, 1988; Rogers *et al.*, 1990). Activation of the presynaptic input gives rise to an all-or-nothing, suprathreshold e.p.s.p. (Blackmann *et al.*, 1969; Holman *et al.*, 1971; Tabatabai *et al.*, 1986; Snider, 1987; Purves *et al.*, 1988). Physiologically these ganglia appear to function as simple relays. The simplicity of the anatomical arrangement has permitted us to investigate the mechanisms by which opioids modulate synaptic transmission. In the mouse hypogastric ganglion activation of either μ- or δ-opioid receptors depressed the nicotinic e.p.s.p., apparently by altering stimulus-secretion coupling in the presynaptic nerve terminal. The selective activation of δ- and μ-receptors by DSLET and DAMGO respectively was confirmed by the selective antagonism of responses to DSLET but not DAMGO by the δ-receptor antagonist ICI 174864 (Cotton *et al.*, 1984).

Table 2 Effect of barium and quinine on the depression by [D-Ser^2,Leu^5,Thr^6]enkephalin (DSLET) and [D-Ala^2,NMePhe^4,Gly-ol^5]enkephalin (DAMGO) of the e.p.s.ps in mouse hypogastric ganglion neurones

	% *inhibition*				% *inhibition*			
	Control	*Barium*	*Ratio*	(n)	*Control*	*Quinine*	*Ratio*	(n)
DSLET (100 nM)	68.8 ± 5.0	52.4 ± 6.3*	0.74 ± 0.05	(5)	72.7 ± 9.1	52.7 ± 5.8	0.76 ± 0.14	(3)
DAMGO (300 nM)	60.6 ± 7.1	31.6 ± 5.0*	0.55 ± 0.11	(5)	63.0 ± 10.4	37.7 ± 13.0	0.60 ± 0.15	(3)

In the neurones used in this study stimulation of the preganglionic fibre with single pulses (0.017 Hz) in the presence of hexamethonium (1 mM) evoked subthreshold, all-or-nothing e.p.s.ps. Barium (1 mM) or quinine (100 μM) was added to the superfusate for 15 min before reapplying DSLET or DAMGO. Data are given as mean ± s.e.mean. The asterisk indicates statistically significant difference ($P < 0.05$) from control.

Our experiments suggest that μ- or δ-receptor activation depressed the e.p.s.p. by an action on the preganglionic nerve terminal. Neither μ- nor δ-agonists had any effect on the resting membrane potential or input resistance of the postganglionic neurone, suggesting that the depression of the e.p.s.p. was not secondary to the activation of a postganglionic conductance. Also, the response of the postganglionic neurone to exogenous acetylcholine was not depressed by concentrations of DSLET and DAMGO which were supramaximal for the inhibition of the e.p.s.p.

Furthermore, the results of the analysis of the effects of opioids on the quantal content of the e.p.s.ps support a presynaptic locus of action. The distribution of e.p.s.p. amplitudes observed was consistent with a Poisson distribution, as the range of amplitudes did not exceed that predicted by the value of m. Also, the number of e.p.s.ps measuring between zero mV and the amplitude of the quantal unit was approximately half the value of the first term of the calculated distribution (Del Castillo & Katz, 1954). Opioids were shown to depress the mean amplitude of the e.p.s.p. in a concentration-dependent manner. However, this was due to a reduction in the number of quanta constituting an e.p.s.p. and not due to a reduction in the amplitude of the response evoked by a single quanta. This is entirely consistent with an action at a presynaptic locus, as opposed to a reduction in the response of the postganglionic neurone to acetylcholine.

Opioids have long been known to depress neuro-effector transmission in the mouse vas deferens (Henderson *et al.*, 1972). Thus, both the preganglionic and postganglionic terminals of the sympathetic outflow to the vas deferens possess opioid receptors. However, the distribution of opioid receptor subtypes is different in that the preganglionic terminals bear only μ- and δ-receptors, whereas postganglionic terminals in the mouse vas deferens bear μ-, δ- and κ-receptors (Ramme & Illes, 1986). The potency of μ-agonists to depress transmitter release from preganglionic nerve terminals and postganglionic terminals is similar whereas, δ-receptor agonists are 20–40 fold more potent at the postganglionic terminals suggesting that there is a larger reserve of δ-receptors on postganglionic terminals (Rogers & Henderson, unpublished observations).

In autonomic ganglia, a number of workers have previously shown that opioids and opioid peptides depress ganglionic transmission by inhibiting transmitter release (Konishi *et al.*, 1979; 1986; Dun & Karczmar, 1979; Fileccia & Julé, 1983; Katayama & Nishi, 1984; Kennedy & Krier, 1987; Balayadi *et al.*, 1988; Hirai & Katayama, 1988). However, in the mouse hypogastric ganglion we have shown, for the first time, that both μ- and δ-receptor activation inhibits transmitter release. In addition, we have previously found that e.p.s.ps evoked by focal stimulation arise from the stimulation of a single strong fibre input (Rogers *et al.*, 1990). Thus the present finding that activation of either μ- or δ-receptors depresses the same e.p.s.ps in all neurones tested is strong evidence for the co-localisation of both μ- and δ-receptors on the same preganglionic terminal. The lack of an additive effect of maximally effective concentrations of μ- and δ-agonists when applied simultaneously, supports this conclusion.

It would appear that in the mouse hypogastric ganglion there is a large safety factor for synaptic transmission, typical of other simple ganglia, which apparently prevents the opioids from blocking transmission. Activation of opioid receptors did not, in general, completely block synaptic transmission. Large e.p.s.p./action potential complexes remained suprathreshold for action potential initiation in the presence of opioids, despite considerable reduction in e.p.s.p. amplitude. E.p.s.ps that were just suprathreshold for action potential initiation were rendered subthreshold by opioids but this type of synaptic response was only rarely observed. This raises the question as to whether the opioid receptors present on these nerve terminals have any physiological significance. Enkephalin-like immunoreactivity has been detected in baskets of nerve fibres surrounding these ganglion neurones (M. Costa, J.B. Furness & G. Henderson, unpublished observations); thus an endogenous ligand for μ- and δ-receptors is present in the vicinity of the receptors.

We have attempted to define whether opioid activation of a potassium conductance in the preganglionic terminals underlies the depression of the e.p.s.p. Electrophysiological experiments monitoring somatic membrane responses have shown that both μ- and δ-receptors are functionally coupled to the activation of a potassium conductance (North & Tonini, 1977; Pepper & Henderson, 1980; Werz & Macdonald, 1983; North & Williams, 1985; Mihara & North, 1986). Excitability testing on nerve processes in the cortex (Nakamura *et al.*, 1982) and spinal cord (Carstens *et al.*, 1979) has detected an increase in membrane conductance following local application of opioids. The somatic potassium conductance activated by opioids can be blocked by extracellularly applied barium or quinine (North & Williams, 1983; 1985; Cherubini *et al.*, 1985; Cherubini & North, 1985). In the presence of barium the inhibition of the e.p.s.p. by DAMGO in the mouse hypogastric ganglion was reduced by 45%, whilst that produced by DSLET was reduced by only 25%. Interestingly, in the presence of quinine there was only a slight, non-significant reduction in the μ- and δ-receptor mediated inhibition of the e.p.s.p. Thus, it seems unlikely that an increase in potassium conductance is the sole mechanism by which μ- and δ receptor activation results in inhibition of transmitter release. It has also been demonstrated that both μ- and δ-receptor activation directly reduces neuronal, voltage-dependent calcium conductances (Tsunoo *et al.*, 1986; Hescheler *et al.*, 1987; North *et al.*, 1988; Bean, 1989; McFadzean & Docherty, 1989; Seward *et al.*, 1989). It is of note that in the low calcium solution used in our quantal release studies, the inhibition of the e.p.s.p. was greater at lower concentrations of DSLET and DAMGO than that observed in normal Krebs. This would be consistent with opioids having a direct inhibitory action on calcium influx in the preganglionic nerve terminals. However, we cannot exclude other mechanisms for inhibition of transmitter release which do not involve potassium or calcium conductances, as has recently been described for the inhibitory neuropeptide Phe-Met-Arg-Phe-NH_2 (Man-Son-Hing *et al.*, 1989).

In conclusion, in the mouse hypogastric ganglion, both μ- and δ-receptor activation results in a reduction of transmitter release from the same preganglionic nerve terminals. The mechanism by which opioids inhibit transmitter release does not appear to be entirely by an increase in the potassium conductance of the preganglionic terminal.

Supported by a grant from the MRC: H.R. held an MRC Research Studentship.

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(Received October 3, 1989
Revised May 25, 1990
Accepted June 18, 1990)

Br. J. Pharmacol. (1990), **101**, 513–520

Characterization of 5-HT_3 and 'atypical' 5-HT receptors mediating guinea-pig ileal contractions *in vitro*

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1 Neuronal 5-hydroxytryptamine (5-HT) receptors mediating contraction of guinea-pig ileal segments have been characterized *in vitro* by the use of methysergide to block 5-HT_1-like and 5-HT_2 receptors. Concentration-response curves to 5-HT were biphasic (first phase, defined as those responses occurring between 1 nM and 0.32 μM 5-HT, $-\log EC_{50} = 7.15 \pm 0.08$; second phase, defined as these responses occurring between 0.32 μM and 32 μM 5-HT, $-\log EC_{50} = 5.32 \pm 0.03$) but monophasic to 5-methoxytryptamine ($-\log EC_{50} = 7.0 \pm 0.08$) and 2 methyl 5-HT ($-\log EC_{50} = 5.2 \pm 0.13$). The maximal response of the first phase to 5-HT and the maximal response to 5-methoxytryptamine were $30 \pm 4\%$ and $35 \pm 5\%$ respectively of the maximum response to the second phase of the 5-HT concentration-effect curve (set at 100%). In contrast, the maximal response to 2-methyl-5-HT equalled that obtained with 5-HT (second phase).

2 The responses comprising the second phase of the concentration-effect curve to 5-HT were antagonized by 1 μM ICS 205-930, ondansetron, granisetron, quipazine, N-methyl-quipazine and (**R,S**)-zacopride and the following pK_B values, with 5-HT as the agonist, were obtained at the 5-HT_3 receptor: ICS 205-930 7.61 ± 0.05, ondansetron 6.90 ± 0.04, granisetron 7.90 ± 0.04, (**S**)-zacopride 8.11 ± 0.06, (**R,S**)-zacopride 7.64 ± 0.11, and (**R**)-zacopride 7.27 ± 0.06.

3 Under conditions of 5-HT_1-like, 5-HT_2 and 5-HT_3 receptor blockade, the following rank order of agonism was observed: 5-HT > 5-methoxytryptamine = renzapride > (**S**)-zacopride > (**R,S**)-zacopride > 5-carboxamidotryptamine > BRL 24682 > (**R**)-zacopride > metoclopramide > 2-methyl-5-HT $\gg$ sulpiride. 8-Dihydroxydiphenylaminotetralin (8-OHDPAT), GR 43175, N,N-dipropyl-5-carboxamidotryptamine, ondansetron, ICS 205-930, granisetron, quipazine and N-methyl-quipazine were inactive as agonists and antagonists. Relative to 5-HT, (**R,S**)-zacopride acted as a partial agonist (intrinsic activity, $\alpha = 0.80$; $-\log EC_{50} = 6.3 \pm 0.12$; $-\log K_A = 6.1 \pm 0.03$) as did (**R**)-zacopride ($\alpha = 0.4$, $-\log EC_{50}$ 5.7 ± 0.08, $-\log K_A = 5.5 \pm 0.11$). (**S**)-zacopride acted as a full agonist ($-\log EC_{50} = 6.9 \pm 0.03$). ICS 205-930 (3 μM) antagonized competitively responses to 5-HT, 5 methoxytryptamine, (**R,S**)- and (**S**)-zacopride and 5-carboxamidotryptamine yielding $-\log K_B$ estimates ranging from 6.1–6.5.

4 It is concluded that two different 5-HT receptors mediate excitatory neuronal responses in the guinea-pig ileum. 5-HT_3 receptors mediate the second phase of the biphasic concentration-response curve, whereas a receptor with properties distinct from the 5-HT_1-like, 5-HT_2 and 5-HT_3 subtypes mediates the initial phase of the concentration-response curve. This receptor, which exhibits a close similarity to the 5-HT_4 subtype is: (1) stimulated by 5-methoxytryptamine but not 2-methyl-5-HT; (2) stimulated selectively by certain substituted benzamides; (3) recognizes the optical isomers of zacopride and (4) is blocked by relatively high concentrations ICS 205-930 ($pK_B = 6.0$–6.5) but not ondansetron, granisetron, quipazine or N-methyl-quipazine.

Introduction

Gaddum & Picarelli (1957) showed that directly and indirectly mediated responses to 5-hydroxytryptamine (5-HT) receptors evoked contractions of guinea-pig ileum. They termed these receptors D and M, respectively. The indirect responses are sensitive to atropine and involve the release of acetylcholine (Clarke *et al.*, 1989). 5-HT receptors have been most recently classified as 5-HT_1-like, 5-HT_2 and 5-HT_3 (Bradley *et al.*, 1986). The 5-HT_3 subtype, which corresponds to the M receptor (see Clarke *et al.*, 1989 for discussion), is selectively antagonized in the guinea-pig ileum by ICS 205-930 ($pA_2 = 8.0$, Richardson *et al.*, 1985), renzapride (BRL 24924; $pA_2 = 7.3$, Sanger, 1987), granisetron (BRL 43694; $pA_2 = 7.8$, Sanger & Nelson, 1989), zacopride ($pA_2 = 8.5$, Smith *et al.*, 1988) and ondansetron (GR 38032F; $pA_2 = 7.0$, Butler *et al.*, 1988).

Studies on guinea-pig isolated longitudinal ileal muscle strips (Kilbinger & Pfeuffer-Freidrich, 1985; Buchheit *et al.*, 1985) have revealed a biphasic concentration-response curve to 5-HT which is sensitive to tetrodotoxin (TTX) and it has been suggested that two different 5-HT receptors may modulate excitatory neuronal activity. The second component of the biphasic concentration-response curve to 5-HT is due to stimulation of 5-HT_3 receptors but the receptor mediating the initial, high potency, phase of the curve has not been extensively characterized. The initial phase, however, has been shown to be insensitive to blockade by ICS 205-930 (up to 1 μM), granisetron and ondansetron (Buchheit *et al.*, 1985; Sanger & Nelson, 1989; Butler *et al.*, 1985) confirming the lack of involvement of 5-HT_3 receptors. Similar experiments undertaken in the presence of methysergide (Costa & Furness, 1976; Buchheit *et al.*, 1985) indicate that the responses at the initial, high potency phase appear to be unrelated to stimulation of 5-HT_1-like or 5-HT_2 receptors.

Taken together the data on the unstimulated ileum (Buchheit *et al.*, 1985) suggest that a 5-HT receptor which acts to modulate excitatory neuronal activity, is present in the ileum but is distinct from 5-HT_1-like, 5-HT_2 or 5-HT_3 subtypes as currently defined (Bradley *et al.*, 1986; Myelcharane, 1989). The aim of the present study was to examine this 'orphan' 5-HT receptor on the isolated, quiescent ileum of the guinea-pig.

Methods

Portions of ileum (dissected 1.5 cm distal to the ileocaecal junction) were removed from male, Dunkin-Hartley guinea-pigs (350–400 g) which had been killed by CO_2 asphyxiation.

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Whole segments of ileum were gently flushed intraluminally with warm Tyrode solution (pH 7.4, 37°C) and prepared by the method described by Edinburgh Staff (1968). The composition of the Tyrode solution was (mM): NaCl 136.9, KCl 2.7, $MgCl_2 . 6H_2O$ 1.2, NaH_2PO_4 1.2, glucose 10.0, $NaHCO_3$ 25.0 and $CaCl_2 \cdot 6H_2O$ 2.5. It was continually gassed with 5% O_2/95% CO_2. Methysergide was added at a concentration of 1 μM as described by Smith et al. (1988) in order to exclude any potential effects of 5-HT_1-like or 5-HT_2 receptor stimulation. Portions (1.5 cm) of intact ileum were placed in Tyrode solution under 1.0 g tension and allowed to equilibrate for 60 min. During this period the bathing solution was replaced every 15 min. Preliminary experiments (R.M. Eglen, unpublished observations) showed that the tissues responded with an initial contracture when exposed to methysergide. The contracture did not persist and the baseline tension was re-attained within 5 min. The inclusion of 1 μM methysergide did not significantly affect the potency of 5-HT, a finding which is in agreement with that of Buchheit et al. (1985). However, all experiments were conducted in the presence of methysergide because Costa & Furness (1972) have demonstrated the converse.

Ileal responses were measured by determining changes in isometric tension (mg), with a Hugo Sachs K30 force transducer and a Graphtec-Watanabe Linearecorder WR3101.

In all experiments, tissues were exposed to 50 mM KCl for 3 min to obtain an estimate of the maximal size of contraction of the preparation (4.81 $\pm$ 0.06 g, mean $\pm$ s.e.mean, $n = 103$). The tissues were washed and allowed 15 min to re-attain baseline tension. Concentration-response curves were then constructed, in a non-cumulative fashion, to 5-HT (1 nM–32 μM) with an agonist exposure period of 30 s on a 5 min dose-cycle. After the final agonist exposure, the tissues were washed and left for 60 min. During this 60 min recovery period, the bathing solution was replaced every 15 min after which another agonist concentration-response curve was constructed.

In studies with antagonists, the ileum was equilibrated with a particular antagonist during the 60 min recovery period, and the antagonist was retained in the bath during construction of the subsequent concentration-response curve. Only one antagonist was exposed to each ileal segment, and parallel control studies were always undertaken to correct for changes in sensitivity of the tissue to 5-HT.

To obtain an accurate estimation of the dissociation constants of antagonists at the 5-HT_3 receptors, the technique of Fozard (1985) was employed. The preparations were placed in 10 μM 5-methoxytryptamine for the duration of the experiment, which selectively removed the initial component of the 5-HT concentration-response curve, the remaining 5-HT responses being mediated through 5-HT_3 receptors. Preliminary experiments showed that inclusion of 5-methoxytryptamine did not affect responses of the preparation to carbachol, histamine, substance P, 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP) or KCl (data not shown).

In separate studies, the effect of catecholamine depletion on 5-HT responses was assessed in animals that had been pretreated with reserpine (5 mg kg^{-1}, i.p.) 18 h before they were killed, a procedure that has been previously shown to remove the responses to tyramine (Eglen & Whiting, 1989).

Analysis of results

The methods used to determine agonist potencies ($-\log EC_{50}$) were similar to those described by Buchheit et al. (1985). The potencies from monophasic concentration-response curves were characterized by non-linear iterative fitting procedures (Parker & Waud, 1971) calculated with RS1 software (BBN Software Products Corp., Cambridge, MA, U.S.A.). Biphasic concentration-response curves were characterized as follows: the maxima for each phase of the curve were determined graphically, and the $-\log EC_{50}$ values were calculated with respect to the two phases, defined as follows. The $-\log EC_{50}$ values determined at the initial phase (defined as those responses occurring between 1 nM and 0.32 μM 5-HT) were denoted as $-\log EC_1$ and the maximal response denoted as max_1. The $-\log EC_{50}$ value determined at the second phase (defined as those responses occurring between 0.32 μM 5-HT and 32 μM 5-HT) was denoted as $-\log EC_2$ and the maximal response as max_2.

In some experiments, the dissociation constants for partial agonists were calculated according to the method of Barlow et al. (1967). Equiactive concentrations of 5-HT and the partial agonist were plotted in a double reciprocal fashion, with the former values plotted on the ordinate scale and latter values plotted on the abscissae. The intercept with the ordinate scale and slope of the resulting straight line were determined by linear regression. The dissociation constant (expressed as the $-\log K_A$) was calculated from the relationship:

$$-\log K_A = -\log\left(\frac{\text{slope}}{\text{intercept}}\right).$$

Antagonists

The antagonist dissociation constants were determined in two ways. Firstly, where the concentration-response curves were dextrally shifted in parallel, with no reduction in maxium response, the pA_2 values were calculated by the method of Arunlakshana & Schild (1959). The slope and the intercept of the resulting straight line with the abscissae were determined by linear regression. Secondly, in experiments where a single concentration of antagonist was used, the method employed was that described by Furchgott (1972). The $-\log K_B$ values were calculated according to the following relationship:

$$-\log K_B = -\log\left(\frac{[\text{antagonist}]}{\text{dose-ratio} - 1}\right).$$

Statistics

Statistical differences were assessed by Student's t test, with $P < 0.05$ being considered as significant; all values quoted are the mean $\pm$ s.e.mean from 6–10 experiments.

Drugs used

The following compounds were synthesized in the Institute of Chemistry, Syntex Research: ($\pm$)-pindolol, atenolol, BRL 24682 (4-amino-N(N-methyl-8-azabicyclo[3.2.1]-5-chloro-2-methoxy benzamide), renzapride (BRL 24924 ($\pm$)-endo-4-amino-5-chloro-2-methoxy-N-(1-azabicyclo[3.3.1]non-4-yl) benzamide), granisetron (BRL 43694; N-endo-9-methyl-9-azabicyclo [3.3.1] non-3-yl)-1-methyl-indazole-3-carbexamide), 5-carboxamido-tryptamine, N,N-dipropyl-5-carboxamido-tryptamine, ondansetron (GR 38032F, 1,2,3,9-tetrahydro-3-[(methyl-imidazol-1-yl)methyl]-9-methyl-4H-carbazol-4-one), GR 43175 (3-[2-dimethyl-amino]ethyl-N-methyl-1H-indole-5-methane- sulphonamide), N-methylquipazine, rauwolscine, (**R,S**)-zacopride, (**S**)-zacopride and (**R**)-zacopride. Reserpine (Serpasil) was purchased from Ciba-Geigy. Methysergide was generously donated by Sandoz, Research Institute, N.J., U.S.A. ICS 205-930 ((3α-tropanyl)-1H-indole-3-carboxylic acid ester), 8-OHDPAT (8-dihydroxydiphenylaminotetralin), sulpiride, metoclopramide and quipazine were purchased from Research Biochemicals Ltd. 5-Methoxytryptamine was obtained from Aldrich, and the remaining compounds were obtained from Sigma Chemical Co. Ltd.

Results

Studies conducted in the presence of 5-HT_1-like and 5-HT_2 receptor blockade

The contractile responses to 5-HT were rapid in onset and accompanied by a fade phenomenon, particularly at high

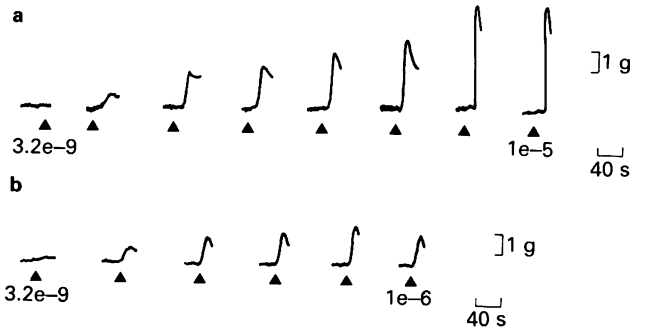

Figure 1 Recordings of representative responses to 5-hydroxytryptamine (5-HT) in the segments of whole ileum from guinea-pig. Shown are responses to 5-HT in the absence (a) or presence (b) of 0.1 μM ICS 205-930. Methysergide (1 μM) was present throughout each experiment. Concentrations of 5-HT were added, at points indicated by the closed triangles, in 3 fold molar incremental increases in concentration. The exposure period was 30 s.

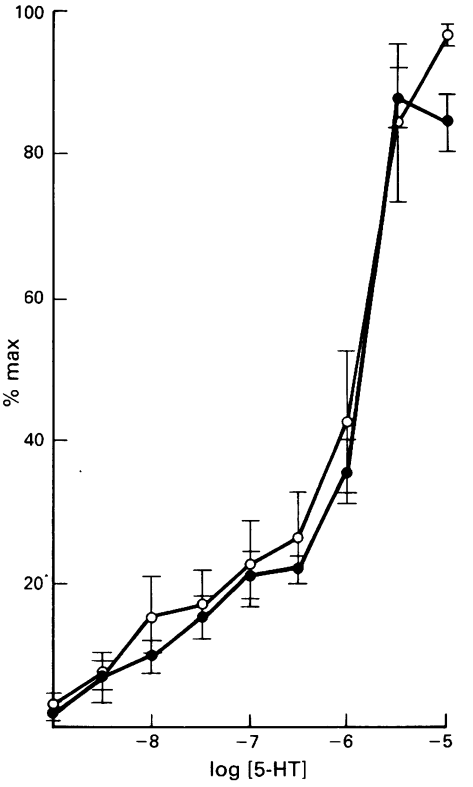

Figure 2 Concentration-response curves to 5-hydroxytryptamine (5-HT) in segments of whole ileum: (○) initial concentration-response curve; (●) second curve to 5-HT, after an interval of 60 min. Values are mean, from 6–10 preparations with the vertical bars indicating s.e.mean.

(>1 μM) concentrations (Figure 1a). The concentration-response curve to 5-HT was biphasic, consisting of an initial phase occurring between 5-HT concentrations of 3.2 nM and 0.32 μM and a second phase which occurred between 1 μM and 32 μM 5-HT. The $-\log EC_1$ value for 5-HT was 7.15 ± 0.08, and the $-\log EC_2$ value was 5.32 ± 0.03. The maximal responses at each phase were: $max_1 = 33.4 \pm 0.02\%$; $max_2 = 100\%$. No evidence of desensitization was evident with a 60 min recovery period between consecutive 5-HT concentration-response curves. In time control experiments, the first concentration-response curves exhibited a mean $-\log EC_1$ of 7.20 ± 0.11 and a mean $-\log EC_2$ of 5.35 ± 0.08, whereas the second concentration-response curve exhibited a $-\log EC_1$ of 7.23 ± 0.06 and a $-\log EC_2$ of 5.29 ± 0.11 (Figure 2). The maximal responses of either phase were also similar in the two curves.

The data obtained with 5-HT in the absence and presence of various antagonists are summarised in Tables 1 and 2. The effect of tetrodotoxin, which inhibits neuronal depolarization by blocking sodium channels (Gershon, 1967) and morphine, which inhibits acetylcholine release (Ganatra *et al.*, 1979), were studied. In the presence of tetrodotoxin (1 μM), the responses to 5-HT in the initial phase of the curve were abolished (Table 1). However, the second phase of the concentration-response curve was shifted slightly to the right but the maximum response was greatly reduced (Table 2). Similar results were

Table 1 Effect of antagonists on 5-hydroxytryptamine (5-HT) responses occurring in the initial phase of the concentration-response curve

	Control		*+ Antagonist*	
Antagonist	$-\log EC_1$	$\%\ max_1$	$-\log EC_1$	$\%\ max_1$
TTX (1 μM)	7.52 ± 0.03	36 ± 3	abolished	
Morphine (10 μM)	7.38 ± 0.11	22 ± 4	abolished	
Atropine (0.1 μM)	7.51 ± 0.06	43 ± 3	7.35 ± 0.10	12 ± 2^b
Atropine (1 μM)	7.62 ± 0.08	25 ± 8	abolished	
Physostigmine (0.1 μM)	7.83 ± 0.11	28 ± 5	7.52 ± 0.18^a	90 ± 18^b
Propranolol (1 μM)	7.48 ± 0.09	38 ± 4	7.35 ± 0.13	35 ± 2
Atenolol (1 μM)	7.53 ± 0.13	35 ± 5	7.52 ± 0.09	36 ± 7
Pindolol (1 μM)	7.61 ± 0.04	36 ± 2	7.59 ± 0.11	38 ± 4
ICI 118551 (1 μM)	7.55 ± 0.07	38 ± 7	7.62 ± 0.14	40 ± 5
Phenoxybenzamine (0.1 μM)[c]	7.54 ± 0.06	38 ± 5	abolished	
Phentolamine (1 μM)	7.52 ± 0.09	38 ± 9	7.43 ± 0.11	34 ± 8
Prazosin (1 μM)	7.55 ± 0.04	35 ± 4	7.48 ± 0.03	34 ± 5
Rauwolscine (1 μM)	7.35 ± 0.08	34 ± 5	6.85 ± 0.04^a	25 ± 3^b
Ondansetron (1 μM)	7.49 ± 0.07	36 ± 4	7.54 ± 0.13	38 ± 5
Granisetron (1 μM)	7.58 ± 0.09	34 ± 8	7.62 ± 0.11	37 ± 6
ICS 205-930 (1 μM)	7.53 ± 0.11	32 ± 6	7.44 ± 0.04	28 ± 4
Quipazine (1 μM)	7.46 ± 0.08	37 ± 9	7.38 ± 0.12	35 ± 6
N-methylquipazine (1 μM)	7.69 ± 0.04	38 ± 7	7.59 ± 0.11	36 ± 9
(R,S)-zacopride (1 μM)	7.78 ± 0.11	35 ± 4	7.32 ± 0.14	8 ± 2^b

Values are mean ± s.e.mean, $n = 6$–10.
[a] Significantly different ($P < 0.05$) from control $-\log EC_1$ value.
[b] Significantly different ($P < 0.05$) from control $\%\ max_1$ value.
[c] Phenoxybenzamine was allowed 60 min to equilibrate and the second curve to 5-HT was constructed in the presence of phenoxybenzamine.

Table 2 Effect of antagonists on 5-hydroxytryptamine (5-HT) responses occurring in the second phase of the concentration-response curve

	Control		*+ Antagonist*	
Antagonist	$-\log EC_2$	% max_2	$-\log EC_2$	% max_2
TTX (1 μM)	5.81 ± 0.06	100	5.61 ± 0.11[a]	12 ± 2[b]
Morphine (10 μM)	5.73 ± 0.08	100	5.24 ± 0.08[a]	38 ± 4[b]
Atropine (0.1 μM)	5.68 ± 0.11	100	5.18 ± 0.12[a]	34 ± 8[b]
Atropine (1 μM)	5.75 ± 0.03	100	5.32 ± 0.11[a]	18 ± 4[b]
Physostigmine (0.1 μM)	5.52 ± 0.04	100	5.43 ± 0.17	141 ± 19[b]
Propranolol (1 μM)	5.69 ± 0.08	100	5.63 ± 0.05	100
Atenolol (1 μM)	5.71 ± 0.11	100	5.73 ± 0.08	100
Pindolol (1 μM)	5.75 ± 0.04	100	5.69 ± 0.14	100
ICI 118551 (1 μM)	5.77 ± 0.07	100	5.81 ± 0.11	100
Phenoxybenzamine (0.1 μM)[c]	5.72 ± 0.08	100	5.75 ± 0.09	30 ± 4[b]
Phentolamine (1 μM)	5.73 ± 0.04	100	5.88 ± 0.06	100
Prazosin (1 μM)	5.68 ± 0.11	100	5.73 ± 0.04	100
Rauwolscine (1 μM)	5.63 ± 0.04	100	5.59 ± 0.08	100
Ondansetron (1 μM)	5.64 ± 0.14	100	abolished[a]	—
Granisetron (1 μM)	5.66 ± 0.18	100	abolished[a]	—
ICS 205-930 (0.1 μM)	5.73 ± 0.08	100	abolished[a]	
Quipazine (1 μM)	5.78 ± 0.12	100	abolished[a]	
N-methyl-quipazine (1 μM)	5.81 ± 0.18	100	abolished[a]	—
(R,S)-zacopride (1 μM)	5.68 ± 0.07	100	abolished[a]	—

Values are mean ± s.e.mean, $n = 6$–10.
[a] Significantly different ($P < 0.05$) from control $-\log EC_2$ value.
[b] Significantly different ($P < 0.05$) from control % max_2 value.
[c] Phenoxybenzamine was allowed 60 min to equilibrate and the second curve to 5-HT was constructed in the presence of phenoxybenzamine.

seen with morphine (10 μM) in that the initial phase of the concentration-response curve to 5-HT was abolished (Table 1), whereas the second phase was shifted to the right and the maximum was reduced (Table 2).

Pre-exposure of the tissues to 0.1 μM phenoxybenzamine for a 15 min period (when followed by a 45 min washout period) was without significant effect on either component of the concentration-response curve to 5-HT (data not shown). However, when phenoxybenzamine (0.1 μM) was equilibrated with the tissue for 60 min and a second concentration-response curve was constructed in its presence, the initial portion of the curve was abolished and the second phase reduced by 70%. In order to assess the effect of phenoxybenzamine on direct muscarinic receptor stimulation, identical experiments were performed with the muscarinic agonist carbachol instead of 5-HT. A parallel dextral shift in the concentration-response curve to carbachol was obtained (control $-\log EC_{50} = 6.52 \pm 0.04$; plus phenoxybenzamine $-\log EC_{50} = 5.53 \pm 0.08$).

Since phenoxybenzamine also possesses α-adrenoceptor antagonist activity, further experiments were undertaken in which different α-adrenoceptor antagonists were used: phentolamine (a non-selective adrenoceptor antagonist), prazosin (an α_1-adrenoceptor selective antagonist) and rauwolscine (an α_2-adrenoceptor selective agonist). There was no significant effect on either phase of the concentration-response curve to 5-HT established in the presence of either 1 μM phentolamine or prazosin (Table 1). In the presence of 1 μM rauwolscine, however, the initial phase of the 5-HT curve was slightly but significantly shifted to the right. The maximal response was also reduced whereas the second phase was unaffected (Tables 1 and 2). The effects of the β-adrenoceptor antagonists, propranolol and pindolol (non-selective β-adrenoceptor antagonists), atenolol (β_1-adrenoceptor selective) and ICI 118551 (β_2-adrenoceptor selective) were also studied on the 5-HT responses. At 1 μM there was no significant effect of these compounds on either phase of the 5-HT concentration-response curve (Tables 1 and 2).

Atropine (0.1 μM) markedly affected the concentration-response curve to 5-HT, in that both phases were shifted dextrally and the maximal responsed were reduced (Figure 3). Atropine at 1 μM completely abolished the initial component and markedly reduced the second component. Conversely, physostigmine (0.1 μM) potentiated the maxima of both phases of the concentration-response curve to 5-HT (Figure 4). The potency of 5-HT on the initial phase was slightly but significantly reduced, whilst no effect was seen on the second phase (Tables 1 and 2).

The concentration-response curve to the selective 5-HT_3 agonist, 2-methyl-5-HT was uniphasic ($-\log EC_{50} = 5.4 \pm 0.03$), and the maximum response was not significantly different from that attained with 10 μM 5-HT. The concentration-response curve to 5-methoxytryptamine was also uniphasic ($-\log EC_{50} = 7.1 \pm 0.08$), and the maximum response (35 ± 5%) was not significantly different from that attained by 1 μM 5-HT. The responses to 5-methoxytryptamine were abolished in the presence of 0.1 μM atropine.

Maximal responses to 2 methyl 5-HT were reduced by 80 ± 5%. Conversely, in the presence of physostigmine, the maxima, although not the potency, of responses to 5 methoxytryptamine were enhanced to 63 ± 8% of the 5-HT (10 μM)

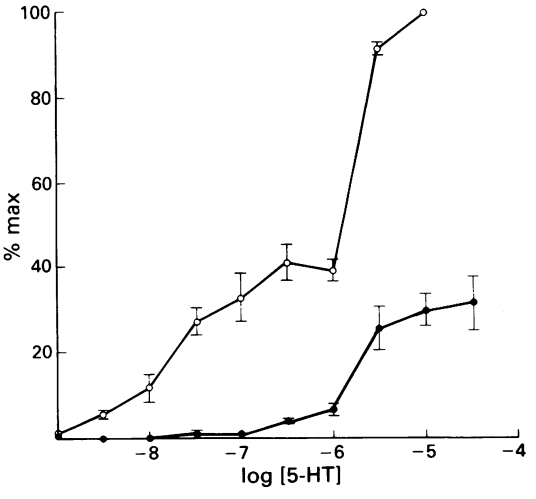

Figure 3 Concentration-response curves to 5-hydroxytryptamine (5-HT) in the absence (○) or presence of 0.1 μM (●) atropine. Values are mean, from 6–10 preparations, with vertical bars indicating s.e.mean.

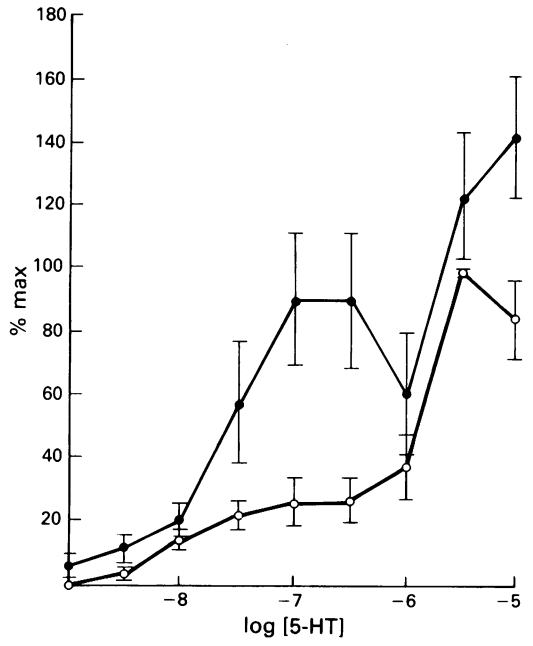

Figure 4 Concentration-response curves to 5-hydroxytryptamine (5-HT) in the absence (○) and presence (●) of 0.1 μM physostigmine. Values are mean, from 6–10 preparations, with vertical bars indicating s.e.mean.

maximal response. Furthermore, the maximal response to 2 methyl 5-HT was increased to 113 ± 4% of the maximal 5-HT (10 μM) response by physostigmine. Ondansetron, quipazine, N-methyl quipazine and granisetron did not affect the responses to 5-HT over the initial component of the concentration-response curve, whereas they antagonized the second phase of the curve. These data are shown in Tables 1 and 2. Similar results were observed in the presence of 0.1 μM ICS 205-930. However, at higher concentrations of ICS 205-930 (> 1 μM), the initial phase of the curve to 5-HT was dextrally shifted in a non-parallel fashion.

The effects of (R,S)-zacopride (10 nM–1 μM) were similar to ICS 205-930, in that the second component of the concentration-response curve was abolished, and, in addition, concentration-dependent reductions in the initial portion of the 5-HT concentration-response curve were also observed (Figure 5). During the 60 min exposure of the tissues to zacopride (0.1–10 μM), a contracture of the tissues was observed which was maximal after 5 min and declined to baseline tension levels after 40 min. This effect was not observed

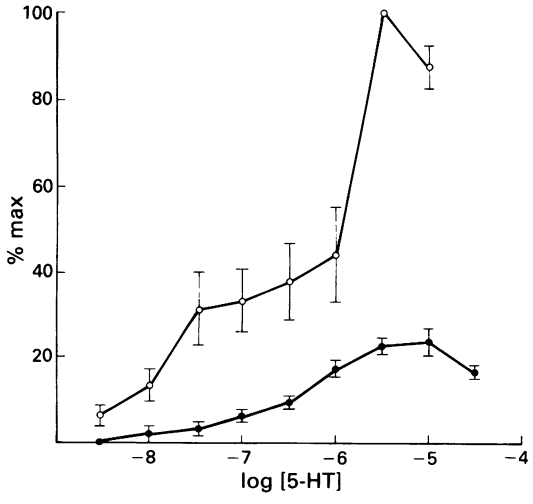

Figure 5 Concentration-response curves to 5-hydroxytryptamine (5-HT) in the absence (○) and presence (●) of 0.1 μM (R,S)-zacopride. Values are mean, from 6–10 preparations, with vertical bars indicating s.e.mean.

with either ICS 205-930, ondansetron, quipazine, N-methylquipazine or granisetron at the concentrations studied (1 nM–10 μM) (Table 3). The effects seen with (R,S)-zacopride may not represent receptor blockade, but desensitization since (R,S)-zacopride acts as an agonist at the site mediating the initial component (see below).

In the presence of 10 μM 5-methoxytryptamine and 1 μM methysergide, the concentration-response curve to 5-HT was uniphasic with no evidence of an initial component. Under these conditions, pA_2 values versus 5-HT were obtained with ICS 205-930, ondansetron, granisetron, (R,S)-zacopride, (R)-zacopride and (S)-zacopride (Table 4). All compounds exhibited Schild slopes which were not significantly different from unity, with the exception of (R,S)-zacopride. At concentrations of granisetron above 0.32 μM, the concentration-response curves to 5-HT were shifted dextrally in a non-parallel fashion, and were accompanied by a depression in the maximum. When the unity constraint was imposed, the following rank order of dissociation constants was found: (S)-zacopride > granisetron > (R,S)-zacopride = ICS 205-930 > (R)-zacopride > ondansetron (Table 4).

Table 3 Potencies ($-\log EC_{50}$) and maximal responses (% max) relative to 5-hydroxytryptamine (5-HT) of agonist eliciting contractile responses of guinea-pig ileum

Agonist	$-\log EC_{50}$	% max
5-HT	7.5 ± 0.08	1.0
5-methoxytryptamine	7.0 ± 0.03	1.0
Renzapride	7.0 ± 0.06	0.8
(S)-zacopride	6.9 ± 0.03	1.0
(R,S)-zacopride	6.3 ± 0.12	0.8
(R)-zacopride	5.7 ± 0.08	0.4
5-carboxamidotryptamine	6.1 ± 0.12	0.6
BRL 24682	5.9 ± 0.11	0.4
Metoclopramide	5.5 ± 0.12	0.6
Sulpiride	>4.0	—
2-methyl-5-HT	inactive	—
N,N,-dipropyl-5-carboxamidotryptamine	inactive	—
GR 43175	inactive	—
B-OHDPAT	inactive	—
Ondansetron	inactive	—
ICS 205-930	inactive	—
Granisetron	inactive	—
Quipazine	inactive	—
N-methyl-quipazine	inactive	—

Values are mean ± s.e.mean, n = 4–8. All agonists studied between 1 nM–10 μM. All studies conducted in the presence of 1 μM methysergide and 1 μM ondansetron to exclude 5-HT_1-like, 5-HT_2 and 5-HT_3 receptor function.

Table 4 pA_2 values and Schild slopes of antagonists at 5-HT_3 receptors in guinea-pig ileum

Antagonist	pA_2	*Slope*	pK_B
ICS 205-930	7.62 ± 0.10	0.98 ± 0.12	7.61 ± 0.05
Ondansetron	6.95 ± 0.10	0.88 ± 0.11	6.91 ± 0.04
Granisetron[a]	7.84 ± 0.07	1.09 ± 0.08	7.90 ± 0.04
(R,S)-zacopride	7.86 ± 0.24	1.23 ± 0.15[b]	7.64 ± 0.11
(R)-zacopride	7.15 ± 0.09	1.18 ± 0.14	7.27 ± 0.06
(S)-zacopride	7.96 ± 0.14	1.15 ± 0.16	8.11 ± 0.06

Values are mean ± s.e.mean, n = 8–16. All experiments were conducted in the presence of 10 μM 5-methoxytryptamine in order to desensitize selectively the initial phase of the 5-HT concentration-response curve. pK_B values are those calculated after imposing the unity constraint.

[a] At concentrations of granisetron above 0.32 μM, the maximum and slope of the concentration-response curves were reduced. Consequently, the pA_2 values were calculated with concentrations up to and including 0.32 μM.

[b] Significantly different from unity.

Table 5 Equilibrium dissociation constants ($-\log K_B$) for ICS 205-930 (3 μM) at 5-HT receptors mediating ileal contractions in the presence of 1 μM methysergide and ondansetron (1 μM)

Agonist	$-log\ K_B$
5-HT	6.31 ± 0.12
5-Methoxytryptamine	6.02 ± 0.09
(S)-zacopride	6.26 ± 0.13
(R,S)-zacopride	6.52 ± 0.14
5-Carboxamidotryptamine	6.10 ± 0.05

Values are mean ± s.e.mean, n = 4–8.

Studies conducted in the presence of 5-HT$_1$-like, 5-HT$_2$ and 5-HT$_3$ receptor blockade

In order to characterize further the initial phase of the concentration-response curve to 5-HT, the preparations were placed in Tyrode solution containing both 1 μM methysergide and 1 μM ondansetron, to exclude responses at 5-HT$_1$-like, 5-HT$_2$ and 5-HT$_3$ receptors (Buchheit *et al.*, 1985; Richardson *et al.*, 1985). Under these conditions the concentration-response curve to 5-HT was uniphasic (Figure 1b). The potency ($-\log EC_{50} = 7.2 \pm 0.12$) and maximal response (34.5 ± 6%) to 5-HT were not significantly different from the potency and maximal response to 5-HT at receptors mediating the initial phase of the concentration-response curve, when determined in the absence of ondansetron. The responses to 5-HT were highly reproducible, with no evidence of desensitization occurring between 2 consecutive concentration-response curves when established after a 60 min interval (curve 1, $-\log EC_{50} = 7.2 \pm 0.05$; curve 2, $-\log EC_{50} = 7.3 \pm 0.08$. The maximum responses of the two curves were not significantly different).

The responses to 2-methyl 5-HT were abolished in the presence of both methysergide (1 μM) and ondansetron (1 μM) whereas responses to 5-methoxytryptamine were not significantly affected ($-\log EC_{50} = 7.1 \pm 0.08$; maximum = 31 ± 4%). The potencies of 5-HT, 5-methoxytryptamine and 2-methyl 5-HT were also unaffected following reserpine pretreatment in terms of both EC_{50} and maximal responses at both phases of the concentration-response curve (data not shown).

The potencies ($-\log EC_{50}$ values) and intrinsic activities, relative to the maximum response to 5-HT (% maximum response) of a number of agonists at receptors mediating the contractile response in the presence of 1 μM ondansetron and 1 μM methysergide, are shown in Table 3. The rank order of agonist potency was 5-HT > 5-methoxytryptamine = (S)-zacopride = renzapride > (R,S)-zacopride > 5-carboxamidotryptamine > BRL 24682 > (R)-zacopride > metoclopramide ≫ sulpiride = N,N,dipropyl-5-carboxamidotryptamine = 8-OHDPAT = GR 43175 = 0. Only 5-HT, 5-methoxytryptamine and (S)-zacopride acted as full agonists. None of the 'inactive' compounds antagonized responses to 5-HT after equilibration for 60 min at a concentration of 1 μM. As (R,S)-zacopride and (R)-zacopride acted as partial agonists (Table 4), the dissociation constants ($-\log K_A$) were calculated by the method of Barlow *et al.* (1967). The $-\log K_A$ values for (R,S)- and (R)-zacopride were 6.1 ± 0.08 and 5.5 ± 0.11, respectively. The responses to (R,S)-zacopride and its isomers were abolished in the presence of 0.1 μM atropine, whereas the maximal response was significantly potentiated in the presence of 0.1 μM physostigmine (98 ± 4%). Atropine (0.1 μM) also abolished the contractile responses to all the other 'active' agonists (data not shown).

In separate experiments, the responses to 5-HT, 5-methoxytryptamine, (S)-zacopride, (R,S)-zacopride and 5-carboxamidotryptamine were studied in the absence and presence of a high concentration of ICS 205-930 (3 μM: relative to its equilibrium dissociation constant at 5-HT$_3$ receptor; see above). To eliminate completely the possibility of an interaction at 5-HT$_3$ receptors, 1 μM ondansetron was also present throughout the experiment. The concentration-response curves to the agonists were shifted dextrally in a parallel fashion by ICS 205-930. It can be seen that the $-\log K_B$ values of ICS 205-930 obtained with these agonists were very similar (Table 5). In order to study the specificity of action of ICS 205-930, the effect of 10 μM ICS 205-930 against responses to carbachol and DMPP was studied. Responses to these agonists were not significantly affected either in terms of potency or maxima (carbachol control, $-\log EC_{50} = 6.9 \pm 0.06$; in the presence of 10 μM ICS 205-930, $-\log EC_{50} = 7.1 \pm 0.11$; DMPP control, $-\log EC_{50} = 5.6 \pm 0.11$; in the presence of 10 μM ICS 205-930, $-\log EC_{50} = 5.6 \pm 0.14$).

Discussion

The present study has characterized the receptors mediating the indirectly mediated contractile response to the neuronally mediated effects of 5-HT in isolated segments of guinea-pig ileum. This preparation has been shown to exhibit a biphasic concentration-response curve to 5-HT (Fozard, 1985; Buchheit *et al.*, 1985; Butler *et al.*, 1988; Sanger & Nelson, 1989). The second portion of the curve is mediated by stimulation of 5-HT$_3$ receptors (Richardson *et al.*, 1985; Butler *et al.*, 1988; Cohen *et al.*, 1988), whereas the receptor mediating the initial phase of the concentration-response curve remains to be characterized.

The data obtained in the first series of experiments (Tables 1 and 2), in which both phases of the concentration-response curve to 5-HT were studied, are in good agreement with previous results (Buchheit *et al.*, 1985; Butler *et al.*, 1988; Sanger & Nelson, 1989). However, the lack of effect of methysergide while in agreement with data obtained by Buchheit *et al.* (1985) contrasted with that of Costa & Furness (1972) in which an inhibitory effect of methysergide was seen. The receptors mediating both phases of the biphasic concentration-effect curve appear to be neuronally located, in view of their high sensitivity to the sodium channel blocker, tetrodotoxin. The sensitivity of the 5-HT responses to morphine is in agreement with previous studies (Paton, 1957; Schaumann, 1957; Ganatra *et al.*, 1979) and is due to inhibition acetylcholine release. Pre-exposure of the preparations to phenoxybenzamine (0.1 μM) for a brief period (15 min), followed by subsequent washout, did not affect responses to 5-HT over either phase. However, in the maintained presence of phenoxybenzamine responses to 5-HT over the second phase responses to 5-HT were reduced by 70% whilst the responses over the initial phase were abolished. A reasonable explanation for the effect of phenoxybenzamine is that alkylation of postjunctional muscarinic receptors occurred which decreased the indirect effects of 5-HT. The ability of phenoxybenzamine, under similar conditions to those used in the 5-HT studies, to antagonize responses to carbachol is in agreement with this hypothesis.

The lack of effect of the α-adrenoceptor antagonists prazosin and phentolamine on the 5-HT response suggest that these effects were not due to α_1-adrenoceptor blockade. A small, but significant, inhibitory effect was observed with rauwolscine. In addition to its well characterized α_2-adrenoceptor antagonist properties this antagonist has been shown (Kaumann, 1983; Clinschmidt *et al.*, 1985) to antagonize 5-HT responses in the bovine coronary artery and rat stomach fundus. The 5-HT responses of both phases were unaffected in the presence of either propranolol, pindolol, atenolol or ICI 118551, suggesting that β_1- or β_2-adrenoceptors do not participate in the responses to 5-HT. Therefore, the receptors mediating both phases of the concentration-effect curve to 5-HT may differ from those shown to mediate positive chronotropic responses in guinea-pig atria (Eglen & Whiting, 1989). In this latter preparation 5-HT responses are non-competitively antagonized by pindolol and atenolol.

The present studies on the second component of the 5-HT concentration effect curve are in accord with the literature in

that this component appears to be mediated by 5-HT_3 receptor stimulation. The 5-HT_3 antagonists (ICS 205-930, ondansetron, granisetron, quipazine and N-methyl-quipazine) all antagonized the second phase of the concentration-response curve to 5-HT as well as responses to the 5-HT_3 agonist, 2-methyl 5-HT. Conversely, the inclusion of 5-methoxytryptamine in the Tyrode solution selectively abolished the initial phase of the concentration-response curve to 5-HT in agreement with a previous report (Fozard, 1985). The curve to 5-HT became monophasic in the presence of 5-methoxytryptamine, allowing unambiguous estimations of the effect of antagonists at the remaining 5-HT_3 receptors. The pA_2 values obtained under these conditions for ICS 205-930, ondansetron, granisetron, (**R,S**)-zacopride and its constituent enantiomers were in good agreement with other values in guinea-pig ileum reported in the literature (see Introduction for references and Fozard, 1988 for review). The present study shows (**S**)-zacopride to about 10 fold greater than (**R**)-zacopride, in terms of affinity at the 5-HT_3 receptor (8.11 versus 7.27, respectively). These values are similar to the pA_2 estimate for (**R,S**)-zacopride in guinea-pig ileum (Smith *et al.*, 1988) but the isometric ratio is considerably larger than that reported in binding studies in guinea-pig ileum with [^{3}H]-zacopride (**R/S** ratio = 1.4; Pinkus *et al.*, 1989a). Isometric ratios for zacopride (**R/S**) of 28, 42, 6 and 12 were obtained in rabbit ileum, vagus nerve, rat ileum and rat brain respectively (Pinkus *et al.*, 1989a,b).

The data are in agreement with the hypothesis that the biphasic nature of the concentration-response curve to 5-HT is mediated through two distinct receptors, the second phase being mediated by a 5-HT_3 receptor. The finding that the initial phase of the concentration-effect curve to 5-HT is unaffected by ondansetron, granisetron, quipazine and N-methyl quipazine show that, in this respect, they are more selective 5-HT_3 antagonists than either (**R,S**)-zacopride or ICS 205-930. Higher concentrations (0.1 μM–10 μM) of these latter two compounds also inhibited the initial phase of the 5-HT concentration-response curve suggesting effects independent of an interaction at the 5-HT_3 receptor.

Stimulation of the receptor mediating the initial phase of the concentration-response curve appears to be due exclusively to an enhancement of acetylcholine release. The antagonism of the response by atropine, morphine and phenoxybenzamine and its potentiation by physostigmine is in support of this proposal and with previous reports (Buchheit *et al.*, 1985; Sanger & Nelson, 1989). The second, 5-HT_3 receptor-mediated phase of the concentration-response curve also involves the release of acetylcholine, in view of the sensitivity of 5-HT and 2-methyl 5-HT responses to atropine, physostigmine and phenoxybenzamine (this study; Cohen *et al.*, 1985; Fox & Morton, 1989). A residual component of the 5-HT_3-mediated response, which was observed in the presence of atropine (this study), may involve the release of a second neurotransmitter. Buchheit *et al.* (1965) have proposed this to be substance P, although this has been disputed.

The inclusion of 1 μM ondansetron in the Tyrode solution, to inhibit 5-HT_3 function, enabled the effects of agonists to be selectively measured at the 5-HT receptor mediating the initial phase of the curve. The responses to 5-HT, 5-methoxytryptamine, (**S**)-zacopride, (**R,S**)-zacopride and 5-carboxamidotryptamine were competitively antagonized by ICS 205-930 with similar $-\log K_B$ values, indicating that these agonists interacted at the same receptor site. The concentration of ICS 205-930 required to elicit a significant dextral shift (3 μM), whilst relatively high, was specific in that no effect was observed directly at muscarinic or nicotinic receptors, as judged by the lack of effect on the responses to carbachol and DMPP.

The 5-HT receptor mediating the initial phase of the biphasic concentration-effect curve recognized optical isomers of zacopride i.e., (**S**)-zacopride was more potent than (**R**)-zacopride as an agonist. This stereoselectivity was the same for antagonism of the 5-HT_3 receptor. In terms of affinity, (**R,S**)-zacopride was approximately 100 fold more selective for ileal 5-HT_3 receptors (pA_2 = 7·9–8·5; Smith *et al.*, 1988; Cohen *et al.*, 1989; this study), in comparison to the receptor mediating the initial phase of the response curve to 5-HT. The structurally related benzamides, BRL 24682 and metoclopramide were weaker agonists whilst other 5-HT_3 antagonists, ondansetron, ICS 205-930, granisetron, quipazine and N-methyl-quipazine were inactive as agonists. These data indicate that the agonist actions of (**R,S**)-zacopride, renzapride, BRL 24682 and metoclopramide were independent of their 5-HT_3 antagonist properties. In addition, 5-methoxytryptamine, a potent agonist at receptors mediating the initial phase, is devoid of 5-HT_3 agonist and antagonist properties (Fozard, 1985; this study).

Taken together these data further suggest that the site mediating the first phase of the biphasic curve to 5-HT is distinct from the 5-HT_3 receptor. The differential affinity of ICS 205-930 at the 5-HT_3 receptor (7.6) and at the former site (6.3) is in accordance with this hypothesis. Since this site was also insensitive to methysergide it appeared to be different from 5-HT_1-like and 5-HT_2 receptors. The contractile response to 5-carboxamidotryptamine, but not to N,N-dipropyl-5-carboxamidotryptamine, observed at the receptors mediating the initial phase, also suggests that the receptor is dissimilar to the 5-HT_1-like receptor. This postulate is also consistent with the lack of contractile responses with the 5-HT_1-like and 5-HT_{1A} agonists, GR 43175 and 8-OHDPAT, respectively.

The pharmacology of the receptor mediating the initial phase of the response to 5-HT is similar to the pharmacology of the 5-HT receptor which mediates an increase in the 'twitch' response of the field-stimulated guinea-pig ileum (Sanger, 1987; Craig & Clarke, 1989). This latter response is insensitive to 5-HT_1-like, 5-HT_2 and 5-HT_3 antagonists, but sensitive to high concentrations (0.3 μM and greater) of ICS 205-930 (Craig & Clarke, 1989). It should be noted that whilst the $-\log K_B$ values for ICS 205-930 observed in the present study were similar to those reported by Craig & Clarke (1989), the potency of 5-HT at receptors mediating the contractile response in the unstimulated ileum was less than in the field-stimulated ileum ($-\log EC_{50}$ values: present study, = 7.3; field stimulated tissue, = 8.5; Craig & Clarke, 1989). Potent responses to 5-HT, equivalent to the first phase of the biphasic concentration-response to 5-HT, have also been obtained in the field-stimulated guinea-pig ileum (Sanger, 1987; Craig & Clarke, 1989). 'Twitch' responses to electrical stimulation were enhanced by low concentrations (1 nM–1 μM) of 5-HT (Kilbinger & Pfeuffer-Friedrich, 1985; Sanger, 1987; Craig & Clarke, 1989) and by 5-HT_3 antagonists of the substituted benzamide class, including renzapride (Sanger, 1987), zacopride (Craig & Clarke, 1989) and cisapride (Neya *et al.*, 1985; Schuurkes *et al.*, 1985). No enhancement was obtained with ICS 205-930 (Sanger, 1987) or granisetron (Sanger & Nelson, 1989). (**R,S**)-zacopride was a partial agonist at receptors in the quiescent ileum, but a full agonist in the field-stimulated ileum. These differences may reflect differences in effective receptor reserves between the two preparations rather than receptor differences *per se*.

The receptor mediating the contractile responses of the first phase is clearly not a 5-HT_1-like, 5-HT_2 or 5-HT_3 receptor, when defined by the criteria of Bradley *et al.* (1986). It most resembles the putative 5-HT_4 receptor recently described by Dumuis *et al.* (1988, 1989) which is present in both guinea-pig hippocampus and mouse colliculi neuronal culture. It should be noted that the term 5-HT_4 receptor has not been officially recognized by the Serotonin Club Receptor Nomeclature Committee (July 1990). The stimulation of this latter receptor leads to an increase in adenylate cyclase activity, a response which is insensitive to 5-HT_1-like, 5-HT_2 or 5-HT_3 ligands, but is antagonized by high concentrations of ICS 205-930 (pK_i values = 6.0 and 6.3 in mouse embryo colliculi neuronal cultures and guinea-pig hippocampus slices, respectively; Dumuis *et al.*, 1988). In addition, 5-methoxytryptamine (Dumuis *et al.*, 1988) and renzapride (Dumuis *et al.*, 1989) are also potent

agonists in these preparations. Since the receptor mediating the initial phase of the responses to 5-HT in the quiescent ileum also exhibits this profile it is our contention that 5-HT_4 receptors also mediate this response.

In conclusion, indirect excitatory responses to 5-HT in whole segments of guinea-pig ileum appear to be mediated by two distinct receptors. The pharmacological profiles of these receptors suggest stimulation of 5-HT_4 and 5-HT_3 subtypes. Definitive evidence for this hypothesis, however, awaits the development of potent and selective 5-HT_4 receptor antagonists.

The authors wish to thank Miss C.A. Machado for assistance in the pA_2 studies and Dr D.E. Clarke for his advice in the preparation of the manuscript.

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(Received December 4, 1989
Revised May 28, 1990
Accepted June 22, 1990)

Br. J. Pharmacol. (1990), **101**, 521–526

Influence of phorbol esters, and diacylglycerol kinase and lipase inhibitors on noradrenaline release and phosphoinositide hydrolysis in chromaffin cells

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1 We have investigated the modification of catecholamine efflux and inositol phosphate formation in cultured adrenal chromaffin cells by tetradecanoyl phorbol acetate (TPA) and inhibitors of diacylglycerol kinase (R 59 022) and diacylglycerol lipase (RG 80267), the two principal pathways of diacylglycerol metabolism.

2 TPA (1 nM to 1 μM) elicited a slow, calcium-dependent, sustained release of noradrenaline, which was partially blocked by the dihydropyridine calcium channel blocker (−)-202 791 and potentiated by the channel enhancer (+)-202 791.

3 R 59 022 enhanced noradrenaline efflux at 30 and 50 μM, while the lipase inhibitor RG 80267 failed to elicit release.

4 Neither R 59 022 nor RG 80267 affected bradykinin- or histamine-stimulated release, but both drugs substantially attenuated nicotine- and high K^+-stimulated release.

5 Pretreatment for 10 min with TPA (but not the relatively inactive 4-methoxyTPA) or the non-phorbol protein kinase C stimulator mezerein potently inhibited bradykinin- and histamine-stimulated accumulation of total [^{3}H]-inositol phosphate; inhibition of [^{3}H]-inositol phosphate formation was also seen with 24 h TPA treatment.

6 Neither R 59 022 nor RG 80267, separately or together, affected bradykinin-stimulated [^{3}H]-inositol phosphate formation.

7 Thus while the mechanism exists for inhibition of formation of inositol phosphates by stimulation of protein kinase C, these studies failed to show that this mechanism is activated by agonists acting on phospholipase C linked receptors.

Introduction

Chromaffin cells maintained in primary culture release catecholamines in response to activation of a variety of cell surface receptors. This includes receptors which, in addition to stimulating release of noradrenaline, stimulate hydrolysis of polyphosphoinositides, generating inositol phosphates and diacylglycerol, such as receptors to bradykinin, histamine, angiotensin II and prostaglandin (Livett & Marley, 1986; Zimlichman *et al.*, 1987; Noble *et al.*, 1988; Plevin & Boarder, 1988; Koyama *et al.*, 1988; Owen *et al.*, 1989a; Plevin *et al.*, 1990). The generation of diacylglycerols which may activate protein kinase C (PKC) is likely to be of significance in stimulus-secretion coupling. The clearest indication of this is the demonstration that PKC activating phorbol esters such as tetradecanoyl phorbol acetate (TPA) can themselves stimulate release, in a calcium-dependent manner, both in intact (Brocklehurst *et al.*, 1985; Pocotte *et al.*, 1985) and permeabilised cells (Knight & Baker, 1983; Pocotte *et al.*, 1985; Brocklehurst & Pollard, 1985). The studies with permeabilised cells demonstrate that PKC activation may play a role in exocytosis at the level of fusion of granules with the cell membrane, while Bittner & Holz (1990) have shown that more than one mechanism may be responsible for enhancement of release from permeabilized cells.

A second possible role for PKC in stimulus-secretion coupling may be at the level of the receptor. Activation of phospholipase C (PLC) may cause a diacylglycerol-mediated stimulation of PKC which feeds back to inhibit the agonist-induced activation of PLC. Evidence for such a mechanism falls into two categories. Firstly, the inhibition of agonist-induced stimulation of PLC by exogenous PKC activators such as phorbol esters. Secondly, evidence that inhibitory feedback loop is activated by an agonist, such as the enhancement of agonist-stimulated PLC by prior down-regulation of PKC, or attenuation of agonist-stimulated PLC by blocking diacylglycerol breakdown. Studies in a variety of preparations have shown that phorbol esters can attenuate agonist-stimulated formation of inositol phosphates; in chromaffin cell preparations it has been shown that histamine-induced inositol phosphate accumulation can be attenuated by phorbol ester (Wan *et al.*, 1989). Several recent studies have provided evidence that such a feedback loop is activated by agonists acting on PLC-linked receptors in platelets, smooth muscle and epithelial cells (Helper *et al.*, 1988; King & Rittenhouse, 1989; Pfeilschifter *et al.*, 1989; Crouch & Lapetina, 1989).

In the present study, we used bovine cultured adrenal chromaffin cells to characterize the effect of phorbol esters and inhibitors of diacylglycerol metabolism on release of catecholamines. We demonstrated the inhibition of agonist-stimulated PLC by phorbol esters and, also, sought to provide evidence for the regulation of inositol phosphate responses by agonist-stimulated diacylglycerol production.

Methods

Freshly obtained bovine adrenal medullae were digested with collagenase/protease as described by Marriott *et al.* (1988). Chromaffin cells were purified to about 90% by centrifugation and differential plating and cultured on 24-well 'Primaria' plates in complete medium as described earlier (Owen *et al.*, 1989a). Cells for release studies were washed twice with HEPES-buffered balanced salt solution (BSS) containing (mM): NaCl 125, KCl 5.4, $NaHCO_3$ 16.2, N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid 30, NaH_2PO_4 1, $MgSO_4$ 0.8, $CaCl_2$ 1.8 and glucose 5.5, gassed with 95% O_2: 5% CO_2 and buffered to pH 7.4. Release was measured by incubating the cells at 37°C in BSS in the presence or absence of drugs. A preincubation period of 10 min, in the presence or absence of drugs, was introduced where appropriate. At the

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end of the incubation period the medium was removed, centrifuged and the supernatant was acidified with 0.1 M HCl. The cell contents were extracted into 0.1 M HCl and the noradrenaline and adrenaline contents of both supernatant and cell extract were estimated by high pressure liquid chromatography with electrochemical detection. Similar patterns of release were seen for both noradrenaline and adrenaline, but only the release of noradrenaline is shown, expressed as either pmol per well or as % of cell content. Each result is the mean of quadruplicate determinations from a single cell preparation which has been repeated in similar or identical form three or more times on different cell preparations.

To estimate stimulation of total inositol phosphate formation, cells were incubated with 1 μCi *myo*-[2-^{3}H]-inositol (15 Ci mmol^{-1}) at 37°C for 32–40 h in 0.5 ml of modified BSS containing (mM): NaCl 125, KCl 5.4, $NaHCO_3$ 16.2, NaH_2PO_4 1, $MgSO_4$ 0.8, $CaCl_2$ 1.8, glucose 5.5, GIBCO non-essential and essential amino acids at 1%, glutamine 27 mg 100 ml^{-1}, streptomycin 5000 μg 100 ml^{-1}, penicillin 5000 iu 100 ml^{-1}, cytosine arabinoside 5 μM, in 5% CO_2 95% air. Cells were then washed, and preincubated with 10 mM lithium chloride in BSS in the presence or absence of drugs for 10 min. Drugs were then added for the duration of the incubation period as appropriate in BSS plus lithium. The reaction was stopped with cold methanol. Chloroform extraction was followed by isolation of [^{3}H]-inositol phosphates on Dowex-1 (Cl^{-1}) essentially as described by Rooney & Nahorski (1986).

Dihydropyridine, phorbol esters and diacylglycerol kinase and lipase inhibitors were diluted with BSS from stock solutions in dimethylsulphoxide (DMSO).

Materials

Cell culture supplies were from GIBCO, Paisley, Scotland except for Primaria plates (Falcon) which were from Becton-Dickinson, Oxford. *myo*-[2-^{3}H]-inositol was from New England Nuclear. The diacylglycerol kinase inhibitor R 59 022 (6-[2-[4-[(4-fluorophenyl)phenylmethylene]-1-piperidinyl]-ethyl]-7-methyl-5*H*-thiazolo[3,2-a]pyrimidin-5-one) was purchased from Janssen, Olen, Belgium. The diacylglycerol lipase inhibitor RG 80267 (1,6-*bis*(cyclohexyloximinocarbonylamino) hexane) (Revlon) was a kind gift of Rorer Central Research, Washington, Pasadena, USA, while the isomers of 202 791 (4-(benzoxadiazolyl)-1,4-dihydro-2,6-dimethyl-5-nitro-3-pyridin-carbonate-isopropylester) were kindly donated by Sandoz Ltd, Basle, Switzerland. Other chemicals and drugs were from Sigma Chemical Co. plc, Poole, Dorset, U.K. or Fisons plc, Loughborough, U.K.

Results

Effect of TPA, diacylglycerol kinase inhibitor and diacylglycerol lipase inhibitor on noradrenaline release

The time course, dose-response curve and calcium-dependence for the release of noradrenaline in response to TPA is shown in Figure 1a,b and c, respectively. The rate of release was characteristically slow and consequently subsequent experiments used a 30 min incubation. The EC_{50} for TPA was between 8 and 35 nM (3 determinations). Consistent with previous observations, phorbol ester-stimulated release was dependent on added extracellular calcium (Figure 1c), being maximal at 0.3 mM. The dependence on extracellular calcium suggests that calcium entry may play a role in TPA-stimulated release, thus the effects of two dihydropyridine calcium channel drugs were studied (Table 1). The calcium channel blocker (−)-202 791 reduced noradrenaline release in response to TPA while its stereoisomer, (+)-202 791, a calcium channel enhancer, caused a small release alone but had a greater than additive effect when combined with TPA.

The endogenous PKC activator diacylglycerol is metabolised through either phosphorylation to phosphatidic acid, by diacylglycerol kinase (DG kinase), or deacylation at the 2 position to monoacylglycerol, by diacylglycerol lipase (DG lipase). It was therefore of interest to see whether comparable effects to TPA could be produced by inhibition of one or both of these pathways. Hence, we investigated the effect of a DG kinase inhibitor (R 59 022) and a DG lipase inhibitor (RG 80267) on noradrenaline release. Neither the vehicle alone (dimethylsulphoxide 0.3%) nor the DG lipase inhibitor RG 80267 (up to 50 μM) had any effect on release over a period of 30 min. The DG kinase inhibitor R59 022 did induce enhanced efflux at 30 μM and above (Figure 2a), after incubation periods of over 8 min (Figure 2b). The DG lipase inhibitor failed to stimulate release at any time up to 60 min (Figure 2b).

Since bradykinin and histamine stimulate inositol phosphate production in chromaffin cells as well as release (Plevin & Boarder, 1988), then they must also stimulate formation of diacylglycerol. It was therefore possible that inhibition of dia-

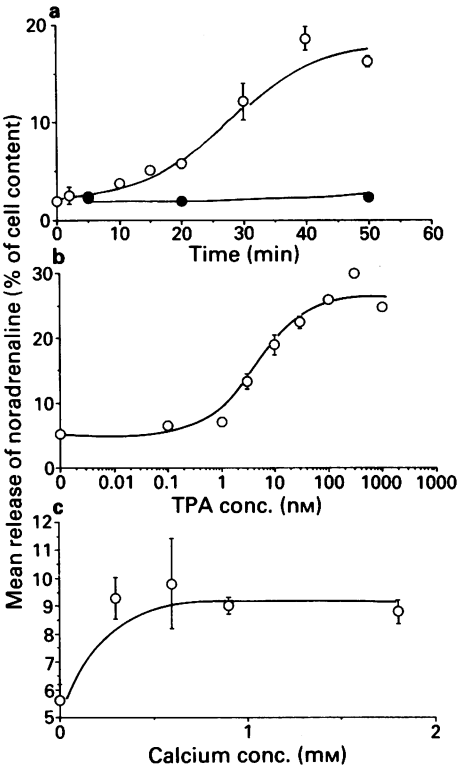

Figure 1 Characteristics of tetradecanoyl phorbol acetate (TPA)-stimulated release of noradrenaline. (a) Release in response to incubation for increasing time with 1 μM TPA (○) or dimethylsulphoxide control (●). (b) Release after 30 min incubation with increasing concentrations of TPA. (c) Release in response to incubation for 30 min with 100 nM TPA in increasing concentrations of extracellular calcium. Basal release in the absence of added calcium was 3.15 ± 0.25%. Release is expressed as % of total noradrenaline cell content. Values are means and vertical bars show s.e.mean of quadruplicates from a single experiment.

Table 1 Effect of dihydropyridine on tetradecanoyl phorbol acetate (TPA)-stimulated release

	Control	*TPA* (100 nM)
Control	27.8 ± 1.3	125.0 ± 7.9
(−)-202 791	31.3 ± 0.5	77.2 ± 7.4
(+)-202 791	54.8 ± 3.2	219.9 ± 9.8

Data shown are pmol noradrenaline released per well over a 30 min incubation period, with dihydropyridine present during a 10 min preincubation as well as during the incubation period. Dimethyl sulphoxide was present at 0.01% throughout. Values are means ± s.e.mean of quadruplicates from a single experiment.

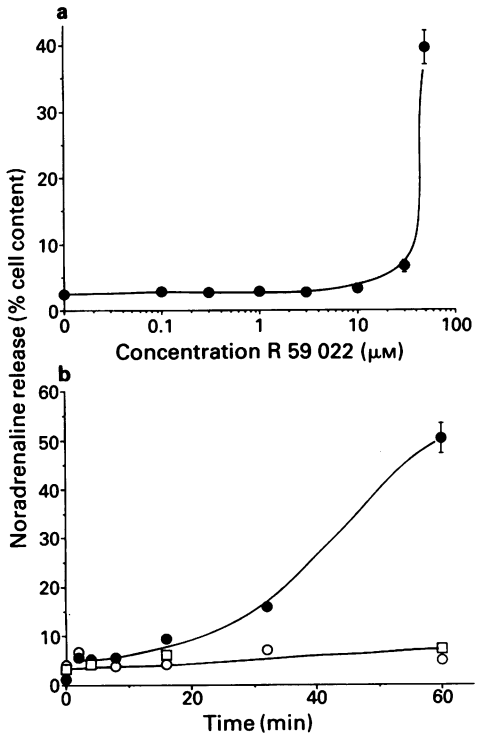

Figure 2 Release of noradrenaline in the presence of inhibitors for diacylglycerol (DG) kinase and diacylglycerol lipase. (a) Noradrenaline release (% of cell content) in response to increasing concentrations of the DG kinase inhibitor R59 022 (●). The dimethylsulphoxide vehicle up to 0.3% had no effect on release. (b) Release (% of cell content) after various times of incubation with 30 μM RG 80267 (○), 30 μM R 59 022 (●) or 0.3% dimethylsulphoxide (□). Values are means and vertical lines show s.e.mean of quadruplicates from a single experiment.

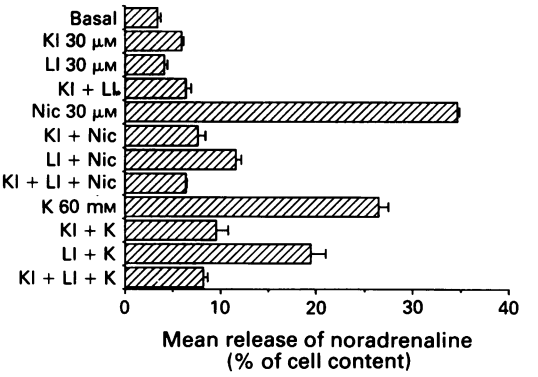

Figure 3 Noradrenaline release in response to nicotine (Nic) and high K^+ (K): effect of diacylglycerol (DG) kinase (KI) and DG lipase (LI) inhibitors. The inhibitors were present where appropriate during a 12 min preincubation period as well as during the 6 min incubation period, in the presence or absence of 30 μM nicotine or 60 mM K^+, during which release was measured. Both inhibitors were present at 30 μM, and dimethylsulphoxide at 0.3% was included where inhibitors were not added. Values are means and horizontal bars show s.e.mean of quadruplicates from a single experiment.

cylglycerol breakdown would modify release by these agents. It was found that bradykinin-stimulated release was unaffected by either the kinase inhibitor or the lipase inhibitor, added separately or together at 30 μM (data not shown). The histamine-stimulated release was reduced by 30 μM kinase inhibitor (data not shown); this was, however, the expected consequence of the antihistamine nature of the drug and was unrelated to its metabolic effects.

Noradrenaline release stimulated by either nicotine or high K^+ (depolarization) was inhibited by preincubation with either inhibitor (30 μM). This is illustrated by results from one such experiment shown in Figure 3; with a stimulation period of 6 min it was found that the nicotine-evoked stimulation could be almost eliminated by either R 59 022 or RG 80267. The high K^+-stimulated release was similarly affected by the kinase inhibitor R 59 022, but was reduced to a lesser extent by the lipase inhibitor RG 80267.

Thus, it appears that neither inhibitor of diacylglycerol metabolism inhibited bradykinin-stimulated release, but they both substantially attenuated release stimulated by nicotine and high K^+ depolarization.

Effect of TPA, diacylglycerol kinase inhibitor and diacylglycerol lipase inhibitor on agonist-stimulated inositol phospholipid breakdown

In the experiments to investigate the effects of TPA on agonist-stimulated formation of total inositol phosphates (in the presence of lithium), the pretreatment time with the phorbol ester was either 10 min (to stimulate PKC) or 24 h (to downregulate PKC). In each case the phorbol ester was also present during the 30 min incubation period in the presence of the agonist. We have previously shown that histamine and bradykinin stimulation of total inositol phosphate formation is linear for 30 min (Plevin & Boarder, 1988). The influence of 1 μM TPA pretreatment for 10 min and 24 h is illustrated by the results in Tables 2 and 3 respectively. Surprisingly, both pretreatment periods produced a substantial loss in agonist-stimulated formation of inositol phosphates. The effect of 10 min TPA pretreatment was characteristically greater with histamine stimulation than with bradykinin stimulation, while 24 h TPA pretreatment almost eliminated (histamine) or substantially reduced (bradykinin) the inositol phosphate response to agonists (Table 3).

We investigated the effects of different TPA concentrations, of the relatively inactive 4-methoxy TPA and of the structurally disparate PKC activator mezerein on bradykinin- and

Table 2 Effect of tetradecanoyl phorbol acetate (TPA) pretreatment for 10 min on bradykinin and histamine stimulation of total inositol phosphate formation

	DMSO control	*TPA (1 μM)*
Control	4625 ± 319	3979 ± 426
Bradykinin	9854 ± 664	4934 ± 401
Histamine	26546 ± 734	5494 ± 637

Data shown are d.p.m. of [^{3}H]-inositol phosphate accumulated in the presence of 10 mM lithium during a 30 min incubation period. TPA was present where appropriate, with dimethyl sulphoxide (DMSO) vehicle at 0.1%, during a 10 min preincubation as well as the incubation period. Values are means ± s.e.mean of quadruplicates from a single experiment.

Table 3 Effect of 24 h pretreatment with tetradecanoyl phorbol acetate (TPA) on bradykinin- and histamine-stimulated total inositol phosphate formation

	DMSO Control	*TPA (1 μM)*
Control	2391 ± 135	2001 ± 244
Bradykinin	9049 ± 1794	6875 ± 657
Histamine	22239 ± 7943	4118 ± 945

Data shown are d.p.m. of [^{3}H]-inositol phosphate accumulated in the presence of 10 mM lithium during a 30 min incubation period. TPA was present, where appropriate, with dimethylsulphoxide (DMSO) vehicle at 0.1% during a 24 h preincubation as well as the incubation period. Values are means ± s.e.mean of quadruplicates from a single experiment.

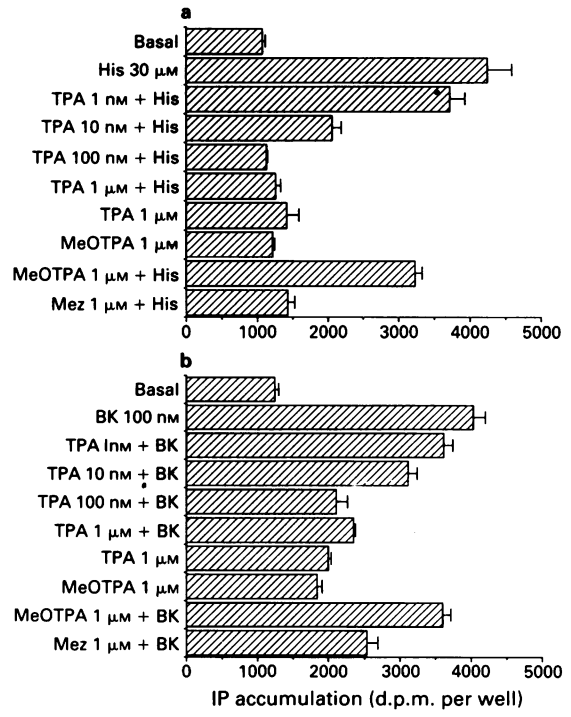

Figure 4 Total [^{3}H]-inositol phosphate (IP) formation in response to stimulation for 30 min with (a) 30 μM histamine (His) or (b) 10 nM bradykinin (BK). Tetradecanoyl phorbol acetate (TPA) was present at the concentrations shown, 4-methoxy TPA (MeOTPA) 1 μM or mezerein (Mez) 1 μM were present during a 10 min preincubation as well as the 30 min incubation period. Values are means and horizontal bars show s.e.mean of quadruplicates from a single experiment.

histamine-stimulated formation of inositol phosphates. These results are illustrated in Figure 4. When stimulation was with histamine substantial effects of TPA could be seen at 10 nM, with essentially maximal effects at 100 nM. 4-MethoxyTPA at 1 μM was no more effective than TPA at 1 nM. Mezerein (1 μM), while having no effect of its own, effectively inhibited histamine-stimulated formation of inositol phosphates. A very similar pattern of results was obtained when stimulation was with bradykinin (Figure 4b). These results are consistent with the regulation by PKC of histamine- and bradykinin-stimulation of inositol phosphates.

The indication that phorbol ester stimulation of PKC might regulate the chromaffin cell response to histamine and bradykinin led us to investigate the role of endogenously activated PKC, using the inhibitors of diacylglycerol kinase (R 59 022) and lipase (RG 80267). When included before and during incubations with bradykinin, R 59 022 was without effect (Table 4). Results of experiments in which the effect of R 59 022 on histamine stimulation was investigated are not presented since it is known that R 59 022 is an H_1-receptor antagonist (de Chaffoy de Courcelles *et al.*, 1985). RG 80267 had no effect on either bradykinin- or histamine-stimulated inositol phosphate formation. A combination of the two inhibitors also failed to influence the accumulation of inositol phosphate in response to bradykinin.

Discussion

The initial observation of Knight & Baker (1983), who used permeabilized cells to show that TPA induced an increased sensitivity of the exocytotic process for calcium, presumably by activating PKC, was influential in directing attention to the role of stimulation of this enzyme in exocytosis. However, here we are concerned with the role of PKC in the initiation of stimulus secretion coupling at the cell membrane. The time course of TPA-stimulated release described was found to be very slow and sustained and dependent on extracellular calcium, in confirmation of previous observations (Brocklehurst *et al.*, 1985). There are perhaps two explanations for this calcium-dependency. Firstly, incubation in nominally calcium-free medium results in a reduction in free intracellular calcium levels, to which TPA-stimulated release is sensitive (Brocklehurst *et al.*, 1985; Pocotte *et al.*, 1985). Secondly, TPA leads to an enhanced calcium influx, without which TPA-stimulated release cannot occur. We found that in chromaffin cells the release in response to TPA was partly blocked by the dihydropyridine antagonist (−)-202 791. Chromaffin cells have clearly been shown to possess L-type dihydropyridine sensitive voltage gated calcium channels which may mediate stimulus-secretion coupling (e.g. Garcia *et al.*, 1984; Boarder *et al.*, 1988; Owen *et al.*, 1989b) and so one explanation for this observation is that TPA-stimulated release is, in part dependent upon an enhanced opening of these channels caused, directly or indirectly, by stimulated PKC activity. The data we present here contain one additional indication that TPA is affecting calcium entry through L-type channels: the calcium channel agonist (+)-202 791 enhanced the release stimulated by TPA. This is consistent with the demonstration of dihydropyridine-sensitive phorbol ester-stimulated calcium influx in cloned rat pituitary cells (Albert *et al.*, 1987). Effects of TPA on calcium fluxes in other cell types include an enhancement of calcium channel opening in *Aplysia* (De Riemer *et al.*, 1985), facilitation of depolarization enhanced calcium influx in intact adrenal medulla of the rat (Wakade *et al.*, 1986), inhibition of depolarization-enhanced calcium flux in PC12 cells (Harris *et al.*, 1986; Di Virgilio *et al.*, 1986) and attenuation of calcium influx in neutrophils (McCarthy *et al.*, 1989).

An interesting alternative to TPA as an activator of PKC is the inhibition of DG kinase and/or DG lipase, in an attempt to elevate the levels of diacylglycerol, an endogenous activator (alongside calcium) of PKC. The DG kinase inhibitor R 59 022 has been shown to potentiate thrombin-induced diacylglycerol production in platelets and inhibit phosphatidic acid production in neutrophils (de Chaffoy de Courcelles *et al.*, 1985; Mege *et al.*, 1988), while RG 80267 has been shown to increase basal diacylglycerol levels and potentiate hormonal stimulated diacylglycerol production (Sutherland & Amin,

Table 4 Effect of inhibitors of diacylglycerol lipase and kinase on bradykinin- and histamine-stimulated inositol phosphate production

	Control	*RG 80267*	*R 59 022*	*RG 80267 and R 59 022*
Control	2373 ± 343	2816 ± 84	3079 ± 511	2396 ± 469
Bradykinin (100 nM)	6540 ± 376	6261 ± 498	5899 ± 619	5559 ± 304
Histamine (30 μM)	12270 ± 1133	12918 ± 414	*	*

The diacylglycerol lipase inhibitor RG 80267 was 10 μM and the diacylglycerol kinase inhibitor R 59 022 was 50 μM. Data shown are d.p.m. of [^{3}H]-inositol phosphates produced during a 30 min incubation period with the inhibitors present during both this incubation period and a preceding 10 min pre-incubation. Values are means ± s.e.mean of quadruplicates from a single experiment. * Effect of R 59 022 on histamine stimulated [^{3}H]-inositol phosphate production was not valid due to antihistamine nature of the inhibitor.

1982; Chang *et al.*, 1988). In experiments run in parallel with those described here, we have shown that both these metabolic inhibitors increase the accumulation of diacylglycerol in chromaffin cells at 30 μM and 50 μM, with the effect of R 59 022 being greater than that of RG 80627 (Owen & Boarder, unpublished observations). Here we showed that the DG kinase inhibitor was able to enhance release at these concentrations. By contrast, the lipase inhibitor was not able to elicit increased noradrenaline efflux. Combined with the effect of these compounds on diacylglycerol accumulation in chromaffin cells (Owen & Boarder, unpublished observations), these results may indicate a relationship between enhancement of diacylglycerol accumulation and the stimulation of release.

Bradykinin-stimulated noradrenaline release was unaffected by the DG kinase and lipase inhibitors suggesting that accumulation of diacylglycerol plays little role in bradykinin-stimulated release. This is consistent with the differing sensitivity of bradykinin- and TPA-stimulated release to dihydropyridines: the TPA-stimulated release is partially sensitive (this paper) while bradykinin-stimulated release is insensitive (Owen *et al.*, 1989) to dihydropyridine calcium channel blockers. The contrasting observation that nicotine- and high K^+-stimulated release is inhibited by both kinase and lipase inhibitors is most likely due to an effect at the level of calcium entry, since the lack of effect on responses to bradkykinin shows that the exocytotic process is not impaired. The inhibition of nicotine- and high K^+-stimulated release may be through PKC activation and subsequent phosphorylation of a component of calcium entry, or a 'non-specific' membrane effect perturbing channels and/or nicotinic receptors.

A possible role for bradykinin-stimulated PKC in chromaffin cells is modulation of receptors and associated effector mechanisms. For example, we have previously provided evidence that angiotensin II enhances prostaglandin stimulation of adenylate cyclase in cultured adrenal medulla cells, by a mechanism involving diacylglycerol production and the activation of PKC (Boarder *et al.*, 1988). In addition, a number of examples exist in a variety of cell types of inositol phospholipid-linked receptor responses which are down-regulated by phorbol ester-stimulated PKC (e.g. Rittenhouse & Sasson, 1985; Drummond, 1985; Aiyar *et al.*, 1986), with indications that endogenously produced PKC activation may be involved (Helper *et al.*, 1988; King & Rittenhouse, 1989; Crouch & Lapetina, 1989; Pfeilschifter *et al.*, 1989). We showed that the inositol phosphate response to bradykinin and histamine in chromaffin cells was attenuated by 10 min prior activation of PKC. These results indicate that stimulation of PKC can result in a reduced response to these agonists, and raise the possibility that enhanced production of diacylglycerol may play a feedback role in the response. Pretreatment for 24 h with phorbol esters, a protocol which causes loss of PKC activity in a variety of cell types, including PC12 cells and adrenal chromaffin cells (Matthies *et al.*, 1987; Bittner & Holz, 1990), might therefore be expected to enhance the inositol response, as shown recently in different cell types (Helper *et al.*, 1988; Pfeilschifter *et al.*, 1989). However, our observation of reduced responses for both bradykinin and histamine stimulation is difficult to interpret: it may be due to a reduced response to the agonist following a long period of prior PKC stimulation by TPA, or a possible dependency of the response on intact PKC activity in the cells. A further investigation is needed to monitor the changes in PKC occurring in these cells on lengthy treatment with phorbol esters.

As previously discussed, diacylglycerol is metabolised by kinase or lipase pathways, so a further strategy is to potentiate the consequences of increased synthesis of diacylglycerol by inhibiting one or both of these pathways. Agonist-enhanced diacylglycerol formation may only be able to activate a feedback loop when diacylglycerol breakdown is impaired. This would result in a reduced inositol phosphate formation in response to agonists in the presence of the diacylglycerol kinase and/or lipase inhibitors. However, we found no effect of either or both of these inhibitors on bradykinin-stimulated accumulation of inositol phosphates. Furthermore, when histamine was used to stimulate these cells, there was no effect on the DG lipase inhibitor.

These results demonstrate that protein kinase C activation by phorbol esters can inhibit the receptor-stimulated synthesis of inositol phosphates in chromaffin cells. However, the results provide no support for the suggestion that diacylglycerol produced as a result of receptor stimulation in chromaffin cells acts to down-regulate the inositol phospholipid receptor effector system. This may help to explain why in chromaffin cells the agonist stimulation of inositol phosphate production is linear for 45 min (Plevin & Boarder, 1988), while other systems which have this feedback loop desensitize rapidly (e.g. Helper *et al.*, 1988).

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(Received January 22, 1990
Revised May 10, 1990
Accepted July 7, 1990)

Br. J. Pharmacol. (1990), **101**, 527–530

Sodium nitroprusside modulates the fibrinolytic system in the rabbit

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1 We have investigated the effect of sodium nitroprusside (NP) and glyceryl trinitrate (GTN) on fibrinolysis in anaesthetized rabbits *ex vivo* and *in vitro* by measurement of euglobulin clot lysis time (ECLT), plasma levels of tissue plasminogen activator (t-PA) activity, plasma t-PA antigen levels and plasminogen activator inhibitor (PAI-1) activity.

2 *In vivo*, NP ($30\,\mu g\,kg^{-1}$), GTN ($30\,\mu g\,kg^{-1}$) and prostacyclin ($3\,\mu g\,kg^{-1}$) caused similar transient decreases in left ventricular systolic pressure. However, while prostacyclin induced near-maximal inhibition of *ex vivo* platelet aggregation, NP or GTN had no effect.

3 *Ex vivo*, NP caused a significant decrease in ECLT and an increase in plasma t-PA activity.

4 Intravenous co-administration of t-PA ($30\,\mu g\,kg^{-1}$) with NP caused substantial prolongation of plasma t-PA activity, without affecting t-PA antigen levels.

5 In whole blood *in vitro*, NP ($30\,\mu g\,kg^{-1}$) prevented the time-dependent increase in PAI-1 activity and inhibited inactivation of added t-PA ($10\,ng\,ml^{-1}$).

6 We propose that NP exhibited fibrinolytic activity through increased t-PA activity as a result of inhibition of PAI-1 release from platelets. These results could have important therapeutic consequences when t-PA and nitrate treatments are combined.

Introduction

Fibrinolysis is a process by which activated plasmin proteolytically degrades fibrin. Conversion of plasminogen to plasmin is stimulated by a variety of plasminogen activators (PAs), predominantly tissue-PA (t-PA). However, t-PA can be rapidly degraded by the liver, and inactivated upon binding to plasminogen activator inhibitors (PAIs) in the plasma. Therefore, a major control over the release of plasmin and fibrinolysis lies both with t-PA and PAIs.

Platelets, in addition to their procoagulant role, have an inhibitory effect on fibrinolysis, which is mainly due to the release of PAI-1 (Erickson *et al.*, 1984). Prostacyclin (PGI_2) and many of the nitrovasodilators, including sodium nitroprusside (NP) and endothelium-derived relaxing factor (EDRF)/nitric oxide (NO) inhibit platelet aggregation and adhesion (Radomski *et al.*, 1987a,b). Prostacyclin exhibits its anti-platelet activity by increasing platelet cyclic AMP (Tateson *et al.*, 1977; Gorman *et al.*, 1977) while the nitrovasodilators (NO releasing agents) inhibit platelet function by an increase in cyclic GMP (Mellion *et al.*, 1980).

Recently we have found that co-administration of NP with t-PA *in vivo* resulted in significant prolongation of plasma t-PA activity (Korbut *et al.*, 1990). In the present study we further investigate the mechanism of fibrinolytic activity induced by NP or GTN in anaesthetized rabbits.

Methods

Surgical procedure

The studies were performed on male rabbits (New Zealand White), weighing 2.1–3.2 kg, receiving a standard diet and water *ad libitum*. General anaesthesia was induced with sodium pentobarbitone ($20–30\,mg\,kg^{-1}$; Sagatal, May & Baker) administered via the left marginal ear vein and maintained with supplementary doses of anaesthetic as required. In addition, lignocaine (Xylocaine 2%) was administered for local anaesthesia. Body temperature was maintained at 37°C by means of a homeothermic blanket (BioScience, Sheerness, Kent). The trachea was cannulated and the rabbit ventilated with air at a rate of 40–45 strokes min^{-1} and a tidal volume of 14–20 ml by a miniature ventilator (Harvard, Edenbridge, Kent). Polythene cannulae were placed into the left ventricle via the right carotid artery, for withdrawal of blood samples, and the femoral vein or right marginal ear vein for i.v. injections. The left ventricular cannula was connected to a transamerica type 4-422-0001 pressure transducer to monitor left ventricular systolic pressure (LVSP) and heart rate (HR) on a Grass model 7D polygraph (Grass Instruments, Quincy, Mass., USA).

Platelet aggregation ex vivo

Arterial blood (2 ml) was withdrawn from the left ventricle and collected into tri-sodium citrate (3.15% w/v) in a ratio of 9:1 and immediately centrifuged at $1400\,g$ (4000 r.p.m.) for 20 s (Biofuge A; Hereus) to produce platelet-rich plasma (PRP). The blood was further centrifuged at $14900\,g$ (12000 r.p.m.) for 1 min to obtain platelet-poor plasma (PPP). Platelet aggregation was measured in a Payton aggregometer with 0.5 ml PRP. In initial experiments, dose-response curves to adenosine 5'-diphosphate (ADP, $0.2–3.2\,\mu g\,ml^{-1}$) were determined to establish $1.6\,\mu g\,ml^{-1}$ ADP as a sub-maximal dose with respect to aggregation, as measured by peak increase in light transmission.

After a stabilization period of 20–30 min two blood samples were processed and the aggregation to ADP ($1.6\,\mu g\,ml^{-1}$) assessed. At time 0, vehicle or NP, t-PA, NP + t-PA or GTN (all at $30\,\mu g\,kg^{-1}$) were given intravenously. Blood samples were withdrawn at 1, 5, 15, 30 and 60 min after injection, and the PRP challenged with ADP ($1.6\,\mu g\,ml^{-1}$).

Fibrinolytic activity ex vivo

The fibrinolytic activity within plasma samples was assayed *ex vivo*, by measurement of t-PA activity and t-PA antigen levels, using commercially available kits (Biopool; Sweden). In addition, the euglobulin clot lysis time (ECLT) was assessed, based on the method of von Kaulla & Schultz (1958), as described below.

Blood containing tri-sodium citrate (3.15% w/v) in a ratio of 9:1 was centrifuged at $14900\,g$ (12000 r.p.m.) for 1 min

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(Biofuge A; Hereus) to produce PPP. Distilled water was added (14 ml ml^{-1} PPP) and the pH adjusted to pH 5.4 by bubbling with CO_2 gas (approximately 3 min). This procedure causes precipitation of the euglobulin fraction, while the acidity destroys the biological activity of PAI. After a second centrifugation at 14900 g (12000 r.p.m.) for 1 min, the supernatant was discarded and the euglobulin precipitate dissolved in 1 ml buffer (13.4 mM KH_2PO_4, 53.6 mM Na_2HPO_4).

At time 0, 2 u human thrombin (10 μl; 200 u thrombin ml^{-1} 0.05 M $CaCl_2$) was added to 0.3 ml euglobulin fraction to induce clot formation. The fraction was incubated at 37°C and the time for complete lysis to occur recorded.

Fibrinolytic activity in vitro

Rabbit blood was collected into tri-sodium citrate (3.15% w/v) in a ratio of 9:1 and centrifuged at 200 g (low speed) for 8 min (Petalfuge; OrthoDiagnostis Systems) to produce PRP. The PPP was obtained by further centrifugation of PRP at 900 g (high speed).

Whole blood, PRP or PPP (2 ml) was added to plastic tubes and incubated at 37°C with gentle agitation. At time 0, vehicle, NP (0.1–30 μg ml^{-1}), t-PA (1–10 ng ml^{-1}), or NP and t-PA was added and samples taken after 1, 5, 15, 30 and 60 min. The fibrinolytic activity was evaluated by measurement of plasma t-PA activity and PAI-1 activity (Biopool, Sweden).

Drugs

Sodium nitroprusside (Sigma Chem. Co., Poole, U.K.) and glyceryl trinitrate (Lipha, West Drayton, U.K.) were dissolved and administered in saline. Single chain tissue plasminogen activator, specific activity 600,000 i.u. mg^{-1} (Biopool, Sweden) was initially dissolved in $KHCO_3$ (1 M) and diluted and administered in saline. Prostacyclin (a gift from Dr B.J.R. Whittle, Wellcome Research Labs., U.K.) was initially dissolved in Tris buffer (1 M, pH 8.4) and diluted and administered in 1.25% $NaHCO_3$. Sagatal (May & Baker, Dagenham, U.K.) was administered in saline 1:1 and Xylocaine 2% (Astra Pharm., Kings Langley, UK) was administered directly.

Statistical analysis

Results are expressed as the mean ± s.e.mean of n experiments, and analysed by two-way analysis of variance followed by a least significance procedure to determine the nature of the response. A P value of less than 0.05 was considered statistically significant.

Results

Haemodynamic effects

The resting LVSP observed in this study ranged from 75 mmHg to 110 mmHg (97 ± 2 mmHg). During the 60 min experimental period, no changes in LVSP were observed in anaesthetized rabbits treated with vehicle or t-PA (30 μg kg^{-1}). Prostacyclin (3 μg kg^{-1}) caused a transient, but significant, decrease in LVSP (31 ± 5 mmHg) 20 s after administration, that returned to basal pressure within 5 min. Both NP (30 μg kg^{-1}) and GTN (30 μg kg^{-1}) caused a similar fall in LVSP (− 33 ± 3 mmHg and 38 ± 7 mmHg, respectively) at 20 s, which also returned to basal levels within 5 min, although that induced by NP was slightly more prolonged (Figure 1a).

Platelet aggregation ex vivo

Prostacyclin caused almost complete inhibition of ADP-induced platelet aggregation *ex vivo* (93 ± 7%) 1 min after administration. This effect was still significant at 5 min, but had returned to control levels by 15 min. In contrast, NP or GTN had no effect on platelet aggregation *ex vivo* (Figure 1b).

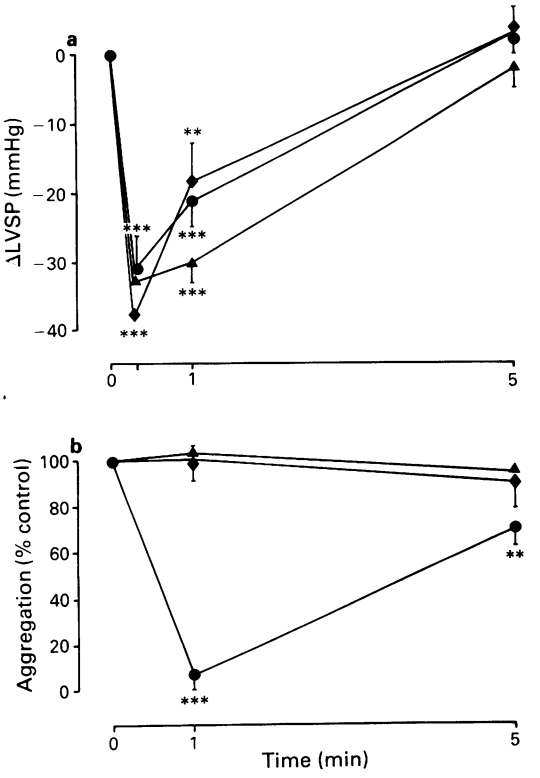

Figure 1 The effect of 3 μg kg^{-1} prostacyclin (●; n = 7), 30 μg kg^{-1} sodium nitroprusside (▲; n = 4) or 30 μg kg^{-1} glyceryl trinitrate (◆; n = 3) administration to anaesthetized rabbits on (a) left ventricular systolic pressure (LVSP) and (b) ADP-induced, *ex vivo* platelet aggregation. Results are expressed as mean and vertical lines show s.e.mean. ** $P < 0.01$; *** $P < 0.001$.

Fibrinolysis ex vivo

Intravenous administration of NP (30 μg kg^{-1}) or GTN (30 μg kg^{-1}) to anaesthetized rabbits caused a gradual decrease in ECLT measured *ex vivo*, although only that induced by NP proved significantly different from control (Figure 2a). In addition, NP (30 μg kg^{-1}), but not GTN (30 μg kg^{-1}), caused an increase in plasma t-PA activity, significantly different from the control 5 min after administration (Figure 2b).

Bolus injection of t-PA (30 μg kg^{-1}) caused an increase in plasma t-PA activity measured in a sample withdrawn after 1 min. This transient activity had returned to basal levels, just detectable by the assay, within 5 min (Table 1). However, while NP alone (30 μg kg^{-1}) had comparatively little effect on plasma t-PA activity (Figure 2b), co-administration of NP with t-PA resulted in considerable prolongation of the plasma t-PA activity measured *ex vivo*. This activity was significantly greater than that obtained with t-PA alone at 5, 15, 30 and 60 min (Table 1).

Using samples from the same experiments, exogenous t-PA caused an immediate increase in plasma t-PA antigen levels 1 min after administration, that decreased exponentially over 60 min. The co-administration of NP with t-PA did not significantly alter the t-PA antigen levels measured with t-PA alone (Figure 3).

Fibrinolysis in vitro

When t-PA (10 ng ml^{-1}) was incubated with citrated whole blood *in vitro* a gradual decline in plasma t-PA activity was observed over 60 min. Co-incubation of NP (3 μg ml^{-1}) with t-PA resulted in a stabilization of the t-PA activity that proved significantly higher than with t-PA alone after 60 min (Figure 4a).

Incubation of citrated whole blood at 37°C resulted in a gradual increase in PAI-1 activity in the plasma. The addition

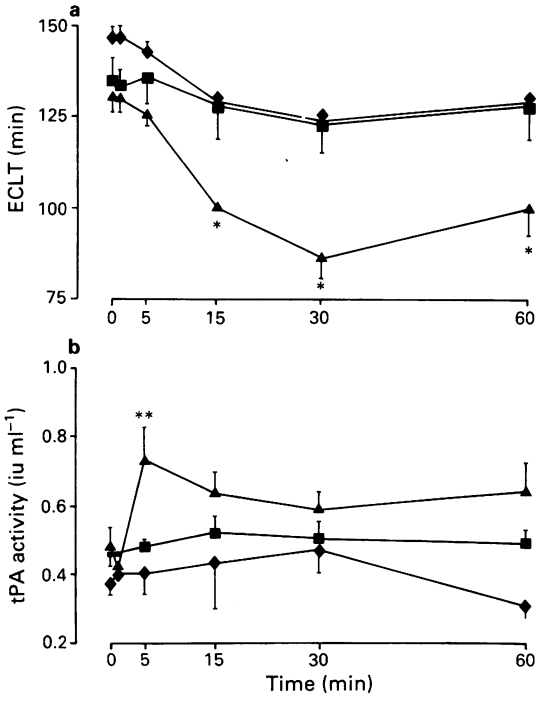

Figure 2 The effect of vehicle (■; $n = 5$), $30\,\mu g\,kg^{-1}$ sodium nitroprusside (▲; $n = 4$), or $30\,\mu g\,kg^{-1}$ glyceryl trinitrate (◆; $n = 3$) administration to anaesthetized rabbits on fibrinolysis, as measured *ex vivo* by (a) euglobulin clot lysis time (ECLT) and (b) tissue plasminogen activator (t-PA) activity in plasma. Results are expressed as mean and vertical lines show s.e.mean. * $P < 0.05$; ** $P < 0.01$.

Table 1 The time-related changes in tissue plasminogen activator (t-PA) activity measured *ex vivo*, following administration of $30\,\mu g\,kg^{-1}$ t-PA alone or in the presence of $30\,\mu g\,kg^{-1}$ sodium nitroprusside (NP) to anaesthetized rabbits at time 0

	t-PA activity (iu ml^{-1})	
Time (min)	*t-PA*	*NP + t-PA*
0	0.8 ± 0.2	0.8 ± 0.2
1	33.8 ± 2.4	32.0 ± 1
5	2.3 ± 0.9	27.0 ± 0.6***
15	0.6 ± 0.1	20.0 ± 0.6***
30	0.5 ± 0.2	8.0 ± 0.6***
60	0.7 ± 0.1	6.0 ± 0.6**

Results are expressed as mean ± s.e.mean; $n = 3$. ** $P < 0.01$; *** $P < 0.001$.

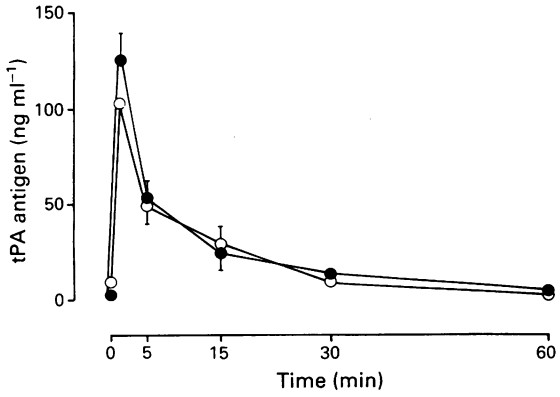

Figure 3 The time-related changes in tissue plasminogen activator (t-PA) antigen levels measured *ex vivo*, following administration of $30\,\mu g\,kg^{-1}$ t-PA alone (●; $n = 3$) or in the presence of $30\,\mu g\,kg^{-1}$ sodium nitroprusside (NP, ○; $n = 3$) to anaesthetized rabbits. Results are expressed as mean and vertical lines show s.e.mean.

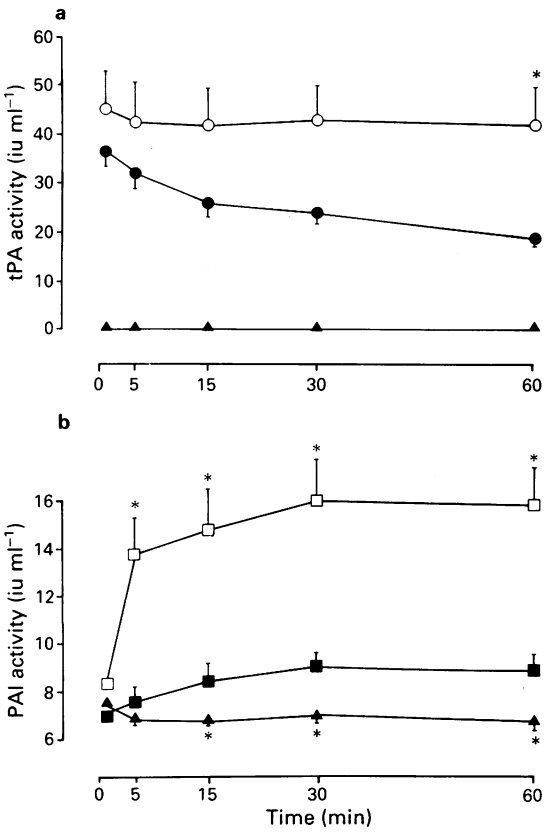

Figure 4 (a) The time-related decrease in tissue plasminogen activator (t-PA) activity following administration of $3\,\mu g\,ml^{-1}$ sodium nitroprusside (NP) (▲; $n = 3$), and $10\,ng\,ml^{-1}$ t-PA alone (●; $n = 3$) or in the presence of $3\,\mu g\,ml^{-1}$ NP (○; $n = 3$) to whole blood *in vitro*. (b) The effect of $30\,\mu g\,ml^{-1}$ NP (▲; $n = 3$) on the time-related increase in plasminogen activator inhibitor (PAI-1) activity in whole blood *in vitro* (■; $n = 3$). The increase in PAI-1 activity in platelet rich plasma (□; $n = 3$) is also represented. Results are expressed as mean and vertical lines show s.e.mean. * $P < 0.05$.

of NP ($30\,\mu g\,ml^{-1}$) to the blood inhibited this increase, maintaining basal levels of PAI-1 throughout the experimental period, and was significantly lower than control at 15, 30 and 60 min (Figure 4b). Incubation of PRP caused a considerable increase in PAI-1 activity, that was not observed with PPP.

Discussion

When administered to anaesthetized rabbits prostacyclin (PGI_2), NP or GTN all caused a transient decrease in LVSP, while only PGI_2 induced inhibition of platelet aggregation *ex vivo*. This emphasizes the weak activity that nitrates display on platelets as compared to their action on vascular smooth muscle (Schafer *et al.*, 1980). In contrast, PGI_2 was equally active on the vasculature and on platelets (Lidbury *et al.*, 1989). Despite the lack of anti-aggregatory activity, NP activated fibrinolysis, as measured by a significant decrease in ECLT and a significant increase in plasma t-PA activity *ex vivo*. To elucidate the mechanism by which NP induces fibrinolysis, exogenous t-PA was administered, in the presence or absence of NP, and the profile of t-PA removal from the circulation determined by measurement of plasma t-pA activity and t-PA antigen levels. The t-PA activity assay measures only free plasma t-PA (exclusive of that bound to PAIs), while the t-PA antigen assay measures total plasma t-PA (inclusive of that bound to PAIs). The t-PA activity measurements demonstrated the rapid removal of t-PA activity from the blood (less than 5 min) by binding to PAIs and degradation by the liver, whereas the plasma t-PA antigen levels decreased somewhat more slowly representing the loss of t-PA due to degradation by the liver only. As demonstrated recently, in the presence of NP, t-PA activity *ex vivo* was greatly prolonged,

while t-PA antigen levels remained unchanged (Korbut *et al.*, 1990). This indicates that NP prevents inactivation of t-PA by PAI, probably by inhibition of PAI-1 release from platelets, which normally accounts for more than 90% of circulating PAI (Booth *et al.*, 1985).

That NP inhibited PAI-1 release was supported by *in vitro* experiments with whole blood. They showed that the time-related decrease in t-PA activity following incubation with t-PA, presumably due to binding to PAIs, was significantly maintained in the presence of NP. Furthermore, the time-related increase in PAI-1 activity seen in whole blood, probably due to release of PAI-1 from platelets, was significantly inhibited by NP.

While we have shown that NP can induce fibrinolysis at lower concentrations than those required for inhibition of platelet aggregation (Lidbury *et al.*, 1989), PGI_2 exhibits fibrinolytic activity (Korbut *et al.*, 1983) at concentrations very similar to anti-aggregatory concentrations (Gorman *et al.*, 1977; Moncada, 1982). Furthermore, we found that the time course of NP-induced fibrinolysis differs from that of PGI_2 (Moore *et al.*, 1988; Korbut *et al.*, 1989). Thus, the mechanism of fibrinolytic activity induced by activators of guanylate cyclase, such as NP, is different from that of activators of adenylate cyclase. Although various hypotheses have been proposed to explain the fibrinolytic activity induced by PGI_2 (Moore *et al.*, 1988), it is accepted that activators of adenylate cyclase produce a long-lasting stimulation of fibrinolysis by an unknown mechanism. Furthermore, due to the lack of effect of NP on platelet aggregation *ex vivo*, these results indicate that NP may induce fibrinolysis while producing minimal changes in platelet cyclic GMP levels. Although our evidence is indirect, fibrinolysis may be stimulated by small increases in cyclic GMP, as with platelet adhesion, whereas larger increases in cyclic GMP are required for inhibition of platelet aggregation. It is tempting to speculate that the fibrinolytic action of NP may be related to the NO-induced anti-adhesive effect on platelets (Radomski *et al.*, 1987b) rather than anti-aggregatory properties. Alternatively, NP may stimulate fibrinolysis via an unknown, cyclic GMP-independent mechanism. Although both GTN and NP cause smooth muscle relaxation via NO-mediated stimulation of soluble guanylate cyclase, only NP activated fibrinolysis *ex vivo*. This may occur because GTN can only generate formation of NO in the presence of thiols, such as cysteine (Feelish & Noack, 1987), whereas NO release from NP is regarded as non-enzymatic/spontaneous. On the other hand we believe that GTN is likely to express fibrinolytic activity, similar to that of NP, at higher concentrations or with repeated administration over a longer period of time. The possibility of a GTN-induced fibrinolytic response is presently under investigation.

NP exhibits fibrinolytic activity through increased t-PA levels that probably result from inhibition of PAI-1 release from platelets. Several studies have shown that platelets, when stimulated by a variety of stimuli such as collagen, thrombin, ADP or adrenaline, are able to release PAI-1 (Murray *et al.*, 1974; Erickson *et al.*, 1984). Irrespective of the mechanism of platelet activation, such release of PAI-1 appears to be quantitative, and in human blood a greater amount of PAI is associated with platelets than with plasma (Kruithof *et al.*, 1986). To our knowledge, no inhibitors of PAI-1 release have been investigated in non-activated platelets. It is important to stress, therefore, that in our experiments the platelets were not stimulated. However, as we observed, the conditions prevailing in experiments *in vitro*, both in whole blood or PRP, might easily result in signficantly raised levels of PAI-1. Moreover, activation of the fibrinolytic system by NP observed in anaesthetized animals, presumably due to inhibition of PAI-1 release from platelets, could suggest that platelets constantly release PAI-1 *in vivo* following surgical procedures. The physiological role of this activation could be to prevent premature lysis of primary, platelet-rich, haemostatic plugs.

Finally, the administration of anti-platelet nitrovasodilators in combination with t-PA may have important therapeutic consequences due to the prolongation of the efficacy of t-PA, perhaps allowing a reduction in the total administered dose. As we have suggested previously (Korbut *et al.*, 1990) the bleeding tendencies and a reduced t-PA requirement associated with angina pectoris patients treated with t-PA (Gold *et al.*, 1987), could result from the prolongation of t-PA activity due to combined treatment with nitrates.

The authors wish to thank Dr R Botting for editorial assistance. The William Harvey Research Institute is supported by a grant from Glaxo Group Research Limited.

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(Received February 16, 1990
Revised June 4, 1990
Accepted June 25, 1990)

Br. J. Pharmacol. (1990), **101**, 531–540

Tolbutamide excites rat glucoreceptive ventromedial hypothallamic neurones by indirect inhibition of ATP-K^+ channels

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1 The sulphonylureas, tolbutamide (0.1–10 mM) and glibenclamide (0.1–100 μM) were shown not to inhibit ATP-K^+ channel currents when applied to inside-out membrane patches excised from rat cultured cerebral cortex or freshly-dispersed ventromedial hypothalmic nucleus (VMHN) neurones.

2 Saturable binding sites for [^{3}H]-glibenclamide, with similar affinity constants are present in rat cerebral cortex and hypothalamic membranes. The density of binding sites was lower in the hypothalamus than cortex.

3 Intracellular recordings from glucoreceptive VMHN neurones in hypothalamic slices were obtained. In the absence of glucose, tolbutamide (0.1 mM) depolarized these cells, increased membrane resistance and elicited action potentials.

4 Tolbutamide (0.1 mM) inhibited ATP-K^+ channel currents and induced action current activity in cell-attached recordings from glucoreceptive VMHN neurones.

5 Glibenclamide (10–500 nM) had no effect *per se* on glucoreceptive VMHN neurones but did antagonize the actions of tolbutamide.

6 It is concluded that the hypothalamic (and perhaps cortical) sulphonylurea receptors are not directly coupled to ATP-K^+ channels.

Introduction

The sulphonylureas (e.g. tolbutamide and glibenclamide) are used clinically in the treatment of maturity onset (Type 2) diabetes. Their hypoglycaemic properties have been mainly attributed to their actions on pancreatic β-cells where they stimulate insulin release (Lebovitz, 1985). They cause persistent depolarization of β-cells which, coupled with the generation of calcium-dependent action potentials and calcium entry, results in insulin secretion (Howell, 1984). The depolarization is due to a reduction in the β-cell resting K^+ permeability (Henquin & Meissner, 1982). Recent studies, with single channel recording techniques, have shown that the sulphonylureas inhibit the activity of ATP-K^+ channels in insulin-secreting cells (Sturgess *et al.*, 1985; Trube *et al.*, 1986). Thus, they seemingly mimic the actions of nutrient secretagogues like glucose which also act to inhibit ATP-K^+ channel activity (Ashcroft *et al.*, 1984; 1988). However, the actions of the sulphonylureas and the nutrients differ in one important respect, the latter require intracellular metabolism to induce ATP-K^+ channel closure whilst the former act directly to close the channel. This is clearly demonstrated by the ability of tolbutamide and glibenclamide to inhibit ATP-K^+ channels when applied to inside-out or outside-out membrane patches (Trube *et al.*, 1986; Sturgess *et al.*, 1988; Zunkler *et al.*, 1988).

Tritiated sulphonylureas bind reversibly to saturable, high-affinity sites on β-cell membranes. Other oral antidiabetic compounds compete with these sites in a manner that correlates well with their relative hypoglycaemic activities *in vivo* (Giesen *et al.*, 1985). Thus the β-cell is thought to contain specific sulphonylurea receptors and it has been argued that these drugs initiate their pharmacological effects through interactions with these sites. Binding sites for sulphonylureas, seemingly identical to those on insulin-secreting cell membranes, have also been shown to be present in avian and mammalian heart cells (Fosset *et al.*, 1988) and mammalian central neurones (Geisen *et al.*, 1985; Lupo & Bataille, 1987; Bernardi *et al.*, 1988). The presence of ATP-K^+ channels has been well documented in mammalian cardiac cells (Noma, 1983; Trube & Hescheler, 1984; Noma & Shibasaki, 1985) and recently such channels have also been demonstrated in mammalian central neurones (Ashford *et al.*, 1988; 1990a). It has been shown that the sulphonylurea receptor is functionally linked to ATP-K^+ channels in heart cells, as glibenclamide inhibits channel activity in isolated membrane patches (Belles *et al.*, 1987; Fosset *et al.*, 1988). The actions of sulphonylureas on ATP-K^+ channels in central neurones have not been described although it has been shown that 1 μM glibenclamide blocks hyperpolarization induced by anoxia in hippocampal slices (Mourre *et al.*, 1989; Grigg & Andersen, 1989; Ben-Ari, 1990). However, Grigg & Anderson found that, in contrast to its action on other tissues, 0.1 μM glibenclamide had no significant effect.

We have examined the effects of the sulphonylureas, tolbutamide and glibenclamide, on ATP-K^+ channels recorded from inside-out patches obtained from cultured cerebral cortex neurones and ventromedial hypothalmic nucleus (VMHN) neurones and on cell-attached patches from VMHN neurones. In addition, the effects of these drugs on *in vitro* neurones were determined by intracellular recording from glucoreceptive VMHN cells in hypothalamic slices. Preliminary accounts of some of these data have been published in abstract form (Boden *et al.*, 1989; Ashford *et al.*, 1990b).

Methods

Preparation of cerebral cortex and hypothalmic membranes

Male Sprague-Dawley rats (200–500 g) fed *ad libitum* were stunned, decapitated and the cerebral cortex and hypothalamus dissected out on ice and immediately immersed on ice-cold phosphate buffer. The tissues were homogenized in 10 volumes of ice-cold 50 mM Na-K phosphate buffer (37.8 mM Na_2HPO_4, 12.2 mM KH_2PO_4), pH 7.5, by a teflon-glass homogenizer with a motor-driven pestle (5 up and down strokes run at 3000 r.p.m.), and then centrifuged for 30 min at 17000 *g*. The resulting pellet was resuspended in phosphate buffer and then centrifuged again for 30 min at 17000 *g*.

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Finally, this pellet was resuspended in buffer to give a protein concentration of 5–7 mg ml^{-1} and was used the same day. Protein concentration was determined according to the method described by Lowry *et al.* (1951), with bovine serum albumin as standard.

Measurement of ^{3}H-ligand binding

Incubations were carried out in 50 mM Na-K phosphate buffer, pH 7.5, containing the ^{3}H-ligand, inhibitor (where appropriate) and homogenate (1 mg protein) in a total volume of 1 ml (4–5 replicates for each determination). Equilibration was for 2 h at 25°C and was terminated by addition of 4 ml ice-cold buffer. The mixture was filtered immediately through Whatman GF/B glass fibre filters by use of a Shearline R&D (Cambridge, U.K.) 10-place filtration block (25 mm diameter filters). The filters were transferred to scintillation vials and 10 ml scintillator added and allowed to stand overnight before determination of the tritium by liquid scintillation counting. The scintillator was Emulsifier-Safe (Packard)/water 95:5 (v/v). The final concentration of [^{3}H]-glibenclamide in each experiment was determined by counting an aliquot of the equilibrium medium (buffer + ^{3}H-ligand).

Curves for the amount of [^{3}H]-glibenclamide bound versus the concentration of [^{3}H]-glibenclamide, after subtraction of the binding of [^{3}H]-glibenclamide not inhibited by 1 μM glibenclamide, were fitted to the equation:

$$[^3\text{H}]\text{-glibenclamide bound} = \frac{B_{max} \cdot A^n}{A^n + EC_{50}^n}$$

where B_{max} is the maximum binding of [^{3}H]-glibenclamide, A is the concentration of [^{3}H]-glibenclamide, EC_{50} is the concentration of [^{3}H]-glibenclamide giving half-maximal binding and n is the Hill coefficient. The best-fit values of B_{max}, EC_{50} and n were obtained by weighted non-linear regression analysis using the Harwell library routine VBO1A, as implemented on the Cambridge IBM 3081/3084. All points were weighted by the reciprocal of the variance associated with them.

Curves of inhibition of ligand binding were fitted in the same way to the equation:

$$\%\text{ of uninhibited binding of }^3\text{H-ligand} = \frac{100 - NS}{(A/IC_{50})^n + 1} + NS$$

with IC_{50}, n and NS as variables. A is the concentration of inhibitor, IC_{50} the inhibitor concentration giving 50% inhibition of the inhibitor-sensitive binding, n the Hill coefficient and NS the percentage of the binding of the ^{3}H-ligand insensitive to the inhibitor ('non-specific' binding). The affinity constant of the inhibitor (unlabelled ligand) was calculated from the relationship: $K_a = 1/(IC_{50} - [^3\text{H-ligand}])$.

Electrical recording and analysis

Coronal slices (400 μm thick) of hypothalamus were cut from brains of Wistar or Sprague-Dawley rats (100–200 g weight) with a Vibratome (Oxford Instruments). The slices were maintained at room temperature in artificial cerebro-spinal fluid (ACSF). For intracellular recording, the slices were transferred to a recording chamber where they were superfused with ACSF at 37°C. Electrodes were filled with 3 M potassium acetate and had d.c. resistances of 100–150 MΩ when measured in physiological saline. A period of thirty minutes was allowed for equilibration following impalement. Input resistances were derived from the slope of the current-voltage plot obtained by measuring the electrotonic potential during current injection. Pulses, of greater than 100 ms duration, were applied in order to ensure complete capacitance saturation of the membrane. These recording procedures have previously been described in full (Boden & Hill, 1988).

Primary neuronal cultures were obtained from 2–7 day old neonatal rat cerebral cortices. Full details of this procedure have been published elsewhere (Ashford *et al.*, 1988).

For single channel recording from VMHN neurones, cells were acutely dissociated from the VMHN isolated from rat hypothalamic slices. The nuclei were incubated in 0.5 mg ml^{-1} collagenase (Clostridiopeptidase A. Boehringer, Mannheim), 1 mg ml^{-1} trypsin (Type XII, Sigma, Poole, Dorset) in ACSF at room temperature for 1–2 h and then triturated by use of flame-polished Pasteur pipettes of decreasing internal diameter. The dispersed cells were plated onto Sterilin dishes (35 mm) and left for 30 min–1 h before use, by which time the cells had adhered. Single channel currents were recorded, at room temperature, from cell-attached and inside-out membrane patches obtained from cultured or acutely dispersed neurones by standard patch-clamp recording procedures (Hamill *et al.*, 1981). Current recordings were made by either a List Electronic EPC-7 or a Dagan 8900 patch clamp amplifier and stored on magnetic tape (Racal 4DS) for later analysis and reproduction of figures. The potential across the membrane is described following the usual sign convention for membrane potential (i.e. inside negative). Outward current (i.e. current flowing from the intra- to extracellular side of the membrane) is shown as upward deflections on all traces. The data were analysed for current amplitude and open-state probability by computer (Apricot XEN-i 286/45) as described previously (Sturgess *et al.*, 1988; Kozlowski *et al.*, 1989).

Solutions

The ACSF contained (mM): NaCl 128.0, KCl 5.0, NaH_2PO_4 1.2, $CaCl_2$ 2.4, $MgCl_2$ 1.3, $NaHCO_3$ 26.0, D-glucose 10.0, pH 7.4. Before single channel recording the cells (cultured and isolated neurones) were washed thoroughly with normal external physiological salt solution (PSS) consisting of (mM): NaCl 135.0, KCl 5.0, $CaCl_2$ 1.0 $MgCl_2$ 1.0, HEPES 10.0, pH 7.4 with NaOH. For cell-attached and isolated inside-out patch recordings, the patch pipette contained (mM): KCl 140.0, $CaCl_2$ 5.0, $MgCl_2$ 5.0, HEPES 10.0, pH 7.2 with KOH and the batching solution was either the normal external PSS (cell-attached recordings) or an intracellular solution (inside-out recordings) consisting of (mM): KCl 140.0, $MgCl_2$ 1.0, $CaCl_2$ 0.9, EGTA 1.0, HEPES 10.0, pH 7.2 with KOH (free Ca^{2+} concentration of 1 μM). The high concentration of divalent cations (5 mM) in cell-attached experiments was included to maintain stable recordings. The high concentrations were maintained in some inside-out patches as these were excised following cell-attached recordings. In some inside-out experiments the electrode contained the normal external PSS. In experiments where high concentrations of adenosine 5'-triphosphate (ATP) were used, the concentrations of the divalent cations and EGTA were altered in order to compensate for chelation by ATP (as determined by the metal ion/ligand binding programme 'METLIG' (P. England & R. Denton, University of Bristol)). Solutions (with or without drugs) were applied, by gravity feed systems at a rate of 4 ml min^{-1} for slices and approximately 0.5 ml s^{-1} for the isolated cells and membrane patches. The different types of ATP (Na^+ and K^+ salts, vanadium-free) were obtained from Sigma (Poole, Dorset) and [^{3}H]-glibenclamide, glibenclamide and tolbutamide were donated by Hoechst Aktiengesellschaft (Frankfurt, West Germany). All data in the text and figures are presented as mean values ± s.e.mean.

Results

Lack of direct inhibition of ATP-K^+ channels by sulphonylureas

Cultured cortical neurones The incidence of ATP-K^+ channels in rat cultured cortical neurones has been shown to be extremely low (Ashford *et al.*, 1988) and this has also been

borne out in this study. Out of 78 inside-out membrane patches in which the actions of ATP (applied to the intracellular membrane aspect) were tested, only 15 contained an ATP-K$^+$ channel. In agreement with the previous study, the single channel current-voltage relationship for the ATP-K$^+$ channel exhibits pronounced outward rectification at depolarized potentials (as expected from Goldman-Hodgkin-Katz theory) when recorded under an asymmetric potassium ion gradient (normal PSS in electrode and a 140 mM K$^+$-containing PSS in the bath). Furthermore, the reverse potential (approximately −80 mV) and the single channel conductance (~130 pS, measured between −20 mV and +20 mV) were similar to those described previously. Application of ATP to the solution bathing the inside-out membrane patch resulted in a reversible inhibition of channel activity (Figure 1a). This action of ATP was due to a reduction in the open-state probability (P_{open}) of the channel and an apparent decrease in the overall number of functional channels present in the patch. ATP reduced P_{open} in a dose-dependent manner over the range tested (1–10 mM) with half-maximal inhibition obtained at 2–3 mM ATP.

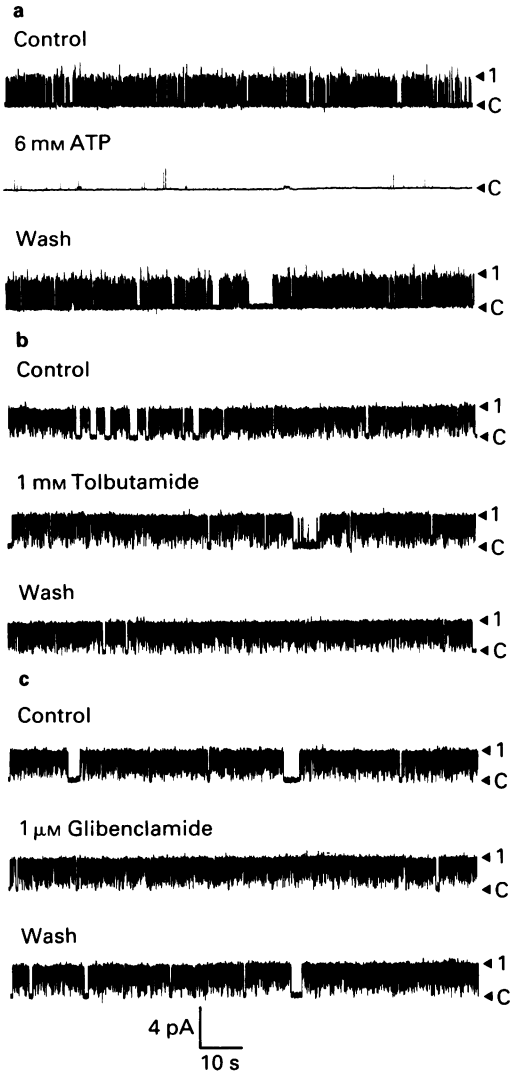

Figure 1 Single channel currents recorded from inside-out membrane patches, excised from rat cultured cerebral cortical neurones, held at a membrane potential of 0 mV. The recording pipette contained 135 mM NaCl and 5 mM KCl and the bath 140 mM KCl and 10^{-6} M calcium. Single channel openings are denoted by upward deflections (outward currents). (a) Inhibition of a potassium selective channel by 6 mM ATP added to the bath solution. Note the effect of ATP is reversible on wash. The values of P_{open} were as follows: control 0.381; 6 mM ATP 0.001; wash 0.365. Application of 1 mM tolbutamide (b) or 1 μM glibenclamide (c) to the bath solution produced no effect upon the activity of the ATP-sensitive potassium channel. The values of P_{open} were as follows: (b) control 0.745; 1 mM tolbutamide 0.689; wash 0.788- (c) control 0.736; 1 μM glibenclamide 0.791; wash 0.752. The data shown in (a) to (c) were obtained from three different inside-out patches. Although not shown, the channel activity in (b) and (c) was inhibited by application of ATP to the bath.

The actions of both tolbutamide (0.5–10 mM, $n = 8$) and glibenclamide (0.1–10 μM, $n = 9$) were tested on ATP-K$^+$ channel activity, recorded from inside-out membrane patches. This patch configuration was chosen because ATP-K$^+$ channels could be identified unequivocally (by testing for inhibition of activity with ATP) and also because the sulphonylureas have been shown to be at least as effective when applied to this side in patches excised from insulin-secreting cells (Sturgess *et al.*, 1988). On each occasion tested, neither tolbutamide nor glibenclamide had any effect on ATP-K$^+$ channel activity (Figure 1b,c) regardless of ionic configuration (i.e. high K$^+$ in bath, Na$^+$ in electrode or high K$^+$ in both). Quantified results for 1 mM tolbutamide and 1 μM glibenclamide are shown in Table 1.

Freshly-isolated VMHN neurones We have recently shown (Ashford *et al.*, 1990a) that ATP-K$^+$ channels are present in glucoreceptive neurones of the VMHN and have suggested that the glucose-induced excitation of these neurones is mediated by inhibition of ATP-K$^+$ channels in a manner identical to that for pancreatic β-cells (Ashcroft *et al.*, 1984; 1988). As far as we have been able to ascertain these hypothalamic ATP-K$^+$ channels are identical, in terms of K$^+$ selectivity, conductance and sensitivity to ATP (Ashford *et al.*, 1990a) to those of the cerebral cortex, except that they occur at a much higher density in freshly dispersed VMHN neurones (approximately 1 in 3 patches). This much-increased success rate in obtaining ATP-K$^+$ channels coupled to identifiable cells (glucoreceptive) meant that a more detailed study of sulphonylurea action could be undertaken.

Thus the actions of tolbutamide and glibenclamide were tested on inside-out membrane patches isolated from freshly dispersed VMHN neurones. On each occasion ($n = 21$) the channel identity was confirmed by testing for inhibition of channel activity with ATP (2–3 mM) either before or after the application of the sulphonylurea. Neither tolbutamide (0.1–10 mM; $n = 11$) nor glibenclamide (1–100 μM; $n = 10$) produced any observable effect on channel activity (Figure 2a,b), even over prolonged exposure periods (up to 20 min tested). The mean change of N_fP_o in the presence of tolbutamide or glibenclamide, was calculated for some of these patches and is shown in Table 1, where it can be seen that there is no significant inhibition induced by these compounds. These results are similar to the data obtained from the cultured cortical cells.

Binding of [^{3}H]-glibenclamide to cerebral cortex and hypothalamic membranes

Although previous studies have demonstrated the presence of saturable [^{3}H]-glibenclamide binding sites in the rat cerebral

Table 1 The lack of effect of sulphonylureas on ATP-K$^+$ currents recorded from inside-out membrane patches

		Mean change in channel activity $N_fP_o(test)/N_fP_o(control)$	
Sulphonylurea		*Cortex*	*VMHN*
Tolb	1 mM	0.96 ± 0.1 (5)	0.93 ± 0.09 (5)
	10 mM	—	1.00 ± 0.06 (4)
Glib	1 μM	0.93 ± 0.14 (5)	0.92 ± 0.20 (3)
	10 μM	—	1.02 ± 0.08 (4)
	100 μM	—	0.97 ± 0.06 (3)

Results are expressed as the mean relative change in N_fP_o ± s.e.mean. The number of patches used are shown in parentheses. Tolb = tolbutamide, Glib = glibenclamide, VMHN = ventromedial hypothalamic nucleus, N_f = number of functional channels in patch and P_o = open-state probability.

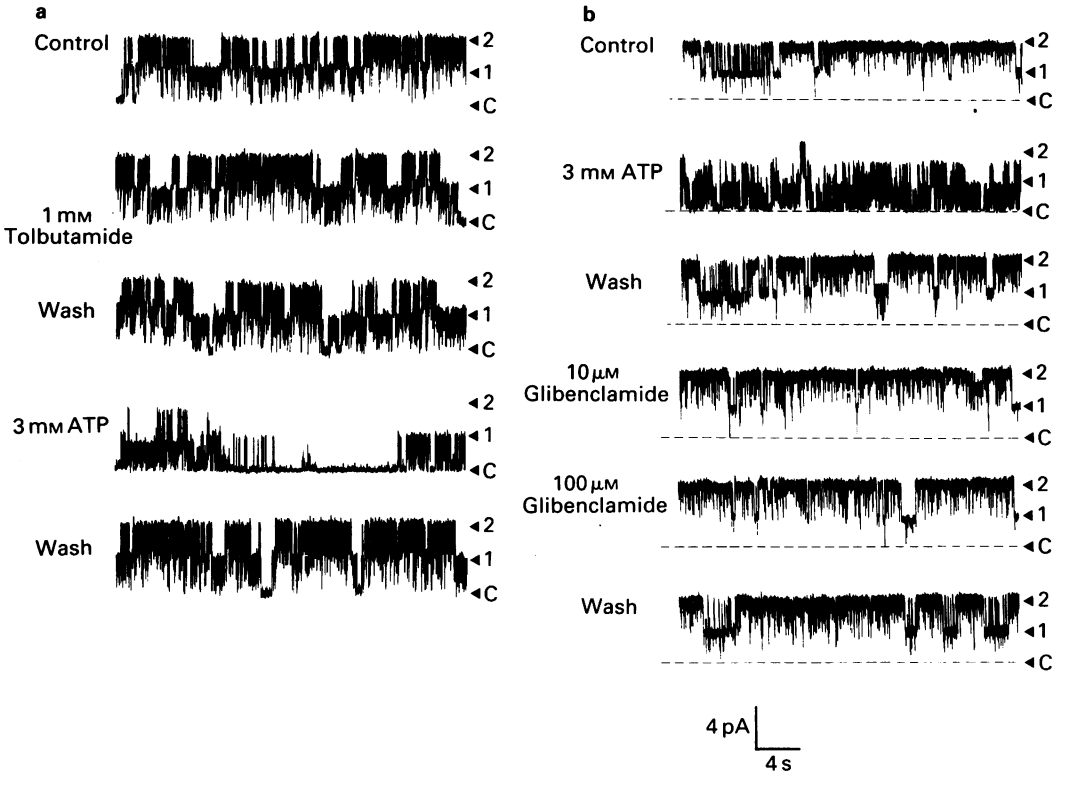

Figure 2 Lack of effect of tolbutamide and glibenclamide on ATP-K^+ channels recorded from inside-out membrane patches from ventromedial hypothalamic neurones. The recording pipette contained 135 mM NaCl, 5 mM KCl and the bath 140 mM KCl and 10^{-6} M calcium. The patch potential was 0 mV and openings are shown as upward deflections (outward currents). (a) Membrane patch containing 2 ATP-K^+ channels. Application of 1 mM tolbutamide had no effect on channel activity, whereas application of 3 mM ATP produced a substantial inhibition of the channel open state probability, which was reversible upon wash. The values of P_{open} were as follows: control 0.728; 1 mM tolbutamide 0.704; wash 0.750; 3 mM ATP 0.350 and wash 0.695. (b) A recording from a separate patch which also contained 2 ATP-K^+ channels. ATP 3 mM applied to the bath produced an inhibition of the channel activity which was reversible on wash, whereas 10–100 μM glibenclamide had no significant effect on channel activity. The P_{open} values were as follows: control 0.841; 3 mM ATP 0.385; wash 0.853; 10 μM glibenclamide 0.865; 100 μM glibenclamide 0.815 and wash 0.875

cortex, such sites have not yet been described in the hypothalamus. Therefore, the demonstration of a lack of action of sulphonylureas on ATP-K^+ channels of cells from this region may simply reflect the absence of binding sites. Thus, binding of [^{3}H]-glibenclamide was investigated in rat hypothalamus and a comparison performed with cerebral cortex.

Saturable binding sites for [^{3}H]-glibenclamide are present in both rat cerebral cortex and hypothalamic neuronal membranes (Figure 3a,b). The affinity constants of the [^{3}H]-glibenclamide binding sites were $2.13 \pm 0.09 \times 10^9\,M^{-1}$ ($n = 5$) in the cerebral cortex and $2.70 \pm 0.15 \times 10^9\,M^{-1}$ ($n = 3$) in the hypothalamus. The values for the Hill slope were 0.95 ± 0.04 ($n = 5$) for cortex and 1.15 ± 0.04 ($n = 3$) for hypothalamus suggesting a single class of binding sites. However, the density of binding sites was lower in the hypothalamus (49.8 ± 0.5 pmol g^{-1} protein) than in the cortex (84.9 ± 1.1 pmol g^{-1} protein). These values for binding densities are generally lower than those obtained recently with a variety of [^{3}H]-sulphonylureas. For example, with [^{3}H]-glipizide in rat cerebral cortex the density was 110 pmol g^{-1} protein (Lupo & Bataille, 1987); [^{3}H]-glibenclamide in rat brain microsomes, 140 pmol g^{-1} protein (Mourre *et al.*, 1989), although values as low as 30 pmol g^{-1} protein have been obtained for [^{3}H]-glibenclamide binding to rat cerebral cortex membranes (Geisen *et al.*, 1985). For comparison, values of 150 pmol g^{-1} protein (Schmid-Antomarchi *et al.*, 1987) and >300 pmol g^{-1} protein (Geisen *et al.*, 1985) have been found for rat insulinoma microsomes and β-cell tumour membranes, respectively. The binding of [^{3}H]-glibenclamide to rat cortical membranes was inhibited by unlabelled glibenclamide (e.g. Figure 3c) with a mean affinity constant of $2.78 \pm 0.25 \times 10^9\,M^{-1}$ ($n = 3$), similar to the value obtained from analysis of saturation curves. Therefore [^{3}H]-glibenclamide binding sites, with similar characteristics to those in cortex, are present in the hypothalamus but at a lower density. The inhibition of [^{3}H]-glibenclamide binding to cortex by tolbutamide is well established (e.g. Geisen *et al.*, 1985) and it is likely that tolbutamide would have a similar affinity for the sulphonylurea binding site in the hypothalamus.

Actions of sulphonylureas on intact VMHN cells

It is well documented that the sulphonylurea receptors are functionally coupled to ATP-K^+ channels in isolated patches of insulin-secreting cells (Sturgess *et al.*, 1985; Trube *et al.*, 1986). The binding data described above, coupled with the evidence that the cortical sulphonylurea receptors appear very similar to those of pancreatic β-cells, make it surprising that tolbutamide and glibenclamide have no effect on this channel in inside-out membrane patches from neurones. One possibility is that the receptor and ATP-K^+ channel are functionally uncoupled in central neurones. However, recently it has been demonstrated (Mourre *et al.*, 1989; Grigg & Andersen, 1989; Ben-Ari, 1990) that high (1 μM) concentrations of glibenclamide prevented anoxia-induced hyperpolarization of hippocampal neurones (an effect involving inhibition of ATP-K^+ channels activated by the anoxic stimulus). This would suggest that some degree of functional coupling must exist.

Therefore we decided to test the actions of the sulphonylureas tolbutamide and glibenclamide on intact cells. In order to determine whether these drugs produced any effect what-

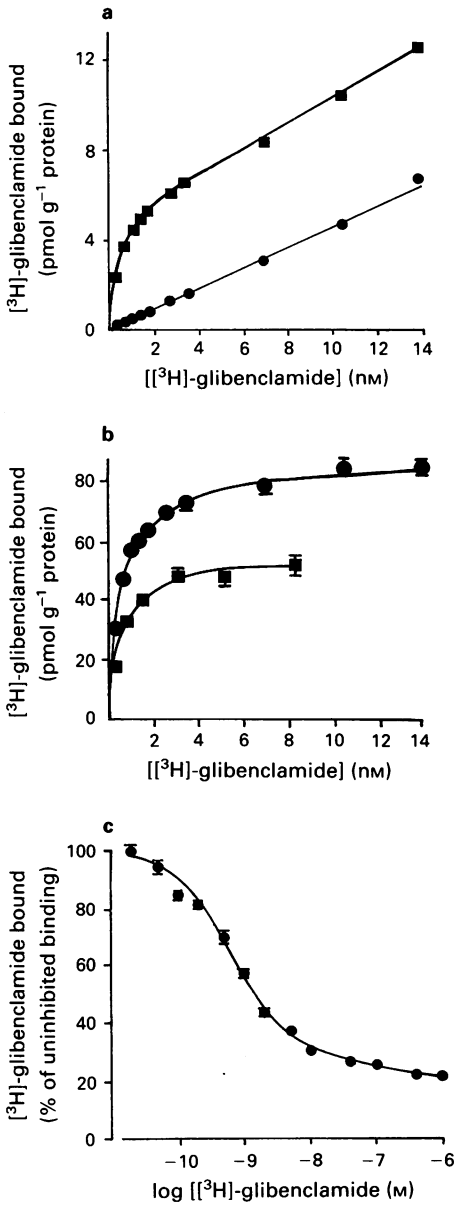

Figure 3 Binding of [^{3}H]-glibenclamide to cerebral cortex and hypothalamic membranes. (a) Saturable [^{3}H]-glibenclamide binding to rat cerebral cortex membranes: (■) total binding; (●) non-specific binding, insensitive to 1 μM glibenclamide. (b) Saturable [^{3}H]-glibenclamide binding to rat cerebral cortex (●) and rat hypothalamus (■). The curve for the cortical data is the difference between the two curves shown in (a). Note the similarity in the shape of the curves and the decreased density of binding sites in the hypothalamus compared to the cortex. (c) An experiment showing the displacement of [^{3}H]-glibenclamide binding to cortex membranes by unlabelled glibenclamide. Each point is the mean of 5 incubations.

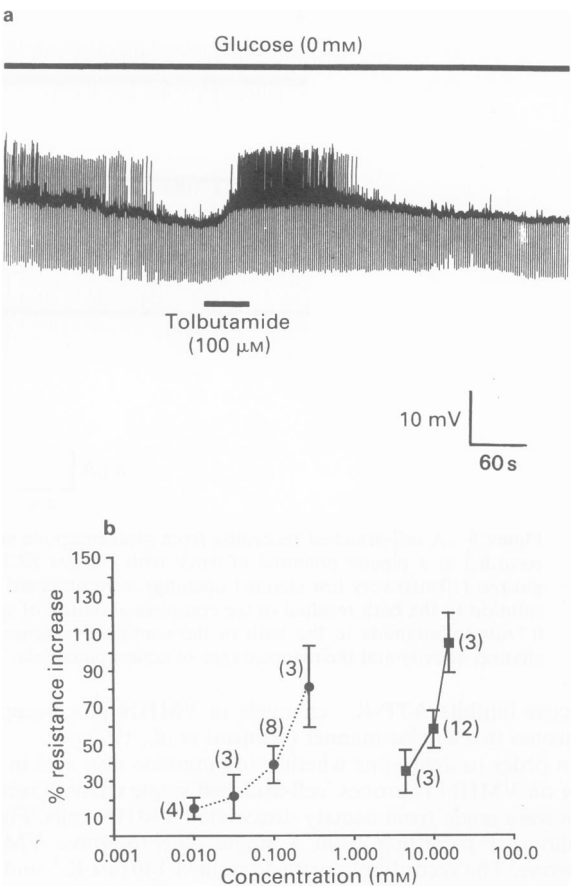

Figure 4 (a) Pen recording showing the effect of tolbutamide (100 μM) on a glucoreceptive neurone located within the ventromedial nucleus of a rat hypothalamic slice. Initial resting potential of the neurone was −62 mV. Removal of extracellular glucose from the perfusing ACSF led to membrane hyperpolarization accompanied by a fall in apparent input resistance as indicated by the reduction in size of electrotonic hyperpolarizing potentials (downward deflections). Upward deflections are action potentials, truncated by the limited frequency response of the pen recorder. Both spontaneous and anode-break action potentials were abolished in glucose-free ACSF. A one minute application of tolbutamide reversed the effect of glucose removal, depolarizing the neurone and leading to the return of action potential firing. Note also the apparent increase in input resistance produced by tolbutamide. All effects of the sulphonylurea were reversed on return to drug-free saline. (b) Summary of data obtained for the concentration-dependent effects of tolbutamide and glucose on the input resistance of glucose-sensitive ventromedial hypothalamic nucleus neurones. The ordinate scale gives the increase in input resistance produced by either tolbutamide (●) or glucose (■); the concentration is plotted on the abscissa scale. All the results were from neurones perfused in glucose-free ACSF but initial impalement and recording was performed in normal glucose. This ensured that only neurones hyperpolarized by glucose removal (i.e. glucoreceptive) were used in the study. The data points show the mean and vertical lines indicate s.e.mean (figures in parentheses are the number of neurones used for each point). Only one concentration of drug was used per neurone.

soever, intracellular recordings from glucoreceptive VMHN neurones in hypothalamic slices were made under current-clamp. Before the application of drug, removal of glucose from the superfusing medium allowed unequivocal identification of the cell, by the decreased membrane resistance, hyperpolarization and the cessation of spontaneous action potential activity (Figure 4a) similar to that found previously (Ashford *et al.*, 1990a). The cell remained quiescent and hyperpolarized throughout the period of exposure to glucose-free PSS. On application of tolbutamide (0.1 mM) to the glucose-free superfusate, the cell depolarized, spontaneous action potential activity returned and there was an increase in membrane resistance. These actions of tolbutamide were reversible on washing-out the drug with glucose-free PSS. Further exposures to the same concentration of tolbutamide produced similar actions. The effects of tolbutamide were concentration-dependent; this is illustrated in Figure 4b, where the increase in membrane resistance elicited by tolbutamide (0.01–0.3 mM) is compared to that of excitatory concentrations (5–20 mM) of glucose. Thus tolbutamide acts on VMHN glucoreceptive cells in a manner identical to that of glucose (Ono *et al.*, 1982; Minami *et al.*, 1986; Ashford *et al.*, 1990a) on these cells and of glucose (Atwater *et al.*, 1978) or sulphonylureas (Ferrer *et al.*, 1984) on pancreatic β-cells. Previous studies have clearly shown that both glucose and tolbutamide inhibit the activity of ATP-K$^+$ channels in pancreatic β-cells (Ashcroft, 1988; Ashford, 1990) and this accounts for their excitatory effects. Recently, we have demonstrated that

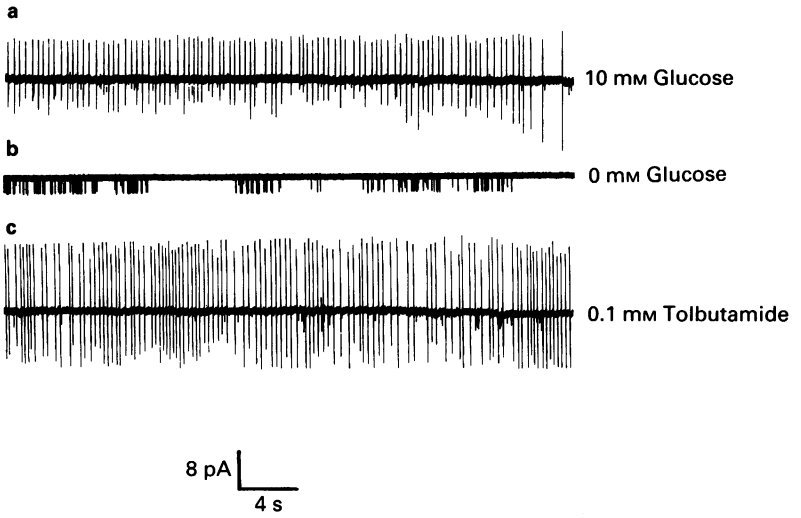

Figure 5 A cell-attached recording from glucoreceptive ventromedial hypothalamic nucleus neurone. Single channel currents were recorded at a pipette potential of 0 mV with 140 mM KCl in the electrode solution, normal saline in the bath. In the presence of glucose (10 mM) very few channel openings were observed and the cell fired action potentials regularly. Application of glucose-free solution to the bath resulted in the complete abolition of spiking activity and the appearance of single channel currents. Addition of 0.1 mM tolbutamide to the bath in the continued absence of glucose produced (after approximately 2 min) complete abolition of channel activity and the reappearance of action potentials. The current records in (a)–(c) were filtered at 0.2 kHz.

glucose inhibits ATP-K^+ channels in VMHN glucoreceptive neurones in a similar manner (Ashford *et al.*, 1990a).

In order to determine whether tolbutamide also acts in this way on VMHN neurones, cell-attached single channel recordings were made from acutely dissociated VMHN cells. Figure 5 shows a recording from a single glucoreceptive VMHN neurone. The recording pipette contained 140 mM K^+ and the pipette potential was zero mV. In the presence of normal bath saline plus 10 mM glucose (Figure 5a) the cell can be observed to spike fairly regularly (seen as extracellularly recorded action currents). Superfusion of the cell with glucose-free saline produced an inhibition of action current activity, usually within 2–3 min. Under these conditions, single channel currents of very short duration (< 10 ms) could, on some occasions, be observed (Figure 5b). Addition of 0.1 mM tolbutamide to the bathing solution (Figure 5c), resulted in the inhibition of this channel activity, when present, and was followed by the re-appearance of action currents ($n = 7$). These data therefore complement the current-clamp slice data and clearly indicate that tolbutamide is capable of exciting glucoreceptive cells.

In order to determine the identity of the channel observed in the above experiments further cell-attached recordings were performed. In these experiments, the cells were kept in normal PSS with no added glucose for approximately 30 min prior to recording, in an attempt to induce a significant level of resting ATP-K^+ channel activity. Figure 6 illustrates a typical cell-attached recording from a VMHN cell under these conditions,

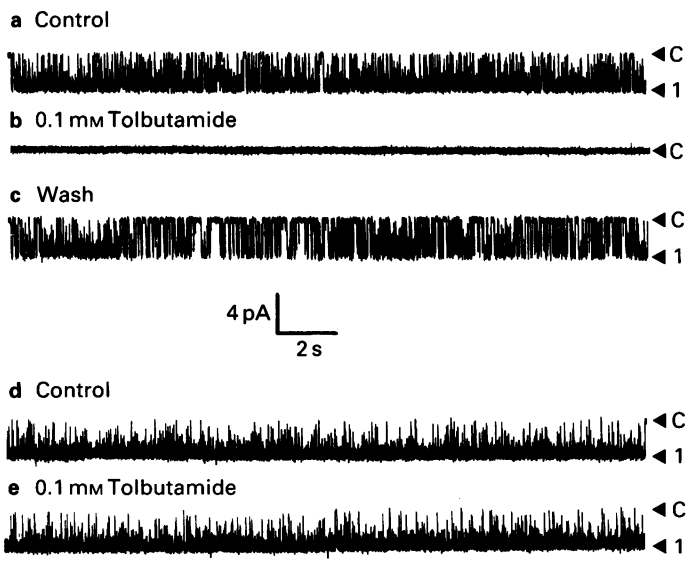

Figure 6 A cell-attached recording from a glucoreceptive ventromedial hypothalamic nucleus neurone. In this experiment the cells had been bathed in zero glucose (for at least 30 min) before the recording. Single channel currents were recorded at a pipette potential of 0 mV in (a)–(c). The electrode contained 140 mM KCl and normal PSS was present in the bath. In (a) under control conditions in the absence of glucose, a single active ATP-sensitive potassium channel can be seen, which was later identified by its conductance and lack of voltage-dependence. Application of 0.1 mM tolbutamide resulted in the cessation of the channel activity and this effect was reversible upon washout of the tolbutamide. Action current activity was not observed on this occasion in the presence of tolbutamide, this was often observed if the cell was exposed to zero glucose for long periods. The P_{open} values were as follows (a) control 0.723; (b) tolbutamide 0.000; (c) wash 0.694. (d) and (e) Recordings from the same membrane patch but after excision from the cell. Under these conditions 0.1 mM tolbutamide produced no inhibition of channel activity. Note also the difference in kinetics between the activity of the ATP-K^+ channel in cell attached conditions and in inside-out conditions. Membrane potential was 0 mV in traces (d) and (e).

clearly showing channel activity with a high level of open-state probability (Figure 6a; see also Figure 2a in Ashford *et al.*, 1990a). Addition of 0.1 mM tolbutamide induced complete closure of this channel, an effect reversible on wash (Figure 6b,c). However, a delay of 1–2 min was usually observed before the sudden termination of channel activity (an action not compatible simply with the superfusion rate, since fluid exchange was less than 30 s). Similar effects were observed on four further patches. Following the reversal of tolbutamide inhibition (1–2 min after the initiation of wash), the membrane patch was excised into the normal saline bathing the cells and an inside-out patch configuration obtained. Under these asymmetric K^+ ion conditions, channel openings are observed as downward deflections (as K^+ leaves the electrode) and it can be seen that the K^+ channel kinetics are quite distinct from that recorded cell-attached (Figure 6d). This reflects the excision of the patch into an ATP-free solution. Application of 0.1 mM tolbutamide to the solution bathing the internal surface of the membrane patch had no effect on channel activity (Figure 6e), even after prolonged contact time (5 min), in agreement with previous data from isolated patches. In a separate experiment (with identical recording conditions to that used in the experiments shown in Figures 5 and 6), the single channel activity recorded cell-attached was completely inhibited by 0.1 mM tolbutamide and the electrode then lifted off the cell (to form an inside-out patch) into the tolbutamide-containing normal external PSS. Channel activity was observed to recover slowly and only after approximately 1 min to regain a high value of P_{open} (Figure 7). Following this recovery, the bath solution was exchanged for an intracellular solution (which resulted in symmetrical 140 mM KCl conditions) and the active channel was found to have a conductance of 142 pS (compare with mean value of 146 pS; Ashford *et al.*, 1990a) and to be inhibited by ATP (data not shown). In this experiment, 3 mM ATP

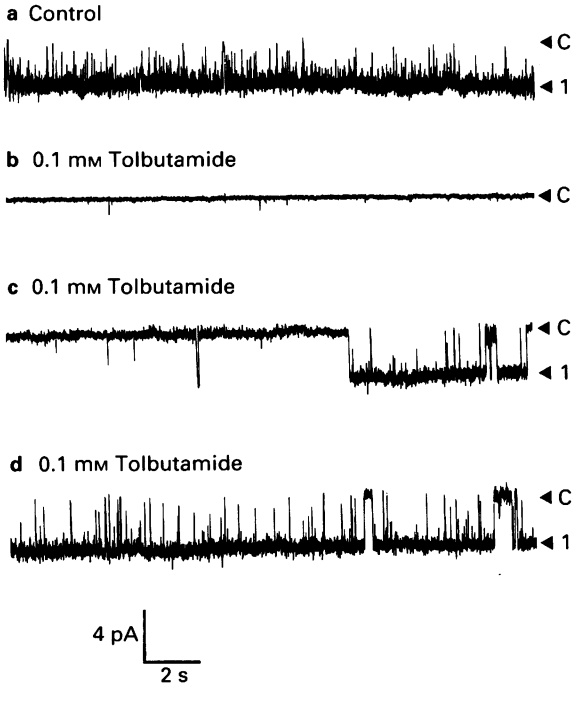

Figure 7 Cell-attached recording from a glucoreceptive ventromedial hypothalamic neurone. The electrode solution was 140 mM KCl, normal PSS was present in the bath. In (a) under control conditions, a single active ATP-K^+ channel can be seen. Application of 0.1 mM tolbutamide (b) produced complete inhibition of channel activity following a 1 min delay after the addition of the drug. In this experiment the electrode was lifted off the cell to form an inside-out patch (c, d) whilst tolbutamide was present in the bath solution, and channel activity returned after a delay (~20 s). Note again the difference in kinetics between the cell-attached recording (a) and the inside-out recording (b).

reduced the P_{open} from a control value of 0.694 to 0.341, but similar results were obtained from two other cells. These data clearly show that tolbutamide (0.1 mM) does reversibly inhibit the ATP-K^+ channel, but only under conditions where the cell is intact.

In contrast to the depolarizing action of tolbutamide on glucoreceptor neurones in VMHN slices, addition of glibenclamide (10–500 nM, $n = 5$) had no effect. This is illustrated in Figure 8a where, after the cell had hyperpolarized and stopped firing in the zero-glucose PSS, application of 100 nM glibenclamide produced no change in input resistance and no significant depolarization (compare with Figure 4 for tolbutamide). In five experiments the mean depolarization obtained with 100 nM glibenclamide was 2.1 ± 2.04 mV with a mean resistance decrease of 7.15 ± 6.14%. However, following glibenclamide treatment, the cell no longer responded to tolbutamide (even after prolonged washing). Figure 8b, clearly shows the effects of the two sulphonylureas on a VMHN glucoreceptive neurone in normal glucose medium. The addition of tolbutamide (0.1 mM) depolarized the cell and induced an increase in action potentials, effects which were both reversible on wash-out of drug and reproducible on re-application of tolbutamide. Glibenclamide (50 nM) had no effect *per se* but blocked the excitatory action of a further application of tolbutamide (0.1 mM).

Discussion

Sulphonylurea receptors (identified as high-affinity binding sites for [^{3}H]-glibenclamide) present in mammalian pancreatic β-cells, cardiac muscle cells and central neurones have similar binding characteristics. The affinity constants for [^{3}H]-glibenclamide are comparable between tissues and similar to the values obtained for the cortex and hypothalamus in the present study. In addition, sulphonylureas displace [^{3}H]-glibenclamide with the same relative potency in all tissues and this also correlates well with their relative hypoglycaemic activity. Furthermore, the sulphonylureas inhibit the activity of ATP-K^+ channels in excised patches from insulin-secreting cells (Ashford, 1990) and heart cells (Belles *et al.*, 1987). These observations have led to the notion that the sulphonylurea receptor and the ATP-K^+ channel are a single entity or, at least, very closely linked (de Weille *et al.*, 1989). This is further supported by the good correlation between the rank order of potency for sulphonylureas to block ATP-sensitive Rb^+ efflux from RINm5F insulinoma cells and their ability to displace [^{3}H]-glibenclamide from receptor sites on the same cells (Schmid-Antomarchi *et al.*, 1987). Indeed, the specificity of glibenclamide for ATP-K^+ channels (no effects have been demonstrated on other K^+ channels; Ashford, 1990) has led to its use as a probe for these channels. For example, glibenclamide-induced block of vasorelaxation and increased $^{86}Rb^+$ efflux of vascular smooth muscle by K^+ channel openers has been taken as strong evidence for the presence of ATP-K^+ channels. This has recently been substantiated for rabbit mesenteric artery at the single channel level, where ATP has been shown to abolish K^+ channel activity, cromakalim to restore activity and glibenclamide to induce block (Standen *et al.*, 1989). Similar arguments have been used to explain sulphonylurea-sensitive $^{42}K^+$ or ^{86}Rb efflux from metabolically exhausted frog skeletal muscle (Castle & Haylett, 1987) and tolbutamide block of a K^+ conductance activated by cromakalim, pinacidil and RP 49356 in human skeletal muscle fibres (Quasthoff *et al.*, 1989; Spuler *et al.*, 1989).

Recently, purification of the [^{3}H]-glibenclamide binding site from pig brain and direct photoaffinity labelling of brain and insulinoma cells membranes have indicated that the binding site is a single polypeptide chain of 140–150 kDa. It has been suggested that this polypeptide incorporates the ATP-K^+ channel (Bernardi *et al.*, 1988; Kramer *et al.*, 1988; de Weille *et al.*, 1989). This proposal may, however, be rather

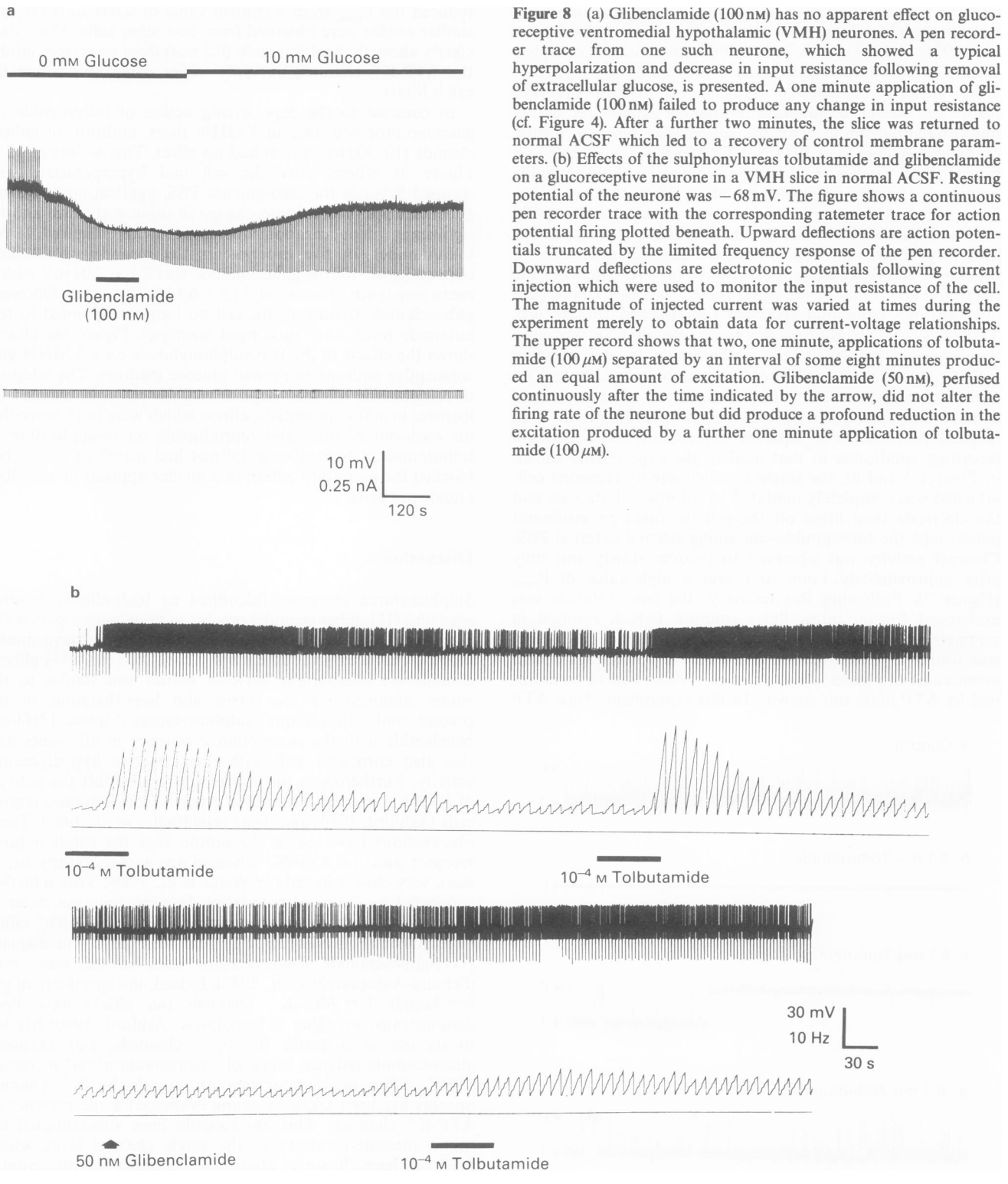

Figure 8 (a) Glibenclamide (100 nM) has no apparent effect on glucoreceptive ventromedial hypothalamic (VMH) neurones. A pen recorder trace from one such neurone, which showed a typical hyperpolarization and decrease in input resistance following removal of extracellular glucose, is presented. A one minute application of glibenclamide (100 nM) failed to produce any change in input resistance (cf. Figure 4). After a further two minutes, the slice was returned to normal ACSF which led to a recovery of control membrane parameters. (b) Effects of the sulphonylureas tolbutamide and glibenclamide on a glucoreceptive neurone in a VMH slice in normal ACSF. Resting potential of the neurone was −68 mV. The figure shows a continuous pen recorder trace with the corresponding ratemeter trace for action potential firing plotted beneath. Upward deflections are action potentials truncated by the limited frequency response of the pen recorder. Downward deflections are electrotonic potentials following current injection which were used to monitor the input resistance of the cell. The magnitude of injected current was varied at times during the experiment merely to obtain data for current-voltage relationships. The upper record shows that two, one minute, applications of tolbutamide (100 μM) separated by an interval of some eight minutes produced an equal amount of excitation. Glibenclamide (50 nM), perfused continuously after the time indicated by the arrow, did not alter the firing rate of the neurone but did produce a profound reduction in the excitation produced by a further one minute application of tolbutamide (100 μM).

presumptive, in particular for neuronal membranes. It is clear from the isolated membrane patch data presented here that the sulphonylurea receptor and the ATP-K^+ channel are not directly linked in either the cortical or ventromedial hypothalamic neurones. Current-clamp and cell-attached recordings from identified (glucoreceptive) ventromedial hypothalamic neurones do indicate that the sulphonylurea receptor and ATP-K^+ channel are functionally linked in intact cells, and that on patch excision this coupling is lost. Because of the paucity of ATP-K^+ channels in the cultured cortical cells and the difficulty in identifying individual cell types we cannot be certain that functional coupling does occur in these cells, as no cell-attached recordings of ATP-K^+ channels were obtained. However, it does seem a reasonable assumption given the similarities of the ATP-K^+ channels and sulphonylurea binding sites between cerebral cortex and hypothalamus.

Hence, despite the biochemical similarity between the sulphonylurea binding sites in these two brain areas and insulin-secreting cells the interaction between tolbutamide and the ATP-K^+ channel differs. In insulin-secreting cells, tolbutamide blocks ATP-K^+ channel activity in inside-out membrane patches (i.e. applied to cytoplasmic side of membrane), suggesting a close association between the sulphonylurea

receptor and the channel (Sturgess *et al.*, 1985; 1988; Trube *et al.*, 1986), whereas for VMHN neurones (and probably cortical neurones) some factor which confers a functional link between the receptor and channel is not present, or is inhibited, in inside-out membrane patches. Alternatively, a structural difference in the ATP-K$^+$ channel of neurones (different amino acid sequence?) underlies this altered sensitivity to sulphonylureas. Such a proposition is strengthened by the different conductance, rectifying properties and ATP-sensitivity of the channel in neurones compared to insulin secretory cells. It is unlikely that the sulphonylurea receptor is accessed only from the extracellular side of the membrane patch, as ATP-K$^+$ channel inhibition readily occurred in the cell-attached recording configuration with tolbutamide present in the bath solution only (i.e. not electrode) and the tight glass-membrane seal is known to be a diffusion barrier to hydrophilic molecules (Sakmann & Neher, 1984). In addition, Zunkler *et al.* (1989), on studying the pH-dependence and time course of sulphonylurea-induced block of ATP-K$^+$ currents in mouse pancreatic β-cells, have concluded that it is the undissociated forms of the drugs which are effective and it is likely that the sulphonylureas gain access to their binding site on the receptor via the lipid phase of the membrane. Furthermore, there is evidence to suggest that the coupling between the sulphonylurea receptor and ATP-K$^+$ channel in cardiac cells may also be labile. Belles *et al.* (1987) showed that tolbutamide has a slow onset of action during whole-cell recordings (in agreement with the delay observed for ATP-K$^+$ channel inhibition in the cell-attached recordings from VMHN neurones presented here) and that about 30% of the isolated patches were not affected by 2 mM tolbutamide. These authors also suggested that some unknown link between the sulphonylurea receptor and ATP-K$^+$ channel was missing in some of the isolated patches. Perhaps the sulphonylurea receptor-ATP-K$^+$ channel linkage exhibits tissue-dependence, with the lability of coupling being in the order of pancreatic β-cells < cardiac cells < central neurones (i.e. reverse of the order for ATP-sensitivity).

A reduction in the effectiveness of tolbutamide at inhibiting ATP-K$^+$ channel activity in inside-out patches compared to whole-cell currents has been demonstrated for mouse (Zunkler *et al.*, 1988) and human (Ashcroft *et al.*, 1989) pancreatic β-cells. It appears likely that this discrepancy is due to the absence of cytosolic ADP in the excised patch, as Zunkler *et al.* (1988) have shown that this nucleotide enhances the channel sensitivity to tolbutamide. Although we have yet to determine the possible modulatory effect of cytosolic ADP on VMHN neurones, it is unlikely that this would account for the total lack of action of 10 mM tolbutamide on inside-out membrane patches. A further indication of the different nature of the link between the sulphonylurea receptor and ATP-K$^+$ channel in neurones compared to β-cells, heart cells and smooth muscle cells is the total lack of inhibition by glibenclamide, not only on excised inside-out membrane patches, but also on intact-cell current-clamp recordings. This latter observation apparently contrasts with data showing that glibenclamide can reverse hyperpolarization in hippocampal cells (Mourre *et al.*, 1989; Grigg & Anderson, 1989; Ben-Ari, 1990). However, these results are not compatible with the data presented here for the following reasons: different brain regions were employed for the slice preparation; the concentrations of glibenclamide used were different; and anoxia was induced by replacing O_2 with N_2 in the ACSF without changing glucose. Furthermore, there is no direct evidence that glibenclamide is acting on ATP-K$^+$ channels. In fact, Ben-Ari (1990) suggests that the anoxic hyperpolarization is due to an activation of calcium-activated K$^+$ channels and not ATP-K$^+$ channels, which are located presynaptically and mediate glutamate release. This lack of inhibition *per se* cannot simply result from insufficient receptor occupancy, as a very low (50 nM) concentration of glibenclamide prevented the excitatory actions of tolbutamide on intact cells. This in fact supports the argument that high-affinity sulphonylurea binding sites are present on these glucoreceptive cells. Thus a 'second generation' sulphonylurea (glibenclamide) acts as an 'antagonist' to the excitation induced by a 'first generation' drug (tolbutamide).

One explanation for the inhibition of ATP-K$^+$ channels on intact cells and not inside-out patches is that tolbutamide stimulates glucose metabolism resulting in increased levels of intracellular ATP. Such an action has been obtained for tolbutamide in normal (Kramer *et al.*, 1983) and diabetic (Tan *et al.*, 1984) rat heart. However, in the present experiments we do not believe, for several reasons, that tolbutamide acts in this way. Firstly, it has been shown that sulphonylureas decrease the ATP content of β-cells (Ashcroft *et al.*, 1973; Kawazu *et al.*, 1980). Secondly tolbutamide depolarizes the VMHN neurones in the absence of glucose and inhibits ATP-K$^+$ channels cell-attached after 30 min exposure to zero glucose (Figure 6). Thirdly, the effect of tolbutamide is 'antagonized' by low concentrations of glibenclamide.

In conclusion, we have shown that ATP-K$^+$ channels present in mammalian central neurones can be inhibited by tolbutamide in cell-attached recordings (but not inside-out patches) and this results in depolarization of glucoreceptive VMHN neurones. Furthermore, glibenclamide (at concentrations which inhibit β-cell ATP-K$^+$ channels) does not depolarize these cells, but does block the action of tolbutamide. Hence, either the CNS sulphonylurea receptors are not functionally identical to those of pancreatic β-cells or heart cells, or the CNS ATP-K$^+$ channel is markedly different in its coupling to these receptors.

We thank Professor C.N. Hales for discussion and comments on the manuscript. This work was supported by the Science and Engineering Research Council.

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(Received February 19, 1990
Revised June 25, 1990
Accepted July 12, 1990)

Br. J. Pharmacol. (1990), **101**, 541–544

Induction of non-specific airway hyperreactivity by potassium channel blockade in rat isolated trachea

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1 The exposure of rat isolated tracheal segment to the K^+-channel inhibitor tetraethylammonium (TEA, 10 mM) for a period of 10–15 min generally produced little or no contractile response.

2 Cooling (10°C) provocation alone usually produced small (10 ± 3% acetylcholine maximum) contractile responses.

3 In the presence of TEA (10 mM, 10–15 min exposure), rat trachea exhibited airway hyperreactivity to acetylcholine, 5-hydroxytryptamine (5-HT) and cooling. It also increased the peak tension induced by 5-HT.

4 TEA-induced airway hyperreactivity to cooling was signficantly inhibited in Ca^{2+}-free Krebs solution suggesting an important role for extracellular Ca^{2+} influx.

5 We conclude that the blockade of potassium channels with TEA induces non-specific airway hyperreactivity to cooling, 5-HT and acetylcholine in rat isolated tracheal segments.

Introduction

Non-specific airway hyperreactivity (AH) to a variety of pharmacological and physical stimuli such as histamine, methacholine, KCl, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and exercise is a hallmark of asthma (Bleeker, 1986; Dixon *et al.*, 1989). There are at least two hypotheses (cooling and/or drying of the airways and transient increase in the osmolarity/osmolality of tracheal mucosal fluid) which have been put forward to explain the underlying cause of exercise-induced asthma (Anderson, 1984). We have recently developed an *in vitro* model of AH to cooling in rat trachea, in which cooling (10°C) itself causes weak (5–10% acetylcholine maximum) contractile responses. However, tracheal segments exposed to threshold/subthreshold concentrations of potassium chloride, chemical mediators such as platelet activating factor (PAF), acetylcholine, adenosine, phospholipase A_2, and phospholipase C, or following recovery from allergic responses, exhibit marked AH to cooling (Chand *et al.*, 1986; 1987; 1988). In the present study we now demonstrate that tetraethylammonium (TEA; a potassium channel inhibitor; a depolarizing agent) induces non-specific airway hyperreactivity to acetylcholine, 5-hydroxytryptamine (5-HT) and cooling in rat trachea.

Methods

Adult male Sprague-Dawley rats, weighing between 241–689 g were killed by CO_2 exposure. A terminal segment of the trachea, 8 to 10 mm long, was dissected from each rat and kept in warm Krebs-Henseleit solution (37°C). Each tissue was cut into two equal segments and set up 'in pairs' in isolated tissue baths containing Krebs-Henseleit solution, maintained at 37°C and gassed with a mixture of 95% O_2 and 5% CO_2. The composition of Krebs-Henseleit solution was (in mM): NaCl 118, KCl 4.7, $CaCl_2 \cdot 2H_2O$ 2.5, $MgSO_4 \cdot 7H_2O$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25.0 and glucose 10.0 (pH 7.4). The tracheal segments were attached to force-displacement transducers (Grass type FT.03C) by two L-shaped stainless steel hooks. Tissues were allowed to equilibrate for 1 to 3 h with washings at 30–60 min intervals. An optimal resting force of about 1 g was readjusted and maintained during the stabilization period.

Following the equilibration period, tissues were exposed to tetraethylammonium (TEA, 10 mM) for 10–15 min and the circulating water in the outer jackets of the isolated tissue baths was switched to a cold water supply (10°C, refrigerated constant temperature circulator, Polyscience Series 9000). The cold-induced contractile responses were recorded isometrically for a period of 15 to 30 min or until a plateau was established. The circulation of heated (37°C) water in the outer jacket of the tissue baths produced immediate relaxation of cold-induced contractions. Fifteen min later, the resting force (if lowered beyond resting level) was readjusted and then tissues were exposed to acetylcholine (ACh, 10^{-3} M) to determine contractility (peak developed tension) in mg. This response was termed the ACh maximum contractile response (ACh max.). The cold-induced responses were expressed as % of ACh max. About 20% of the tracheal segments exhibited contractions to TEA and were not included in this study.

Role of calcium in TEA-induced airway hyperreactivity

After the equilibration period in normal Krebs solution, one tracheal segment of each pair was incubated in Ca^{2+}-free Krebs solution for a period of 120 min with two or three washes. The second tissue was maintained in normal (Ca^{2+}-containing) Krebs solution. Both tissues were exposed to TEA (10 mM) and then to cold (10°C) by changing circulation fluid in the outer jacket of the tissue baths. Fifteen min later, tissues were exposed to ACh (10^{-3} M) at 37°C. The cold responses were expressed as % of ACh max. and compared by paired *t* test.

Induction of non-specific airway hyperreactivity to acetylcholine and 5-HT

After the equilibration period one tissue of each pair was exposed to TEA (10 mM) and another received vehicle (Krebs solution 0.1 ml) for a period of 15 min. The cumulative concentration-effect curves to either ACh or 5-HT were established with effect expressed in mg and also as a % of control ACh max. responses and compared by paired *t* test. The EC_{50}s (μM), as a measure of sensitivity, were calculated from the linear part of the concentration-effect curves; the 95% confidence limits were also determined and compared by the Bliss method (Bliss, 1967).

Drugs and statistics

Tetraethylammonium chloride (TEA), acetylcholine chloride (ACh) and 5-hydroxytryptamine creatinine sulphate (5-HT) were obtained from Sigma Chemical Co., St. Louis, MO,

U.S.A. These drugs were dissolved in normal Krebs solution immediately before use. Drug concentration is expressed as that occurring in the bath fluid.

Statistical significance was determined by comparing cold-induced responses (% ACh max.) as well as peak developed tension (mg) in control (vehicle)-treated and TEA-treated segments by Student's t test for paired observations. Statistical significance was indicated by a P value of 0.05 or less.

Results

The exposure of rat tracheal segments to TEA (10 mM) generally produced little (50–200 mg) or no contractile response in about 80% of the preparations. However, this treatment produced airway hyperreactivity to ACh and 5-HT (Figures 1 and 2; Table 1). In the case of ACh, TEA produced a significant change in sensitivity (a six fold decline in EC_{50}). With 5-HT, it increased both the sensitivity (a seven fold decline of EC_{50}) as well as increasing the peak tension developed to 238% of the control value (Table 1).

Rat isolated tracheal segments responded with poor contractile responses (10 ± 3% of ACh max.) to cold provocation. However, cold provocation produced strong and rapid contractile responses (35.3 ± 4.2% ACh max.; an increase to 264% of the control) in segments exposed to TEA for a period of 10–15 min (Figures 3 and 4).

The exposure of rat tracheal segments to Ca^{2+}-free Krebs solution for a period of two hours produced a significant inhibition of TEA-induced airway hyperreactivity as well as peak developed tension to acetylcholine (Figure 5).

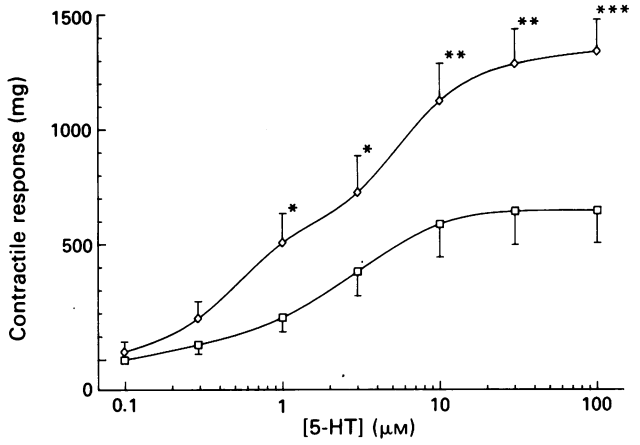

Figure 1 Cumulative concentration-effect curves for 5-hydroxytryptamine (5-HT) in the presence (◇) and absence (□) of tetraethylammonium (TEA, 10 mM, 15–30 min exposure) in rat isolated tracheal segments. Each point represents the mean of values from 12 tissues; vertical lines show s.e.mean. Asterisks indicate significant difference (* $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$) compared with corresponding response in the absence of TEA.

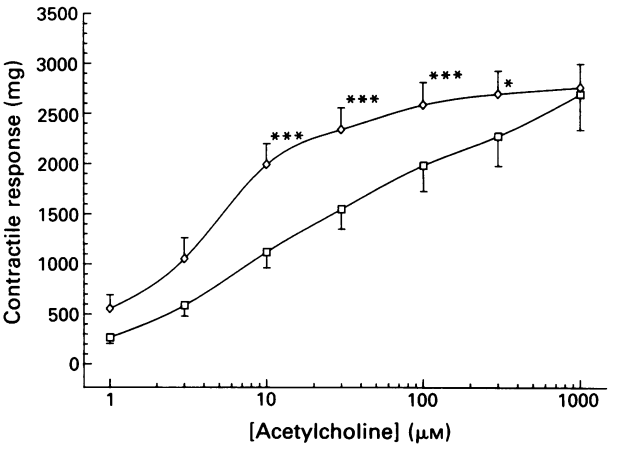

Figure 2 Cumulative concentration-response curves for acetylcholine in the presence (◇) and absence (□) of tetraethylammonium (TEA, 10 mM, 15–30 min exposure) in rat isolated tracheal segments. Each point represents the mean of values from 8 tissues; vertical lines show s.e.mean. Asterisks indicate significant difference (* $P < 0.05$ and ** $P < 0.01$) compared with corresponding response in the absence of TEA.

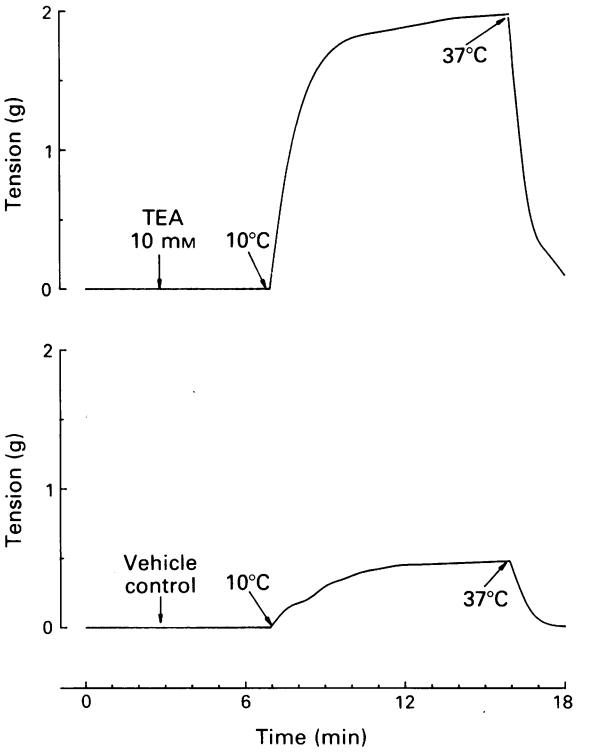

Figure 3 Typical tracings of tetraethylammonium (TEA)-induced airway hyperreactivity to cooling (10°C) in rat tracheal segments.

Table 1 Effect of tetraethylammonium (TEA) on sensitivity and contractility to acetylcholine and 5-hydroxytryptamine (5-HT) in rat isolated tracheal segments

	Agonist			
	Acetylcholine (n = 8)		*5-HT* (n = 12)	
Pretreatment	*Sensitivity* EC_{50} (μM)	*Peak developed tension* (mg)	*Sensitivity* EC_{50} (μM)	*Peak developed tension* (mg)
Krebs solution 0.1 ml	19.5 (16.9–22.6) r = 0.99; slope = 31	2693 ± 357	2.7 (2.1–3.3) r = 0.96; slope = 36	648 ± 141
TEA, 10 mM (15–30 min)	3.4 (1.8–6.4)† r = 0.97; slope = 53	2760 ± 238	0.36 (0.13–0.99)† r = 0.97; slope = 124	1341 ± 138***

Values are means ± s.e.mean.
† $P < 0.05$ as compared to respective controls (Bliss method).
*** $P < 0.001$ as compared to respective controls (paired t test).

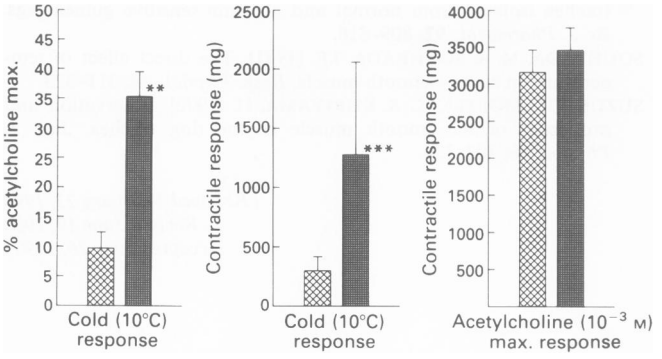

Figure 4 Induction of airway hyperreactivity to cooling provocation (10°C) by tetraethylammonium (TEA, 10 mM, 10–15 min exposure) in rat isolated tracheal segments. Values are means and bars show s.e.mean ($n = 12$). ** $P < 0.01$, *** $P < 0.001$. (▩) Normal Krebs solution, (■) Krebs solution containing TEA (10 mM).

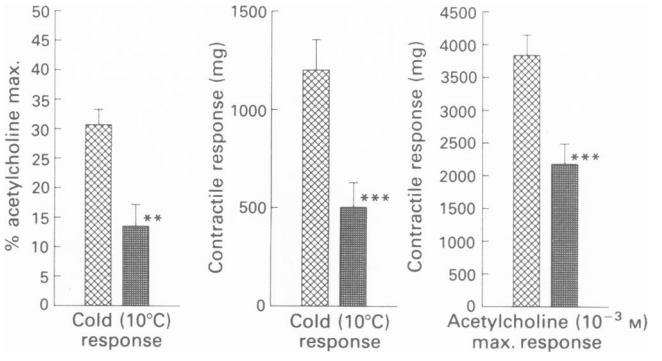

Figure 5 Inhibition of tetraethylammonium (TEA)-induced airway hyperreactivity to cooling and acetylcholine max. (contractility) in Ca^{2+}-free Krebs solution (2 h exposure). All responses to cooling were obtained after 15 min treatment with, and in the presence of, 10 mM TEA. Values are means and bars show s.e.mean ($n = 12$). ** $P < 0.01$, *** $P < 0.001$. (▩) Normal Krebs solution, (■) Ca^{2+}-free Krebs solution.

Discussion

The data obtained in this study clearly demonstrate that the potassium channel inhibitor, TEA, induces non-specific airway hyperreactivity to cooling, ACh and 5-HT in rat tracheal segments. Antigen- and phospholipase A_2 (Chand *et al.*, 1987; 1988)- and TEA (this study)- induced airway hyperreactivity to cooling in this tissue is predominantly mediated via the influx of extracellular Ca^{2+}. The maximum response to ACh (contractility; peak developed tension) utilized 40% intracellular calcium (Figure 5).

TEA increased sensitivity to 5-HT and ACh and also increased peak tension (contractility) to 5-HT. The exact explanation for this differential effect of TEA on maximal responses to ACh and 5-HT in rat trachea is not clear. However, it should be noted that 5-HT exerts only ~25% of the intrinsic activity (contractility or peak tension) of ACh, a full agonist in rat trachea.

Airway hyperresponsiveness is defined as an exaggerated airway narrowing in response to a wide variety of stimuli such as exercise, methacholine, $PGF_{2\alpha}$, histamine, fog, distilled water etc., that have little or no effect in normal subjects. The exact underlying mechanism of bronchial hyperresponsiveness is not yet known. However, bronchial inflammation or inflammatory mediators may exert a priming effect (depolarization) on airway smooth muscle and thus produce a synergistic or exaggerated response to a combination of two or more stimuli, which are individually weak or inert in normal subjects. The data obtained in this study suggest that airway depolarization by TEA simulates a situation in asthmatics, i.e., stimuli (e.g. 5-HT and cooling) with weak activity induce marked constriction after potassium channel blockade by TEA. In guinea-pig trachealis, TEA (0.1–10 mM) failed to produce any leftward shift in the concentration-effect curves for ACh, histamine, KCl and PGE_2 (Boyle *et al.*, 1988). Therefore, due to species-related variations, caution must be exercised when extrapolating results of *in vitro* studies to a disease state(s) in man.

Rat tracheal segments generally did not contract in response to TEA. However, TEA induced slow waves of contraction, occurring at 5–10 min intervals, or produced a sustained contractile response (500–1000 g) in about 20% of the tracheal segments examined. Such tissues were extremely hyperresponsive to low concentrations of ACh (10^{-7}–10^{-6} M). TEA has been shown to depolarize and to induce action potentials and contractions in guinea-pig, bovine and canine trachealis (Kirkpatrick, 1975; Kroeger & Stephens, 1975; Suzuki *et al.*, 1976; Foster *et al.*, 1983). Repeated antigen challenge (repeat sensitization) has been shown shown to produce depolarization of guinea-pig trachealis (McCaig, 1987; Souhrada & Souhrada, 1981). These data and the findings of the present study suggest that the basic fundamental physiological defect in the induction of airway hyperreactivity/hyperresponsiveness in asthmatics may be depolarization (blockade of potassium channels) of the airway smooth muscle cells.

TEA, KCl, subthreshold concentrations of chemical mediators, added exogenously (ACh, 5-HT, PAF, adenosine, etc.) or released endogenously (neuropeptides, PAF, ACh, etc.) during repeated antigen challenge, could produce slight depolarization of airway smooth muscle cells and thus induce non-specific airway hyperresponsiveness, a cardinal sign of asthma (this study, Chand *et al.*, 1986; 1987; 1988; McCaig 1987).

The authors thank Mrs Carolyn Denham for secretarial assistance and Mr John Pillar for graphics. The technical assistance of R. Jakubicki is gratefully acknowledged.

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(Received February 22, 1990
Revised June 19, 1990
Accepted June 26, 1990)

Br. J. Pharmacol. (1990), 101, 545–552

Front-surface fluorometry with fura-2 and effects of nitroglycerin on cytosolic calcium concentrations and on tension in the coronary artery of the pig

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1 By use of front-surface fluorometry and fura-2-loaded strips of the coronary artery of the pig, the effects of nitroglycerin (NG) on cytosolic Ca^{2+} concentrations ($[Ca^{2+}]_i$) and on tension development were measured simultaneously.

2 Both high K^+ depolarization and histamine increased $[Ca^{2+}]_i$ and tension in a concentration-dependent manner. However, the tension development in relation to the $[Ca^{2+}]_i$ increase ($[Ca^{2+}]_i$-tension relation) observed with histamine was much greater than that observed with K^+ depolarization.

3 NG reduced in a concentration-dependent manner both $[Ca^{2+}]_i$ and tension, irrespective of whether the vascular strips were in a resting state or during exposure to high K^+ or to histamine stimulation. However, the extent of reduction in tension (relaxation) was greater than that expected from the reduction in $[Ca^{2+}]_i$ based on the $[Ca^{2+}]_i$-tension relationship observed with K^+-depolarization.

4 In the absence of extracellular Ca^{2+}, NG depleted stored Ca^{2+} and also inhibited Ca^{2+} release from histamine-sensitive stores, but had no effect on the caffeine-sensitive stores. NG inhibited the caffeine-induced tension development with no change in $[Ca^{2+}]_i$.

5 We suggest that NG relaxes the coronary artery of the pig by reducing $[Ca^{2+}]_i$ and also by directly controlling contractile elements through second messengers not related to changes in $[Ca^{2+}]_i$.

Introduction

Changes in cytosolic Ca^{2+} concentrations, $[Ca^{2+}]_i$, play a key role in the excitation-contraction coupling in vascular smooth muscle (Kuriyama *et al.*, 1982; Somlyo, 1985; Sommerville & Hartshorne, 1986, for review). However, unlike skeletal muscle, changes in force in relation to changes in $[Ca^{2+}]_i$ may vary depending on the types of stimulation in vascular smooth muscle (Nishimura *et al.*, 1989a for review). Nitroglycerin (NG) consistently induces relaxation and also inhibits the contraction evoked by various forms of stimulation of vascular smooth muscle. It is generally accepted that NG produces concentration- and time-dependent increases in guanosine 3':5'-cyclic monophosphate (cyclic GMP) levels which are associated temporally with relaxation (Ignarro & Kadowitz, 1987). NG and/or cyclic GMP-mediated systems stimulate sarcolemmal Ca^{2+} ATPase resulting in pump activation and an enhanced Ca^{2+} extrusion (Itoh *et al.*, 1983; 1985; Suematsu *et al.*, 1984; Popescu *et al.*, 1985a,b; Furukawa & Nakamura, 1987; Vrolix *et al.*, 1988), inhibit Ca^{2+} influx (Verhaeghe & Shepherd, 1976; Harder *et al.*, 1979), Ca^{2+} release from stores and increase in Ca^{2+} accumulation into the store (Ito *et al.*, 1980a,b; Imai & Kitazawa, 1981).

Although the above studies on Ca^{2+}-fluxes between anatomical compartments tends to support the notion that NG may decrease $[Ca^{2+}]_i$ in association with relaxation, definite evidence to support this hypothesis has not been obtained, mostly because of difficulties in measuring directly the levels of $[Ca^{2+}]_i$ during contraction and/or relaxation of vascular smooth muscle. In the present study, we have used front-surface fluorometry of the $[Ca^{2+}]_i$ indicator dye, fura-2 (Grynkiewicz *et al.*, 1985), to investigate the effects of NG on $[Ca^{2+}]_i$, developed tension and their relationships in coronary arterial strips of the pig (Kodama *et al.*, 1989; Hirano *et al.*, 1989).

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Tissue preparation

Left circumflex coronary arteries were dissected from the hearts of pigs at slaughter and segments 2–3 cm from the origin were excised and cut longitudinally. The endothelium was removed by rubbing the inner surface with a cotton swab and the adventitia were trimmed away, under a binocular microscope. The medial preparations thus obtained were cut into approximately 0.5 × 4 mm circular strips, 0.1 mm thick.

Vascular strips were loaded with fura-2, in the form of acetoxymethyl ester (fura-2/AM). The strips were incubated in oxygenated (95% O_2:5% CO_2) Dulbecco-modified Eagle's medium containing 25 μM fura-2/AM dissolved in dimethyl sulphoxide (final concentration: 5%) and 2.5% foetal bovine serum for 3–4 h at 37°C. After loading with fura-2, the strips were rinsed with normal physiological salt solution (PSS) for at least 60 min at 37°C to remove the dye in the extracellular space and to equilibrate the strips before starting the measurements.

Measurement of tension development

The strips were mounted vertically in a quartz organ bath and connected to a strain gauge (TB-612-T, Nihon Koden, Japan). During a 60 min fura-2 equilibration period, the strips were stimulated with 118 mM K^+ every 15 min and the resting tension was increased stepwise. After equilibration, the resting tension was adjusted to 250 mg. The responsiveness of each strip to 118 mM K^+ was recorded before starting the experimental protocol. The developed tension was expressed as a percentage, assuming the values in normal (5.9 mM K^+) and 118 mM K^+ PSS to be 0% and 100%, respectively.

Front-surface fluorometry

Changes in the fluorescence intensity of the fura-2-Ca^{2+} complex were monitored with a front-surface fluorometer specifically designed for fura-2 fluorometry (CAM-OF1), with the

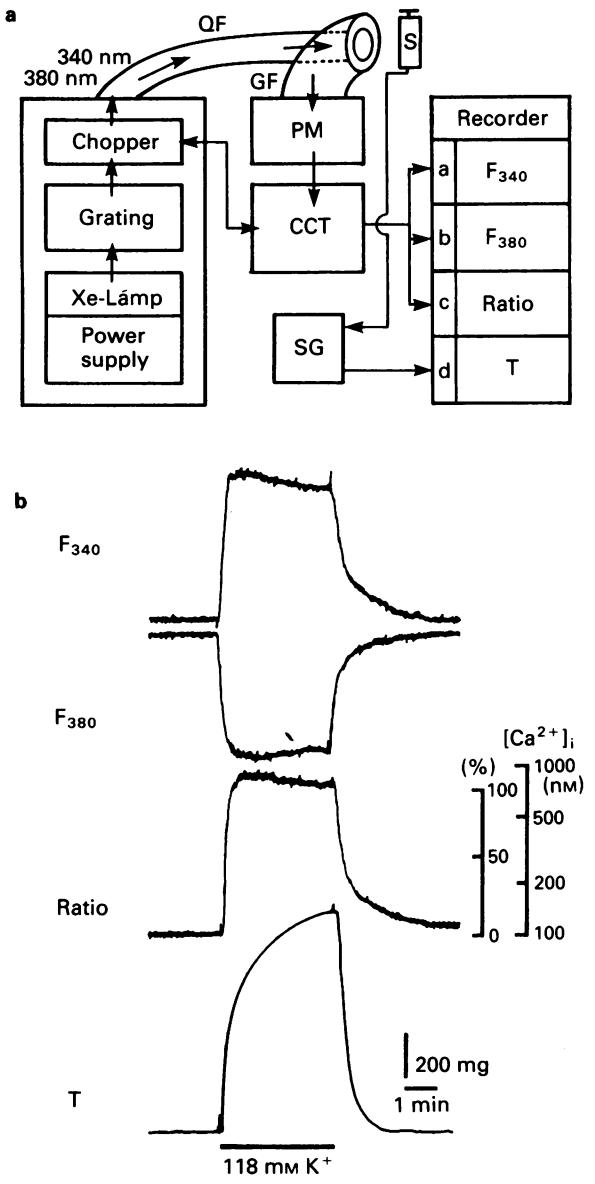

Figure 1 Block diagram of front-surface fluorometry and a representative recording. (a) Schematic block diagram of the front-surface fluorometer. Dual wavelength excitation light (340 nm, 380 nm) was obtained from a spectroscope (Grating) from a Xenon light source. Using a chopper wheel (Chopper), excitation light was alternately (400 Hz) guided through quartz optic fibres (QF) arranged in a concentric inner circle (diameter = 3 mm), and directly illuminated vascular strips (S: 4 mm × 0.5 mm × 0.1 mm). Surface fluorescence of the strips was collected by glass optic fibres (GF) arranged in an outer circle (diameter = 7 mm) and introduced through a 500 nm ± 10 nm band pass filter into a photomultiplier (PM) controlled by a control circuit (CCT). A strain gauge (SG) was used to monitor the developed tension. (b) Representative simultaneous recordings of fluorescence and tension development induced by 118 mM K^+-depolarization. The first and the second traces from the top show changes in 500 nm-fluorescence intensities obtained at 340 nm (F_{340}) and 380 nm (F_{380}) excitations. The third trace shows changes in fluorescence ratio of F_{340} to F_{380} and the lowest trace shows tension development (T).

collaboration of Japan Spectroscopic Co., Tokyo, Japan. A block diagram of the front-surface fluorometry is shown in Figure 1a. Details concerning the fluorometry are given in the legend to Figure 1. Special care was taken to keep the distance between the strip and the end of the optic fibres constant and as short as possible during the measurements.

As shown in Figure 1b, the ratio of the fluorescence intensities (Ratio) at 340 nm-excitation (F_{340}) to that at 380 nm-excitation (F_{380}) was monitored and expressed as a percentage, assuming the values in normal PSS (5.9 mM K^+) and 118 mM K^+ PSS to be 0% and 100%, respectively. The absolute value of $[Ca^{2+}]_i$ was determined by the equation of Grynkiewicz *et al.* (1985).

To examine the effects of dye-loading on contractility of coronary arterial strips of the pig, the responsiveness to 118 mM K^+ of the same strip was determined before and after loading with fura-2. Before loading with fura-2, 118 mM K^+ caused a rapid increase in tension and a maximum steady level was reached at 5 min. After loading with fura-2, 118 mM K^+ caused the same tension development with the same time course as observed before loading (data not shown). Thus, loading the strips with fura-2 produced no change in contractility of the vascular strips.

Solutions

The composition of normal PSS (mM) was: NaCl 123, KCl 4.7, $CaCl_2$ 1.25, $MgCl_2$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 15.5 and D-glucose 11.5; this mixture was bubbled with 95% O_2:5% CO_2, with a resulting pH of 7.4 at 37°C. High external K^+ solution was prepared by replacing NaCl with KCl, isosmotically. The composition of Ca^{2+}-free solution was the same as in normal PSS, except that it contained 2 mM EGTA instead of 1.25 mM $CaCl_2$.

Chemicals

Fura-2 and fura-2/AM were purchased from Molecular Probes (Eugene, OR., U.S.A.) ionomycin was from Calbiochem (Frankfurt, West Germany), histamine was from Wako (Osaka, Japan), nitroglycerin (water soluble form) was obtained from Nihon-Kayaku (Tokyo, Japan) and caffeine and EGTA [ethylenglycol-*bis*-(β-aminoethyl ether)N,N,N',N'-tetraacetic acid] were from DOTITE (Kumamoto, Japan).

Data analysis

The measured values were expressed as mean ± s.d. (n = number of observations). Statistical assessment of the data was made by analysis of variance, Cochran-Cox test, and Student's t test. P values less than 0.05 were considered significant.

To determine EC_{50} (or IC_{50}) values (the concentration that increased (or decreased) $[Ca^{2+}]_i$ and tension to 50% of the maximum response) were determined as follows: for cumulative application of various concentrations of histamine or external Ca^{2+} during high K^+ depolarization, both in the presence and absence of NG (Figures 4b, 6b), EC_{50} values were determined from the concentration-response curve fitted according to a four-parameter logistic model (De Lean *et al.*, 1987). When measurements were performed in a non-cumulative manner (Figures 2b, 3b, 5b), the EC_{50} and the IC_{50} were read directly from the dose-response curve; in this case, the values were approximations.

Results

Effect of nitroglycerin on $[Ca^{2+}]_i$ and tension development induced by K^+-depolarization

As shown in Figure 1b, when the vascular strip was exposed to high external K^+ (118 mM) solution containing 1.25 mM Ca^{2+}, $[Ca^{2+}]_i$ increased rapidly to reach a plateau level during the rising phase of tension development which was maintained with the contraction. The extent of $[Ca^{2+}]_i$ elevation and developed tension induced by high external K^+ (from 5.9 mM to 118 mM) were concentration-dependent (Figure 2c). The levels of $[Ca^{2+}]_i$ observed with under resting conditions (5.9 mM K^+) and during the depolarization (118 mM K^+) were 98 ± 19 nM and 691 ± 95 nM, (n = 6) respectively.

Figure 2a shows representative time courses of the effect of 10^{-5} M NG on $[Ca^{2+}]_i$ and tension in the presence of 40 mM

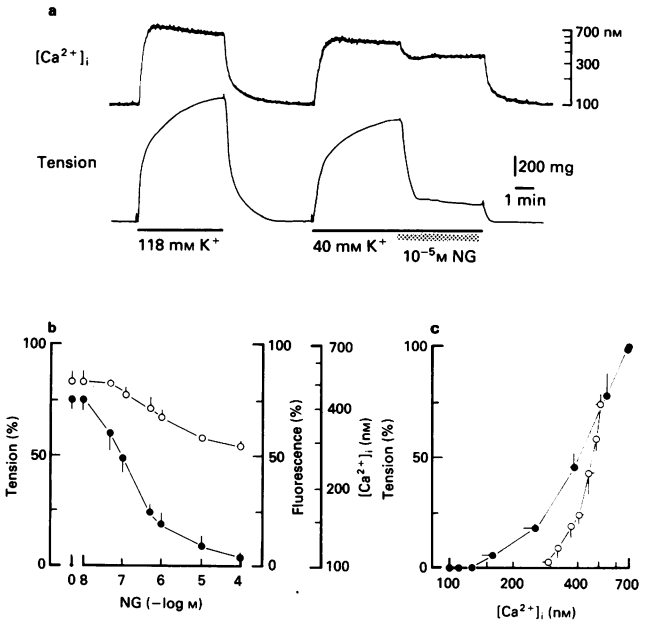

Figure 2 Effect of nitroglycerin (NG) on the increase in $[Ca^{2+}]_i$ and tension development induced by K^+-depolarization. (a) Representative time courses of the effect of 10^{-5} M NG on $[Ca^{2+}]_i$ and tension development during 40 mM K^+-depolarization. (b) Dose-dependent effect of NG on the increases in $[Ca^{2+}]_i$ (○) and tension development (●) induced by 40 mM K^+-depolarization. NG was applied 5 min after application of 40 mM K^+-depolarization. Measurements were performed 5 min after application of NG. Percentage of tension development was obtained by taking values in normal PSS (5.9 mM K^+) and high K^+ solution (118 mM K^+) to be 0% and 100%, respectively. Plots are mean of 5 preparations with s.d. shown by vertical lines. (c) $[Ca^{2+}]_i$-tension relationship (○) obtained from (b). Control $[Ca^{2+}]_i$-tension relationship (●) induced by K^+-depolarization was obtained at plateau levels induced by various concentrations of K^+ (5.9 mM K^+–118 mM K^+).

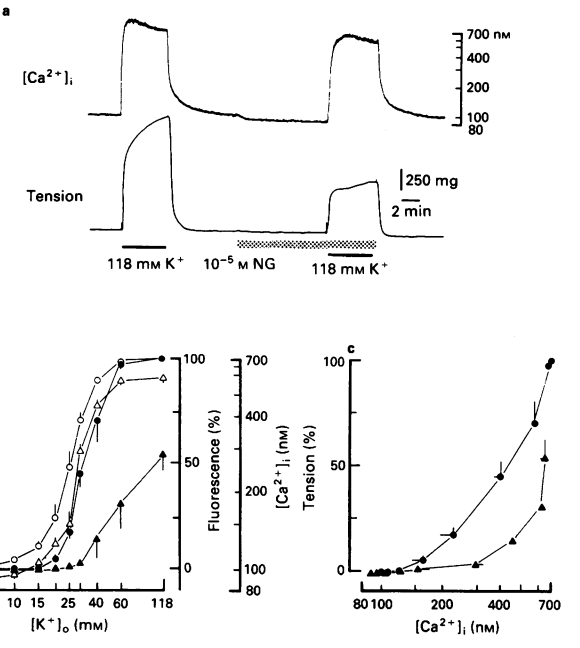

Figure 3 Effect of nitroglycerin (NG) on the increase in $[Ca^{2+}]_i$ and tension development induced by K^+-depolarization. (a) Representative time course of the effect of 10^{-5} M NG on the increase in $[Ca^{2+}]_i$ and tension development induced by 118 mM K^+. (b) Effect of 10^{-5} M NG on the increase in $[Ca^{2+}]_i$ (△) and tension development (▲) induced by depolarization with various concentrations of external K^+. NG was applied 10 min before K^+-depolarization. Controls: the increase in $[Ca^{2+}]_i$ (○) and tension development (●) induced without NG-pretreatment. Plots are mean of 5 preparations with s.d. shown by vertical lines. (c) $[Ca^{2+}]_i$-tension relationship obtained from (b); (▲) with 10^{-5} M NG; (●) control (without NG).

K^+. When the strip was exposed to 40 mM external K^+ solution, plateau levels of $[Ca^{2+}]_i$ and tension were 83 ± 3% (499 nM) and 75 ± 3% ($n = 5$) of the values observed with 118 mM KCl buffer, respectively. Application of 10^{-5} M NG, 5 min after the initiation of depolarization (40 mM K^+) caused rapid and significant reductions in $[Ca^{2+}]_i$ and tension which reached new steady levels within 1 min ($[Ca^{2+}]_i$ = 59 ± 1% (309 nM), $P < 0.01$; tension 9 ± 1%, $P < 0.01$, $n = 5$). As shown in Figure 2b, within the range 10^{-8} and 10^{-4} M, NG induced a concentration-dependent reduction of $[Ca^{2+}]_i$ and tension from the plateau levels evoked by 40 mM K^+. Maximal reduction in $[Ca^{2+}]_i$ and tension was observed with 10^{-4} M NG. IC_{50} values (concentrations of NG which induced 50% of the changes obtained with 10^{-4} M NG) for $[Ca^{2+}]_i$ and tension, were approximately 1.4×10^{-6} M and 5.1×10^{-7} M, respectively. Figure 2c shows the relation between $[Ca^{2+}]_i$ and tension 5 min following the application of NG (steady levels) during 40 mM K^+-depolarization. At each of the $[Ca^{2+}]_i$ concentrations, the levels of developed tension induced by NG were much lower than those observed in K^+-depolarization without NG; thus, the $[Ca^{2+}]_i$ (abscissa scale)-tension (ordinate scale) relationship curve shows a shift to the right with NG.

Figure 3a shows representative time courses of the effect of pretreatment with 10^{-5} M NG on the increase in $[Ca^{2+}]_i$ and tension development induced by 118 mM K^+. When 10^{-5} M NG was applied at rest (5.9 mM K^+) for 10 min, $[Ca^{2+}]_i$ gradually decreased from the resting level of 98.2 nM to a steady level of 89.0 nM. Subsequent application of 118 mM K^+ led to elevations of $[Ca^{2+}]_i$ (576 nM) and to contractions (54 ± 9%), which were significantly lower than those observed without NG-treatment ($[Ca^{2+}]_i$ = 691 ± 95 nM, 100%, $P < 0.01$). As shown in Figure 3b, changes in $[Ca^{2+}]_i$ and contraction induced by high external K^+ were concentration-dependent; EC_{50} values for increasing $[Ca^{2+}]_i$ and contractile response were 25.5 mM and 31.5 mM, respectively. NG (10^{-5} M) inhibited the subsequent increases in $[Ca^{2+}]_i$ and tension induced by depolarization with various concentrations of external K^+ ($P < 0.01$ for both, by two way analysis of variance). The EC_{50} values for the changes in $[Ca^{2+}]_i$ and contraction induced by K^+-depolarization with NG-pretreatment were 28 mM and 56 mM, respectively. Thus, pretreatment with 10^{-5} M NG inhibited the contractile response more potently and effectively than $[Ca^{2+}]_i$, and shifted the $[Ca^{2+}]_i$-tension relationship curve to the right (Figure 3c).

Effects of nitroglycerin on $[Ca^{2+}]_i$ and tension development induced by changes in external Ca^{2+} during high K^+-depolarization

Figure 4a shows representative time courses of changes in $[Ca^{2+}]_i$ and tension development induced by the cumulative application of external Ca^{2+} (0.0125–12.5 mM) during depolarization with 118 mM K^+. In response to the stepwise increment of external Ca^{2+} concentration, $[Ca^{2+}]_i$ and tension increased dose-dependently. When the external Ca^{2+} was 12.5 mM, $[Ca^{2+}]_i$ and contractions were 150 ± 12% (1743 nM) and 140 ± 17%, respectively, of the values observed at 1.25 mM Ca^{2+} during depolarization with 118 mM K^+. EC_{50} values for the elevation of $[Ca^{2+}]_i$ and tension were 0.39 mM and 0.5 mM, respectively (Figure 4b). Pretreatment with 10^{-5} M NG inhibited the increases in $[Ca^{2+}]_i$ and tension development evoked by the cumulative application of external Ca^{2+} ($P < 0.01$ for both, by two way analysis of variance (Figure 4b). EC_{50} values (external Ca^{2+}) for the changes in $[Ca^{2+}]_i$ and tension were 0.54 mM and 3.1 mM, respectively (Figure 4b). As shown in the figure, the inhibition of contraction was greater than that of $[Ca^{2+}]_i$, and as a result, the curve relating $[Ca^{2+}]_i$ and tension development

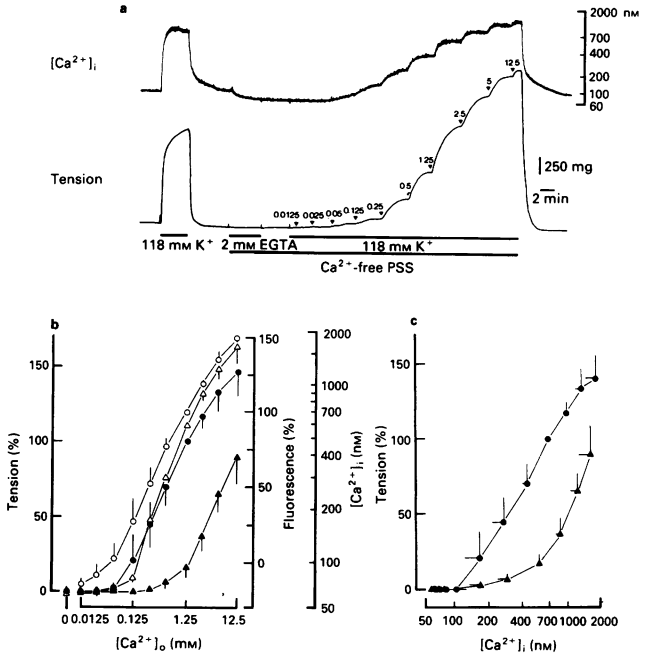

Figure 4 Effect of 10^{-5} M nitroglycerin (NG) on the increase in $[Ca^{2+}]_i$ and tension development induced by the cumulative application of various concentrations (0.0125 mM–12.5 mM) of external Ca^{2+} during 118 mM K^+ depolarization. (a) Representative time course of change in $[Ca^{2+}]_i$ and tension development in the absence of NG. (b) Effect of 10^{-5} M NG on the changes in $[Ca^{2+}]_i$ (△) and tension development (▲) induced by changes in external Ca^{2+} during 118 mM K^+-depolarization. Controls: $[Ca^{2+}]_i$ (○) and tension (●) in the absence of NG-pretreatment. Plots are mean of 4 preparations with s.d. shown by vertical lines. (c) $[Ca^{2+}]_i$-tension relation obtained from (b); (▲) with 10^{-5} M NG; (●) control (without NG).

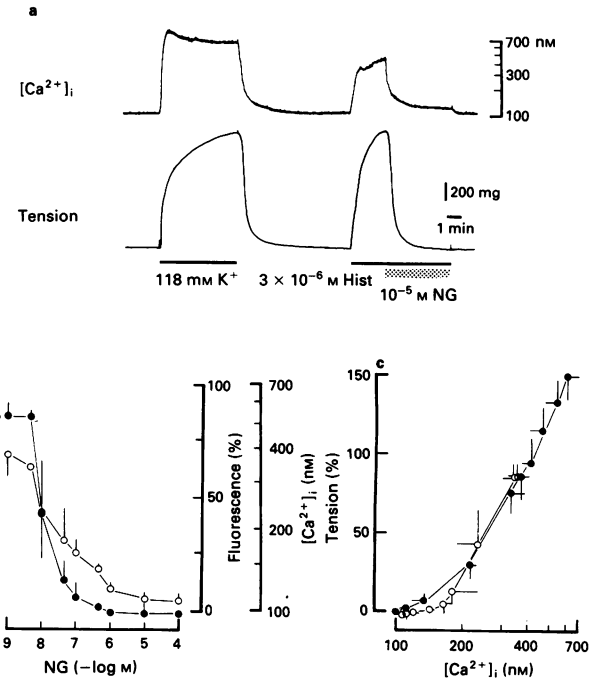

Figure 5 Effect of nitroglycerin (NG) on the increase in $[Ca^{2+}]_i$ and tension development induced by histamine (Hist). (a) Representative time course of the effect of 10^{-5} M NG on $[Ca^{2+}]_i$ and tension development during 3×10^{-6} M histamine application. (b) Dose-dependent effect of NG on the increase in $[Ca^{2+}]_i$ (○) and tension development (●) induced by 3×10^{-6} M histamine. NG was applied 2 min after application of 3×10^{-6} M histamine. Measurements were performed 5 min after application of NG. Plots are mean of 5 preparations with s.d. shown by vertical lines. (c) $[Ca^{2+}]_i$-tension relationship obtained from (b) (○). Control (●) $[Ca^{2+}]_i$-tension relationship obtained by the stepwise application of histamine (10^{-7} M–10^{-4} M) without NG.

was shifted to the right (Figure 4c). With regard to $[Ca^{2+}]_i$ at rest (70 ± 3 nM), 50% of the maximum (497 ± 55 nM), and at the maximum tension development (1743 ± 398 nM), the $[Ca^{2+}]_i$-tension curve obtained by the cumulative application of external Ca^{2+} up to 12.5 mM during depolarization with 118 mM K^+ without NG (solid circles in Figure 4c) overlapped with that noted in vascular strips skinned with saponin (Itoh *et al.*, 1982; Satoh *et al.*, 1987). Whether with or without NG pretreatment, the $[Ca^{2+}]_i$-tension curves in Figure 3c fitted those for the corresponding range of $[Ca^{2+}]_i$ in Figure 4c.

Effect of nitroglycerin on $[Ca^{2+}]_i$ and tension development induced by histamine

Figure 5a shows representative time courses of the effect of 10^{-5} M NG on $[Ca^{2+}]_i$ and tension development in the presence of 3×10^{-6} M histamine. We have reported that the density of histamine H_1-receptors in the coronary artery of the pig is higher than that of other species (Nishimura *et al.*, 1985). Histamine induced rapid rises in $[Ca^{2+}]_i$ and tension; within 3 min, $[Ca^{2+}]_i$ reached peak levels and remained close to these levels during the 20 min observation period. Developed tension also reached peak levels within 5 min and remained at these levels during the 30 min period of observation. Changes in $[Ca^{2+}]_i$ and tension induced by histamine were concentration-dependent and the $[Ca^{2+}]_i$-tension relationship was shifted to the left of that observed with K^+-depolarization (compare Figures 3c and 5c). Thus, for any given increase in $[Ca^{2+}]_i$, histamine induced a greater tension development than did K^+-depolarization. NG markedly, rapidly and concentration-dependently reduced the histamine-induced increases in $[Ca^{2+}]_i$ and tension (Figure 5b). Maximal reduction in $[Ca^{2+}]_i$ and tension were observed with 10^{-4} M NG. IC_{50} values (the concentration of NG which induced 50% of the changes obtained with 10^{-4} M NG) for $[Ca^{2+}]_i$ and tension elevatinos were approximately 2.5×10^{-8} and 10^{-8} M, respectively. The $[Ca^{2+}]_i$-tension curve at 5 min after NG application during histamine stimulation showed a generally good fit with the one obtained during histamine stimulation without NG (Figure 5c).

Figure 6a shows representative time courses of the effect of 10^{-5} M NG on the increases in $[Ca^{2+}]_i$ and tension development induced by 10^{-5} M histamine. When 10^{-5} M NG was applied under basal conditions (5.9 mM K^+), the level of $[Ca^{2+}]_i$ was significantly decreased from 98 nM to 85 nM ($P < 0.01$: Student's t test). Subsequent application of histamine induced elevations in $[Ca^{2+}]_i$ and contraction; however, the peak levels were significantly inhibited ($P < 0.01$ and $P < 0.01$, respectively; Student's t test). Figure 6b shows the effect of 10^{-5} M NG on the subsequent increases in $[Ca^{2+}]_i$ and on tension development induced by cumulative application of various concentrations of histamine. EC_{50} values of histamine for the changes in $[Ca^{2+}]_i$ and contraction without NG treatment were 1.2×10^{-6} M and 2.3×10^{-6} M, respectively. EC_{50} values of histamine for the changes in $[Ca^{2+}]_i$ and tension development with NG treatment were 2.9×10^{-6} M and 6.9×10^{-6} M, respectively. As shown in Figure 6c, the $[Ca^{2+}]_i$ tension curve for histamine with NG treatment was slightly shifted to the right, compared to that without NG treatment.

Effect of nitroglycerin on $[Ca^{2+}]_i$ and tension development induced by caffeine or histamine in Ca^{2+}-free solution containing 2 mM EGTA

Figure 7a shows representative time courses of $[Ca^{2+}]_i$ and tension development induced by repeated applications of 20 mM caffeine in Ca^{2+}-free solution containing 2 mM EGTA. When a strip was exposed to Ca^{2+}-free solution, the level of

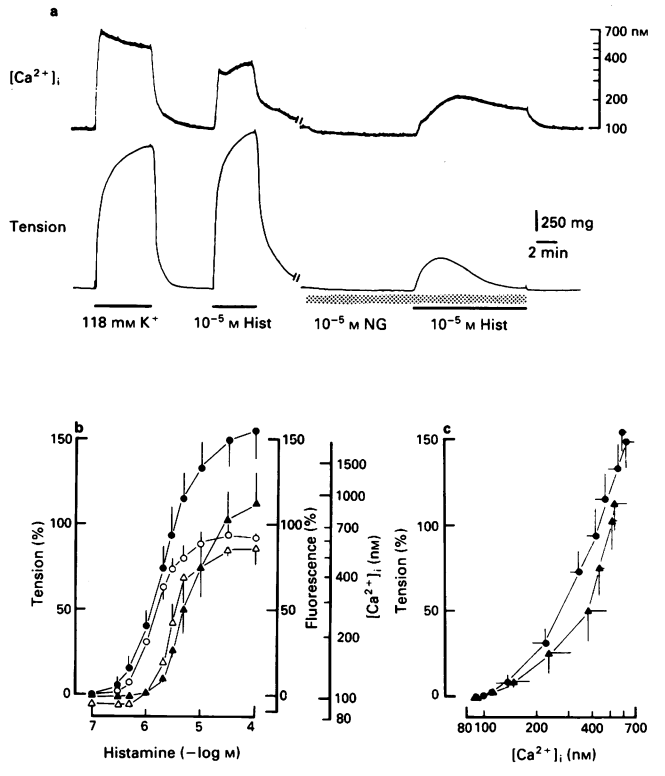

Figure 6 Effect of nitroglycerin (NG) on the increase in $[Ca^{2+}]_i$ and tension development induced by histamine (Hist). (a) Representative time course of the effect of 10^{-5} M NG on the increases in $[Ca^{2+}]_i$ and tension development induced by 10^{-5} M histamine. (b) Effect of 10^{-5} M NG on the subsequent increase in $[Ca^{2+}]_i$ (△) and tension development (▲) induced by histamine. NG was administered 10 min before the cumulative application of various concentrations (10^{-7} M–10^{-4} M) of histamine. Controls: the increases in $[Ca^{2+}]_i$ (○) and tension development (●) induced by histamine without NG-pretreatment. Plots are mean of 5 preparations with s.d. shown by vertical lines. (c) $[Ca^{2+}]_i$-tension relation obtained from (b); (▲) with 10^{-5} M NG; (●) control (without NG).

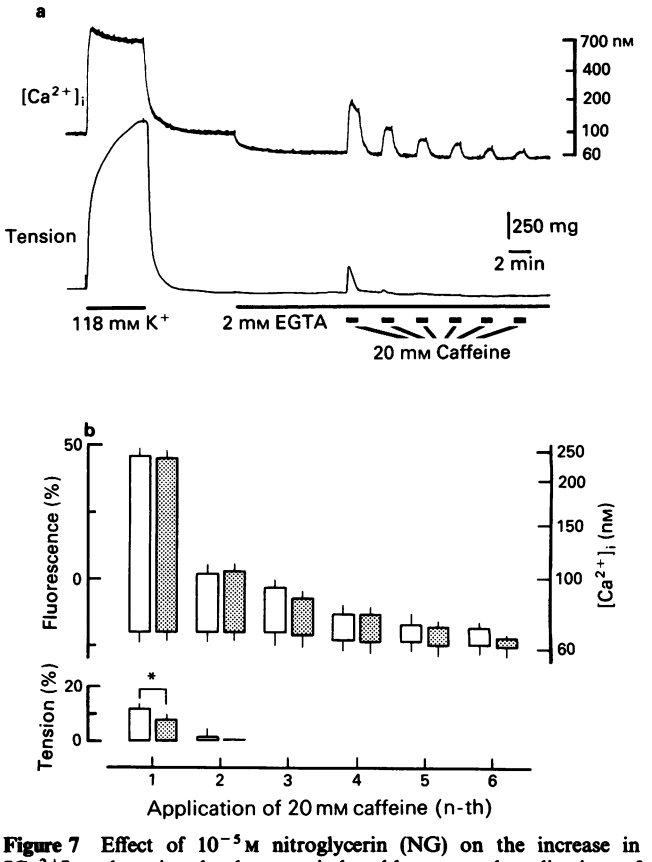

Figure 7 Effect of 10^{-5} M nitroglycerin (NG) on the increase in $[Ca^{2+}]_i$ and tension development induced by repeated applications of 20 mM caffeine in the absence of extracellular Ca^{2+}. (a) Representative time courses of the effect of repeated applications of 20 mM caffeine (at each short bar) in Ca^{2+}-free solution containing 2 mM EGTA. NG was not applied. (b) $[Ca^{2+}]_i$ and tension development in response to repeated applications of caffeine. The bottom and top of each column indicate the $[Ca^{2+}]_i$ and tension development just before and at the peak levels obtained at the n-th (abscissa scale) application of caffeine, respectively, in the absence (open columns) and presence of 10^{-5} M NG (stippled columns). The vertical lines at the bottom and the top of each column show s.d. ($n = 4$). NG was administered 10 min before the application of 20 mM caffeine. * $P < 0.05$

$[Ca^{2+}]_i$ gradually decreased to reach a steady state within 10 min (from 98 nM to 75 nM) with no change in the tension. As shown in Figure 7b, in the absence of extracellular Ca^{2+}, the first application of caffeine induced transient increases in $[Ca^{2+}]_i$ and tension. The peak levels of $[Ca^{2+}]_i$ and tension were $38 \pm 4\%$ (207 nM) and $13 \pm 3\%$, ($n = 5$) respectively. With repeated exposure to 20 mM caffeine for 1 min every 3 min, a series of transient increases in $[Ca^{2+}]_i$ was observed, and the peak levels of $[Ca^{2+}]_i$ were progressively reduced with each application. The peak level of $[Ca^{2+}]_i$ induced by the third application of caffeine was $-4 \pm 8\%$ (93 nM), that is equal to or lower than the resting levels observed in the presence of extracellular 1.25 mM Ca^{2+}. The peak level of devel-

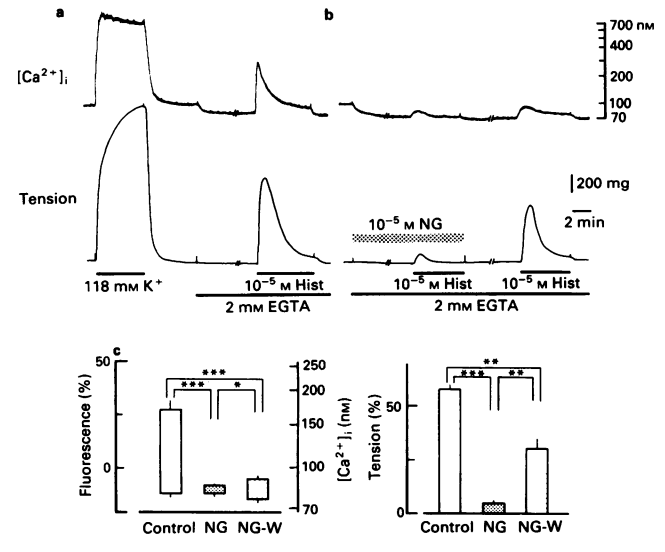

Figure 8 Effect of 10^{-5} M nitroglycerin (NG) on the increase in $[Ca^{2+}]_i$ and tension development induced by 10^{-5} M histamine (Hist) in Ca^{2+}-free solution containing 2 mM EGTA. (a) Representative time course of 10^{-5} M histamine-induced $[Ca^{2+}]_i$ and tension development in Ca^{2+}-free solution containing 2 mM EGTA. (b) Representative time course of the effect of 10^{-5} M NG on $[Ca^{2+}]_i$ and tension development induced by 10^{-5} M histamine in Ca^{2+}-free solution containing 2 mM EGTA. NG was administered 10 min before application of histamine. After the first application of histamine for 5 min, NG was washed out with Ca^{2+}-free solution for 15 min, followed by the second application of histamine. (c) Effect of 10^{-5} M on $[Ca^{2+}]_i$ and tension development induced by 10^{-5} M histamine in Ca^{2+}-free solution containing 2 mM EGTA. Control: $[Ca^{2+}]_i$ and tension development induced by 10^{-5} M histamine in the absence of NG. NG: $[Ca^{2+}]_i$ and tension development induced by the first application of 10^{-5} M histamine in the presence of 10^{-5} M NG. NG-W: $[Ca^{2+}]_i$ and tension induced by the second application of 10^{-5} M histamine after NG had been washed out. The bottom and the top of each column indicate the $[Ca^{2+}]_i$ and tension just before and at the peak levels obtained by the application of histamine, respectively. The vertical bars at the bottom and the top of each column shows mean ± s.d. of 5 preparations. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

oped tension was also progressively reduced by each exposure to caffeine and the third exposure produced little or no response. Thus, it has to be noted that, although $[Ca^{2+}]_i$ significantly and transiently increased at each exposure for up to 6 applications of caffeine, there was no tension development at the third and the later applications when peak $[Ca^{2+}]_i$ levels were below the resting level. NG (10^{-5} M) did not enhance the decrease in $[Ca^{2+}]_i$ observed when vascular strips were exposed to Ca^{2+}-free solution containing 2 mM EGTA. NG (10^{-5} M) had no effect on the $[Ca^{2+}]_i$ transient induced by each application of caffeine, thereby indicating that NG did not affect the Ca^{2+}-release by caffeine nor did it deplete Ca^{2+} stored in the caffeine-sensitive store. However, tension development induced by the first application of caffeine was significantly inhibited by NG ($P < 0.05$; Student's t test). Thus, NG inhibited the caffeine-induced contraction without affecting Ca^{2+} homeostasis.

Figure 8a shows representative time courses of $[Ca^{2+}]_i$ and tension development induced by 10^{-5} M histamine in Ca^{2+}-free solution containing 2 mM EGTA. When the strip was exposed to 10^{-5} M histamine in the absence of extracellular Ca^{2+}, transient increases in $[Ca^{2+}]_i$ and tension were seen. Pretreatment with 10^{-5} M NG in Ca^{2+}-free solution strongly inhibited the elevations of $[Ca^{2+}]_i$ and contraction induced by histamine ($P < 0.001$ and $P < 0.001$, respectively: Student's t test) (Figure 8b,c). However, as shown in Figure 8b,c, when NG was washed out in Ca^{2+}-free solution, the second, subsequent application of 10^{-5} M histamine induced both $[Ca^{2+}]_i$ elevation and contraction, the extent of these changes being greater than those observed during the first application of histamine with NG treatment. In addition, the second application of histamine induced a marked contraction ($30 \pm 8\%$) despite the fact that the peak $[Ca^{2+}]_i$ elevation (91 ± 1 nM) was significantly lower than that of the resting level ($P < 0.001$). The sum of the peak levels of $[Ca^{2+}]_i$ induced by the first and the second applications of histamine was 28 nM, that is a value much smaller than the peak levels (96 nM) seen with the first application of histamine without NG.

Discussion

Using a front-surface fluorometer and fura-2 (Hirano *et al.*, 1989), we have recorded Ca^{2+} transients during the NG-induced relaxation in isolated coronary arterial strips of the pig. Histamine, a receptor stimulating compound, caused a proportionally greater extent of tension development for a given change in $[Ca^{2+}]_i$ than did high K^+-depolarization. Using *Staphylococcus aureus* α-toxin to permeabilize the vascular smooth muscle tissue to ions and small molecules, and assuming that the receptor and signal transduction systems were intact, Nishimura *et al.* (1988) and Kitazawa *et al.* (1989) found that some agonists induced an enhanced sensitivity of myofilaments to $[Ca^{2+}]_i$, through a G-protein mediated pathway other than inositol 1,4,5-trisphosphate-induced $[Ca^{2+}]_i$ elevation. This mechanism may explain why in the present study the histamine-induced contraction was greater than that induced by K^+-depolarization at any given elevation of $[Ca^{2+}]_i$ in intact coronary arterial strips of the pig.

Recently, it has been reported that the $[Ca^{2+}]_i$-tension relation may vary during contraction and that regulatory mechanisms of tension maintenance differ from those related to tension development (Rasmussen *et al.*, 1987; Murphy, 1989). Accordingly, in the present study, to examine the effect on $[Ca^{2+}]_i$ and tension, NG was administered during or prior to the stimulation with K^+ depolarization or histamine. The present study provides evidence that NG actively decreases $[Ca^{2+}]_i$ and relaxes fura-2-loaded coronary arterial strips, regardless of whether the strips are at rest or under conditions where they are stimulated by K^+-depolarization or histamine, or whether NG was administered during or prior to these stimulations. The $[Ca^{2+}]_i$-tension relations obtained both by addition of NG during K^+-depolarization and by K^+-depolarization following NG-pretreatment were shifted to the right from those obtained by K^+-depolarization without NG-treatment. This means that the extent of reduction of developed tension induced by NG is much greater than that expected from the reduction in $[Ca^{2+}]_i$. Regardless of whether NG was applied during or prior to the stimulation with histamine, the $[Ca^{2+}]_i$-tension relation induced by NG fitted with, or was slightly shifted to the right from, the one obtained by histamine stimulation without NG. Thus, also with histamine, the extent of reduction of developed tension induced by NG is much greater than that expected from the reduction of $[Ca^{2+}]_i$ based on the $[Ca^{2+}]_i$-tension relationship obtained by the cumulative application of external Ca^{2+} from 0 to 12.5 mM during depolarization with 118 mM K^+.

The present study showed that NG did not affect the Ca^{2+}-release nor did it increase or deplete the Ca^{2+} present in the caffeine-sensitive store during repeated exposure to caffeine. NG probably has no effect on the uptake and release of Ca^{2+} from the caffeine-sensitive Ca^{2+} store. The observed decrease in caffeine-induced tension development by NG may be due to a mechanism independent of the change in $[Ca^{2+}]_i$. We have reported (Kobayashi *et al.*, 1985) that, in rat aortic vascular smooth muscle cells in primary culture, the caffeine-sensitive Ca^{2+} store was little affected in Ca^{2+}-free solution, and NG did not alter the Ca^{2+} transient induced by the first application of caffeine. This also suggests that NG has no effect on the caffeine-sensitive Ca^{2+} store. However NG enhanced the decrease in $[Ca^{2+}]_i$ in Ca^{2+}-free solution and markedly reduced $[Ca^{2+}]_i$ elevations caused by the second and subsequent applications of caffeine in cultured smooth muscle cells, presumably because of active extrusion of Ca^{2+} from the cell as a result of NG treatment.

In contrast, NG appeared to deplete Ca^{2+} from the histamine-sensitive store and to inhibit the release of Ca^{2+} from this store in coronary arterial strips of the pig in Ca^{2+}-free solution. These findings are compatible with our previous data that NG inhibits the noradrenaline-induced Ca^{2+}-transient in the absence of extracellular Ca^{2+} in primary cultured rat aortic vascular smooth muscle cells (Kai *et al.*, 1990). An active reduction in $[Ca^{2+}]_i$ may result in depletion of Ca^{2+} from the histamine-sensitive store.

Thus, the present study indicates that with the exception of caffeine-induced contraction in Ca^{2+}-free solution, NG actively reduces $[Ca^{2+}]_i$ during the relaxation of the coronary artery of the pig. As exposure of vascular strips to NG in Ca^{2+}-free solution did not enhance the subsequent $[Ca^{2+}]_i$ transient induced by histamine, it seems unlikely that the sequestration of Ca^{2+} into the intracellular Ca^{2+} store can be involved in the mechanism of the $[Ca^{2+}]_i$ reduction induced by NG.

NG increases intracellular cyclic GMP levels, cyclic GMP-dependent protein kinase (G-kinase) activity and phosphorylation of some protein substrates for G-kinase (Ignarro & Kadowitz, 1987). We have reported that 8-Br cyclic GMP, a membrane permeable cyclic GMP analogue, and NG actively decreased $[Ca^{2+}]_i$ irrespective of the level of $[Ca^{2+}]_i$ in cultured vascular smooth muscle cells (Kai *et al.*, 1987; 1990). It has been reported that sarcolemmal Ca^{2+} extrusion ATPase is activated by cyclic GMP or NG (Suematsu *et al.*, 1984; Popescu *et al.*, 1985a,b; Furukawa & Nakamura, 1987; Koh *et al.*, 1987; Vrolix *et al.*, 1988). In the present study, we could not obtain conclusive evidence of this mechanism for the reduction of $[Ca^{2+}]_i$ induced by NG in coronary arterial strips. The present study shows that NG inhibited the release of Ca^{2+} from histamine-sensitive stores, but not from the caffeine-sensitive store. On the other hand, Twort & van Breemen (1988) reported that when skinned vascular smooth muscle cells were fully loaded with Ca^{2+}, cyclic GMP had no effect on the release of Ca^{2+} from the intracellular store, in response to caffeine and inositol 1,4,5-trisphosphate (and hence, histamine). Nishizuka (1983) found that in platelets, cyclic GMP plays a role in negative feed-back control of the

agonist-induced signal transduction by inhibiting the formation of inositol 1,4,5-trisphosphate. In vascular smooth muscle, 8-Br cyclic GMP reduced the hydrolysis of phosphatidylinositol, and hence, decreased the formation of inositol 1,4,5-trisphosphate. However, there is controversy on this point since Sumimoto *et al.* (1987) found that NG and 8-Br cyclic GMP had no effect on the hydrolysis of phosphatidylinositol in arterial smooth muscle. Therefore, it remains to be elucidated whether or not the NG-cyclic GMP-mediated mechanism has any inhibitory effect on the formation of inositol 1,4,5-trisphosphate, which could reduce Ca^{2+}-influx through sarcolemmal Ca^{2+} channels and/or Ca^{2+}-release from storage sites (Berridge & Irvine, 1984a,b).

In the present study, NG relaxed the porcine coronary artery to a greater extent than that expected from the reduction in $[Ca^{2+}]_i$, regardless of whether vascular strips were at rest, depolarized with K^+ or stimulated with histamine. Nishimura & van Breemen (1989b) found that cyclic GMP relaxed arterial smooth muscle with the $[Ca^{2+}]_i$ held constant, thereby suggesting that second messengers exert a direct control on contractile elements. It was also reported that cyclic GMP or G-kinase phosphorylated myosin light chain kinase which resulted in dephosphorylation of the myosin light chain and inhibition of contraction (Axelsson *et al.*, 1979; Kukovetz *et al.*, 1979; Pfitzer *et al.*, 1982; 1983; 1986; Rapoport *et al.*, 1982; 1983). In addition sodium nitroprusside, another nitro-vasodilator, was found to be more effective in relaxing vascular smooth muscle than decreasing $[Ca^{2+}]_i$ (Morgan & Morgan, 1984; Karaki *et al.*, 1988).

In conclusion, NG actively reduces both $[Ca^{2+}]_i$ and tension in strips of coronary artery of the pig, irrespective of whether they are at rest or under stimulation with either high K^+ or histamine. For NG-induced relaxation, the extent of the decrease in tension was greater than that expected from the reduction of $[Ca^{2+}]_i$ based on the $[Ca^{2+}]_i$-tension relation observed with K^+-depolarization. In the absence of extracellular Ca^{2+}, NG depleted stored Ca^{2+} and also inhibited the release of Ca^{2+} from histamine-sensitive stores, but had no effect on caffeine-sensitive stores. NG inhibited caffeine-induced tension development with no change in $[Ca^{2+}]_i$. Thus, NG relaxes the coronary artery of the pig by reducing $[Ca^{2+}]_i$. In addition, and independent of the $[Ca^{2+}]_i$ change, a second messenger, possibly cyclic GMP, may directly influence the contractile elements during NG-induced relaxation.

We thank M. Ohara for critical comments. This work was supported by Grants-in-Aid for Scientific Research on Priority Areas (No. 63624510 and No. 01641532) and for General Scientific Research (No. 01480250) from the Ministry of Education, Science and Culture, Japan and grants from the "Research Program on Cell Calcium Signals in the Cardiovascular System", from Suzuken Memorial Foundation, Tokyo Biochemical Research Foundation, Casio Science Promotion Foundation, Uehara Memorial Foundation, Kanehara Ichiro Memorial Foundation and from Ciba-Geigy Foundation (Japan) for the Promotion of Science.

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(Received February 26, 1990
Revised June 18, 1990
Accepted June 22, 1990)

Br. J. Pharmacol. (1990), **101**, 553–556

An examination of the 5-HT_3 receptor mediating contraction and evoked [^{3}H]-acetylcholine release in the guinea-pig ileum

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1 The relative contributions of two classes of 5-hydroxytryptamine (5-HT) receptor (5-HT_2 and 5-HT_3) to the contractile action of 5-HT, 2-methyl-5-hydroxytryptamine (2-methyl-5-HT) and α-methyl-5-hydroxytryptamine (α-methyl-5-HT) were studied in the guinea-pig ileum longitudinal muscle-myenteric plexus strip (LMMP) preparation. Contractility studies were combined with an analysis of the effects of the three agonists on [^{3}H]-acetylcholine ([^{3}H]-ACh) release from preparations preincubated with [^{3}H]-choline.

2 In contracting the LMMP, 5-HT was approximately one order of magnitude more active than 2-methyl-5-HT and α-methyl-5-HT, with relative activities for 5-HT: 2-methyl-5-HT: α-methyl-5-HT of 1.00: 0.13: 0.10.

3 Ketanserin (1 μM) was without effect on the concentration-response curves for contraction to 5-HT, 2-methyl-5-HT or α-methyl-5-HT, whilst ondansetron (GR38032F; 1 μM) produced a parallel rightward displacement of the upper part of the concentration-response curves to 5-HT and α-methyl-5-HT and of the entire curve to 2-methyl-5-HT.

4 In increasing the spontaneous release of [^{3}H]-ACh from the LMMP, 5-HT was again approximately one order of magnitude more active than 2-methyl-5-HT and α-methyl-5-HT with relative activities for 5-HT: 2-methyl-5-HT: α-methyl-5-HT of 1.00: 0.19: 0.11.

5 Ondansetron (1 μM) greatly attenuated the increase in spontaneous [^{3}H]-ACh release evoked by all three agonists. pK_B estimates of 7.62 ± 0.12 and 7.64 ± 0.09 were obtained for ondansetron antagonism of 5-HT and 2-methyl-5-HT-evoked increases respectively.

6 These data suggest that the contractile action of 5-HT, 2-methyl-5-HT and α-methyl-5-HT in the guinea-pig ileum can, under these conditions, be accounted for largely in terms of 5-HT_3 receptor activation. Estimates for pK_B obtained with ondansetron are in accordance with those previously obtained from contractility studies in this preparation and these findings are discussed in terms of the postulated existence of subtypes of 5-HT_3 receptors.

Introduction

Gaddum & Picarelli (1957) showed that 5-hydroxytryptamine (5-HT) can contract the guinea-pig ileum via an interaction with two different receptor types – the neuronal 'M' receptor and the 'D' receptor located on the longitudinal smooth muscle, subsequently referred to as 5-HT_3 and 5-HT_2 receptors respectively (Bradley *et al.*, 1986). However, literature reports differ considerably with respect to the relative contribution of these pre- and post-junctional receptors to the contractile action of 5-HT in the guinea-pig ileum. For example, whilst Chahl (1983) found that most of the contractile effect of 5-HT was mediated through D (5-HT_2) receptors, others (e.g. Brownlee & Johnson, 1963; Costa & Furness, 1979) concluded that the major component was due to M (5-HT_3) receptor-mediated acetylcholine (ACh) release, although the exact contribution may vary with the region used. This lack of a significant D receptor involvement was also found by Buchheit *et al.* (1985).

However, these various conclusions were all drawn from contractility studies, using 5-HT alone, in which any neurotransmitter-mediated effects are inferred from postjunctional responses of the smooth muscle which is itself influenced by 5-HT. Also, it has been reported that the contractile concentration-response curve to 5-HT in this preparation is biphasic in form, and that substance P release mediates the neuronal actions of 5-HT (Buchheit *et al.*, 1985). Additional actions of 5-HT in the guinea-pig ileum include 5-HT_1-like-receptor-mediated relaxation (Fenuik *et al.*, 1983), a possible 5-HT_3 receptor-mediated inhibitory component (Gunning & Humphrey, 1987), 5-HT_{1a} receptor-mediated inhibition of ACh release (Fozard & Kilbinger, 1985) and non-5-HT_3 neuronal excitation (Sanger, 1987; Craig & Clarke, 1990), which must necessarily complicate interpretation of results from any studies.

Therefore the rationale of the present experiments was to obtain direct estimates of ACh release, in parallel with contractile studies. We have re-examined the relative contribution of 5-HT_2 and 5-HT_3 receptors to the contractile action of 5-HT in the longitudinal muscle-myenteric plexus preparation using the 5-HT_3 receptor agonist 2-methyl-5-hydroxytryptamine (2-methyl-5-HT) (Richardson *et al.*, 1985; Bradley *et al.*, 1986), and α-methyl-5-hydroxytryptamine (α-methyl-5-HT) claimed to be selective for 5-HT_2 receptors (Richardson *et al.*, 1985). Studies of the contractile effects of these three agonists were combined with studies of their ability to increase the release of [^{3}H]-ACh (estimated as tritium overflow) from preparations prelabelled with [^{3}H]-choline, to eliminate as far as possible other complicating actions of 5-HT mentioned above. The involvement of 5-HT_3 receptors in this ACh release was examined by the use of ondansetron (GR38032F) which behaves as a competitive reversible antagonist towards 5-HT_3-mediated contractions in the guinea-pig ileum (Butler *et al.*, 1988). Previous studies of 5-HT-evoked ACh release (Kilbinger & Pfeuffer-Friederich, 1985) used MDL 72222 which has little activity at the 5-HT_3 receptor of this system (Fozard, 1984).

The lower affinity of 5-HT_3 receptor antagonists such as ICS 205-930 and MDL 72222 in the guinea-pig ileum as compared to other preparations including the rabbit isolated heart and the rat or rabbit vagus nerve (Donatsch *et al.*, 1984; Ireland & Tyers, 1987) led to the proposal of 5-HT_3 receptor

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heterogeneity (Fozard, 1984; Richardson & Engel, 1986). More recent 5-HT_3 antagonists such as ondansetron and BRL 43694 (granisetron) also discriminate between the guinea-pig ileum and other preparations (Butler *et al.*, 1988; Sanger & Nelson, 1989). Again, however, such results in the guinea-pig ileum may be compromised by the indirect measurement of 5-HT_3 receptor activation and the complex actions of 5-HT. Here we describe a more direct measure of the affinity of ondansetron for the 5-HT_3 receptor of the guinea-pig ileum through pK_B estimates of its antagonism of 5-HT and 2-methyl-5-HT evoked increases in [^{3}H]-ACh release.

A preliminary account of this work has been communicated to the British Pharmacological Society (Fox & Morton, 1989).

Methods

Male Dunkin-Hartley guinea-pigs (250–500 g) were killed by stunning and exsanguination. The ileum was excised approximately 10 cm from the ileo-caecal junction and strips of longitudinal muscle with adherent myenteric plexus (LMMP) were removed according to the method of Rang (1964) from distal portions of ileum.

Contractility studies

LMMP strips were mounted in 1 ml organ baths containing Krebs solution aerated with 95% O_2/5% CO_2 and maintained at 37°C. Contractile responses were recorded isometrically with Grass FT03B force-displacement transducers with a resting tension of 0.5–1.0 g. In all experiments the bathing solution contained hexamethonium (10 μM), mepyramine (1 μM), guanethidine (1 μM) and ibuprofen (1 μM).

For each experiment four tissues were obtained from each of three different animals and used as twelve preparations studied in parallel. In a balanced block design two of each set of four tissues received either ondansetron (1 μM) or ketanserin (1 μM) throughout, with the remaining two acting as concurrent controls. Concentration-response curves for 5-HT (10 nM–30 μM), 2-methyl-5-HT (0.1 μM–300 μM) and α-methyl-5-HT (0.1 μM–300 μM) were constructed, after at least 30 min equilibration with antagonist, using serial application with 30–45 s contact times and with a 15 min dose-cycle to avoid desensitization. Each preparation received 5-HT and either 2-methyl-5-HT or α-methyl-5-HT to allow direct comparison with 5-HT itself, with concentrations applied in a fully randomized order. Histamine (10 μM) was applied between each 5-HT analogue dose as an internal sensitivity control. All responses were expressed as a percentage of the maximal response to carbachol in individual preparations for the purpose of displaying averaged concentration-response curves. Data from each set of experiments using either ketanserin or ondansetron were combined and expressed in terms of mean response ± s.e.mean for each concentration with agonist activities defined in terms of pD_2 ($-\log_{10}$ EC_{50} (M)) estimates obtained from individual experiments.

^{3}H-overflow studies as an index of acetylcholine release

Four LMMP strips were mounted in perfusion chambers on hypodermic tubing holders (o.d. 0.9 mm) and superfused at a rate of 1 ml min^{-1} with Krebs solution aerated with 95% O_2/5% CO_2 and maintained at 37°C. The tissues were stimulated electrically via a lower platinum electrode and an upper electrode of 0.1 mm annealed stainless steel wire that also served for attachment to the transducer with 1 ms square wave pulses at 0.2 Hz and supramaximal voltage (50–70 V) delivered from Grass S44 stimulators. LMMP preparations were then incubated for 1 h in Krebs solution containing [^{3}H]-choline (0.2 μM; 2.89 GBq mol^{-1}) in 1 ml vials inserted under each preparation within the perfusion chamber and aerated via the muscle holder tubing. During this period electrical stimulation was maintained to enhance uptake of [^{3}H]-choline, with concomitant contractions recorded isometrically with Grass FT03B force-displacement transducers. Superfusion was then resumed in the absence of electrical stimulation with Krebs solution containing hemicholinium-3 (10 μM) and the 5-HT uptake inhibitor citalopram (0.1 μM) and the preparations were washed for 80 min the superfusate being discarded. After this time ^{3}H-overflow had reached a steady level and the superfusate was then collected every 1 min with 3 min agonist application periods and a 15 min dose cycle. Antagonists, when used, were present in the superfusing medium from the start of the post [^{3}H]-choline washout period.

In experiments designed to estimate relative activities of 5-HT, 2-methyl-5-HT and α-methyl-5-HT, each preparation received one agonist at three different concentrations applied as part of a Latin square balanced block design. At the end of every experiment the selective NK_3 receptor agonist senktide, which is known to release ACh from this preparation (Wormser *et al.*, 1986), was applied to each preparation as a positive internal control. Experiments of a similar design were performed with ondansetron (1 μM) present in the superfusing medium of two preparations from the start of the post [^{3}H]-choline washout, with the remaining two acting as concurrent controls.

To test for the possible involvement of other mechanisms or mediators, the effects of hexamethonium (10 μM), mepyramine (1 μM), guanethedine (1 μM) and ibuprofen (1 μM), (which were present throughout the contractility experiments), were assessed against responses to submaximal concentrations of 5-HT, 2-methyl-5-HT and α-methyl-5-HT. Additionally, ketanserin (1 μM) and spiperone (1 μM) were used in these experiments to test for 5-HT_2 and 5-HT_{1a} receptor-mediated influences. The possible involvement of non-5-HT_3 receptors which may mediate contraction or enhance electrically-evoked contractions in this preparation was also examined (see Sanger, 1987; Clarke *et al.*, 1989). Responses to 5-methoxytryptamine (1 μM and 10 μM), BRL 24924 (0.1 μM and 1 μM) and α-methyl-5-HT (3 μM and 30 μM), which are agonists at these receptors (Craig & Clarke, 1990; Eglen *et al.*, 1990; Hill *et al.*, 1990), were tested in the presence of ondansetron (10 μM) to ensure complete antagonism of 5-HT_3 receptors (see Hill *et al.*, 1990).

In experiments to estimate the affinity of ondansetron for the receptor mediating the responses to 5-HT and 2-methyl-5-HT, one preparation acted as a control, whilst the remaining three received ondansetron (0.1 μM, 1 μM or 10 μM) throughout. Each preparation then received three appropriately increased concentrations of agonist applied in a Latin square design to enable shifts in the agonist concentration-response line to be measured.

At the end of each experiment tissues were solubilised in NCS tissue solubiliser (Amersham); these and the superfusate samples were then assayed for radioactivity by liquid scintillation spectrometry. For each preparation the fractional release of initial ^{3}H content was calculated as rate coefficient (min^{-1}) for each collection period (for expression of ACh release in terms of ^{3}H overflow see, Szerb, 1976; Wikberg, 1977; Kilbinger & Pfeuffer-Friederich, 1985). Drug effects were expressed as a percentage increase in average rate coefficient during the 3 min drug treatment period over the average for the preceding 3 min control period. Results from each set of experiments were combined and expressed as mean ± s.e.mean increase in rate of loss in order to display averaged concentration-response curves. For each analogue pD_2 estimates obtained from individual experiments were combined and expressed as mean ± s.e.mean which thus reflects between-preparation variability. The equilibrium dissociation constant estimates for ondansetron expressed as the negative logarithm (pK_B) and s.e.mean, were obtained from individual dose-ratios (x) by direct calculation from the Gaddum-Schild equation ($pK_B = pA_2 = \log_{10}$ $(x - 1)$ $- \log_{10}[A]$) for competition, confirmed by Schild analysis, and assuming a Normal distribution of log $(x - 1)$.

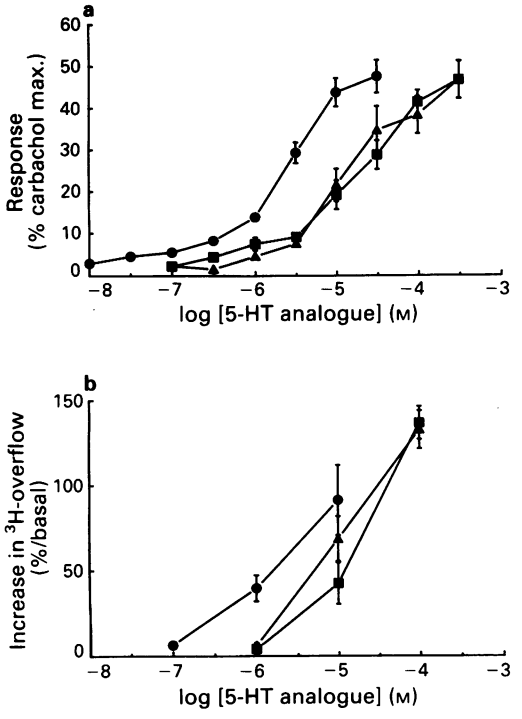

Figure 1 Log concentration-response curves for the effects of 5-hydroxytryptamine (5-HT) (●), 2-methyl-5-hydroxytryptamine (2-methyl-5-HT) (▲) and α-methyl-5-hydroxytryptamine (α-methyl-5-HT) (■). (a) Contractile responses of longitudinal muscle myenteric plexus (LMMP) strips. Results were obtained from preparations which received 5-HT and either 2-methyl-5-HT or α-methyl-5-HT. Each point represents the mean from 24 (5-HT) or 12 (2-methyl-5-HT and α-methyl-5-HT) preparations; s.e.mean shown by vertical bars. (b) Increase in spontaneous outflow of [^{3}H]-acetylcholine from LMMP strips preincubated with [^{3}H]-choline. Results were obtained from experiments in which each preparation received one agonist at three different concentrations. Each point represents the mean from 8 preparations; s.e.mean shown by vertical bars.

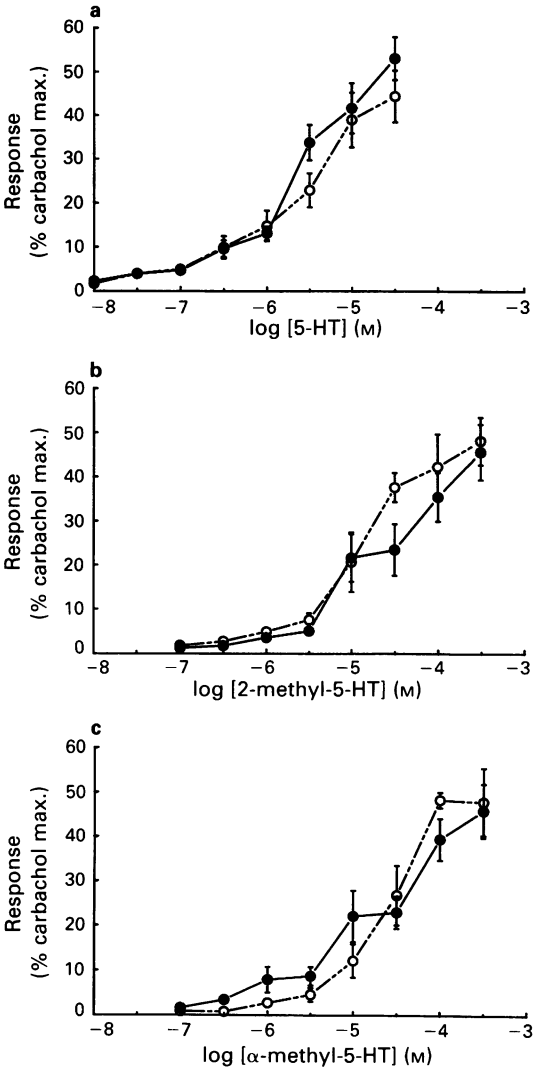

Figure 2 Effects of ketanserin on contractile responses of LMMP strips to (a) 5-hydroxytryptamine (5-HT), (b) 2-methyl-5-hydroxytryptamine (2-methyl-5-HT) and (c) α-methyl-hydroxytryptamine (α-methyl-5-HT). Symbols indicate concurrent control responses (●) or responses in the presence of ketanserin (1 μM) (○). Results were obtained from preparations which received 5-HT and either 2-methyl-5-HT or α-methyl-5-HT. In (a–c) each point represents the mean from 12 (5-HT) or 6 (2-methyl-5-HT and α-methyl-5-HT) preparations; s.e.mean shown by vertical bars.

Drugs

The following drugs were used:- [methyl-^{3}H] choline chloride (Amersham International plc); hemicholinium-3, 5-hydroxytryptamine creatinine sulphate, 5-methoxytryptamine hydrochloride, histamine diphosphate, mepyramine maleate, hexamethonium bromide (all Sigma); guanethedine monosulphate and ibuprofen (Ciba); citalopram (Lundbeck Ltd); senktide (Suc-[Asp^6,MePhe^8]-substance P(6–11); gift, Dr B.N. Williams, Merck, Sharpe and Dohme Laboratories); spiperone 2-methyl-5-HT, α-methyl-5-HT, ketanserin (all RBI); BRL 24924 ([(±)-(endo)]-4-amino-5-chloro-2-methoxy-N-(1-azabicyclo-[3.3.1]-non-4-yl)benzamide hyrochloride; gift, Dr G.J. Sanger, Beecham Pharmaceuticals) and ondansetron (GR38032F), (1,2,3,9-tetrahydro-3-[(methylimidazol-1-yl) methyl]-9-methyl-4H-carbazol-4-one; gift,. Glaxo Group Research Ltd.).

All stock solutions of drugs were prepared in distilled water except for ibuprofen (0.01 M sodium bicarbonate) and senktide (0.01 M acetic acid).

Results

Contractility studies

5-HT, 2-methyl-5-HT and α-methyl-5-HT produced concentration-response curves of similar shape and maxima

Table 1 Comparison of agonist activities for contraction and [^{3}H]-acetylcholine ([^{3}H])-ACh release in the guinea-pig ileum

	pD_2			Relative activities		
Measure	*5-HT*	*2-Me-5-HT*	*α-Me-5-HT*	*5-HT*	*2-Me-5-HT*	*α-Me-5-HT*
Contraction	5.70	4.80	4.70	1.00	0.13	0.10
(s.e.mean)	(0.05)	(0.05)	(0.08)			
[^{3}H]-ACh release	5.73	5.00	4.76	1.00	0.19	0.11
(s.e.mean)	(0.16)	(0.14)	(0.10)			

Activities of 5-hydroxytryptamine (5-HT), 2-methyl-5-hydroxytryptamine (2-Me-5-HT) and α-methyl-5-hydroxytryptamine (α-Me-5-HT) are shown both in terms of pD_2 ($-\log_{10} EC_{50}$) estimates and relative activities calculated from these values. Each pD_2 estimate for contraction represents the mean ± s.e.mean as shown from 24 (5-HT) or 12 (2-methyl-5-HT and α-methyl-5-HT) determinations; pD_2 estimates for [^{3}H]-ACh release are from 8 determinations for each analogue.

which allowed activity comparisons to be made in terms of the location parameter. 5-HT was approximately one order of magnitude more active than 2-methyl-5-HT and α-methyl-5-HT (Figure 1a) and relative activities of the three analogues calculated from averaged pD_2 estimates are shown in Table 1.

Responses to 5-HT, 2-methyl-5-HT and α-methyl-5-HT were unaffected by ketanserin (1 μM) (Figure 2) whilst ondansetron (1 μM) antagonized contractions to all three agonists. This concentration of ondansetron caused a parallel rightward shift of the upper part only of the concentration-response curves to 5-HT and α-methyl-5-HT but of the entire curve to 2-methyl-5-HT (Figure 3).

^3H-overflow studies

5-HT (0.1 μM–10 μM), 2-methyl-5-HT (1 μM–100 μM) and α-methyl-5-HT (1 μM–100 μM) all produced concentration-dependent increases in ^3H-overflow, used as an index of [^3H]-ACh release, which matched in time-course the concurrently recorded contractions of the tissue. Figure 4 shows a typical example of the experiments carried out in this study, including responses to the NK_3 receptor agonist senktide.

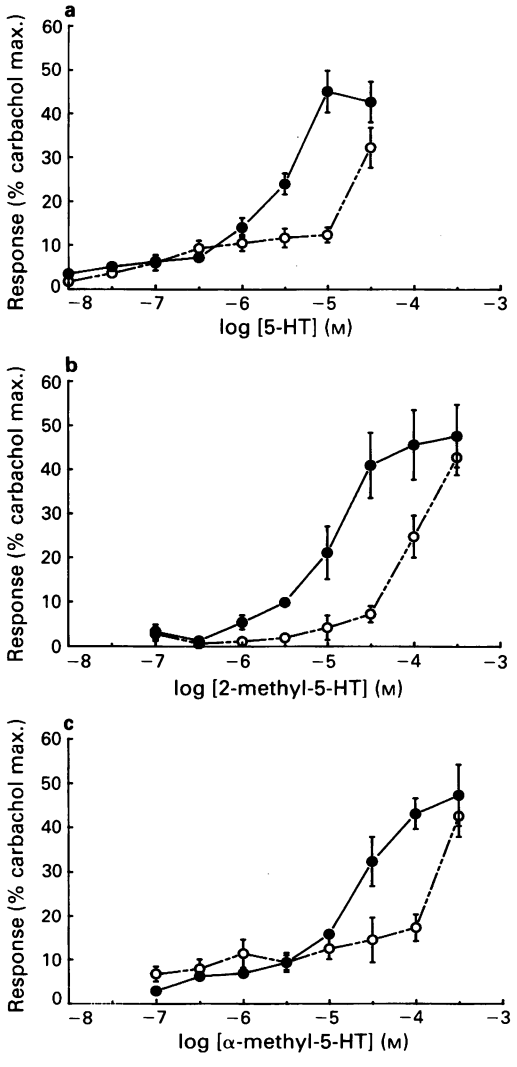

Figure 3 Effects of ondansetron on contractile responses of LMMP strips of guinea-pig ileum (a) 5-hydroxytryptamine (5-HT), (b) 2-methyl-5-hydroxytryptamine (2-methyl-5-HT) and (c) α-methyl-5-hydroxytryptamine (α-methyl-5-HT). Symbols indicate concurrent control responses (●) or responses in the presence of ondansetron (1 μM) (○). Results were obtained from preparations which received 5-HT and either 2-methyl-5-HT or α-methyl-5-HT. In (a–c) each point represents the mean from 12 (5-HT) or 6 (2-methyl-5-HT and α-methyl-5-HT) preparations; s.e.mean shown by vertical bars.

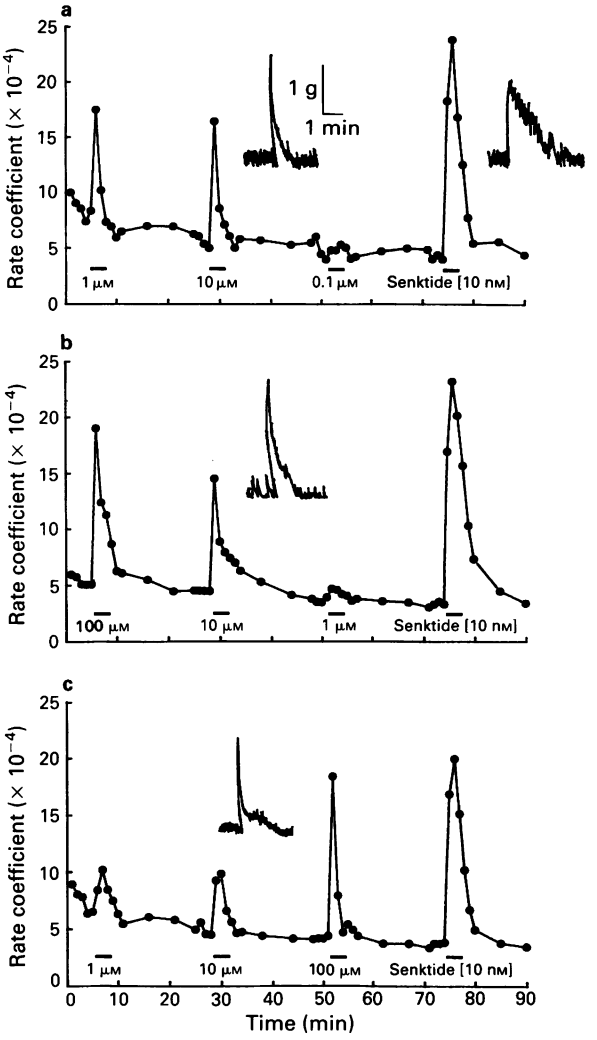

Figure 4 Effects of agonists on outflow of [^3H]-acetylcholine ([^3H]-ACh). LMMP strips of guinea-pig ileum were preincubated with [^3H]-choline and subsequently superfused with Krebs solution containing hemicholinium-3. Perfusate samples were collected every 1 min with 3 min agonist application periods shown by horizontal bars. Individual preparations shown received (a) 5-hydroxytryptamine (5-HT), (b) 2-methyl-5-hydroxytryptamine (2-methyl-5-HT) or (c) α-methyl-5-hydroxytryptamine (α-methyl-5-HT) at the concentrations shown with senktide (10 nM) applied lastly to all preparations. Concurrently recorded contractions are shown for 5-HT (10 μM) and senktide (10 nM) (a), 2-methyl-5-HT (100 μM) (b), and α-methyl-5-HT (100 μM) (c).

Concentration-response lines for the three agonists are essentially parallel (Figure 1b) and relative activity estimates are very similar to those obtained from the contractility studies (Table 1).

Ondansetron (1 μM) greatly attenuated the increases in ^3H-overflow evoked by the three agonists, producing a decrease in response to the highest concentrations used of 5-HT (10 μM), 2-methyl-5-HT (100 μM) and α-methyl-5-HT (100 μM) of 79.5 ± 6.9%, 70.1 ± 2.5% and 87.1 ± 12.9% respectively. Hexamethonium (10 μM), mepyramine (1 μM), guanethidine (1 μM), ibuprofen (1 μM), ketanserin (1 μM) and spiperone (1 μM) were all without effect on responses to each of the three agonists (data not shown). Additionally, no increase in ^3H-overflow was detected with either 5-methoxytryptamine, BRL 24924 or α-methyl-5-HT in the presence of ondansetron (10 μM) (data not shown).

Increasing concentrations of ondansetron gave parallel rightward shifts in the concentration-response lines for 5-HT and 2-methyl-5-HT (Figure 5a) and pK_B estimates obtained from individual dose-ratios were 7.62 ± 0.12 against 5-HT

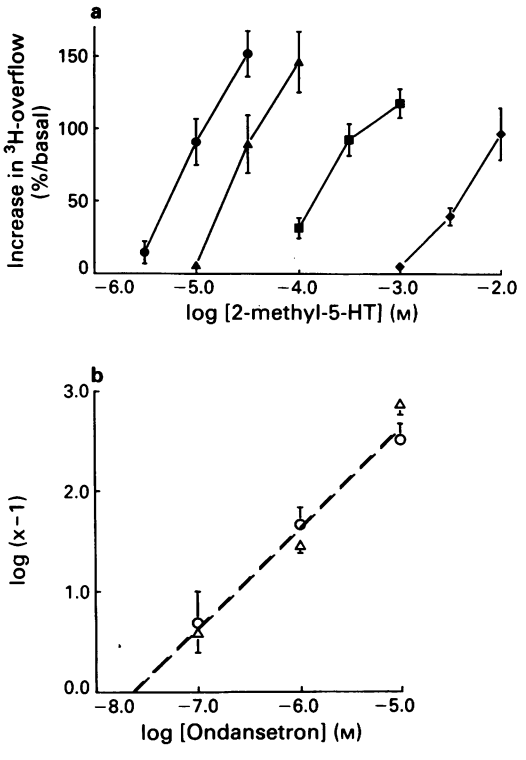

Figure 5 Ondansetron antagonism of 2-methyl-5-hydroxytryptamine-(2-methyl-5-HT) and 5-hydroxytryptamine-(5-HT) evoked increases in outflow of [^{3}H]-acetylcholine. (a) Parallel displacement of averaged concentration-response curves to 2-methyl-5-HT by ondansetron at three concentrations. Results were obtained from preparations receiving 2-methyl-5-HT alone (●) or in the presence of ondansetron at 0.1 μM (▲), 1 μM (■) or 10 μM (◆). Each point represents the mean from 5 preparations; s.e.mean shown by vertical bars. (b) Schild plot analysis of individual competition experiments of ondansetron against 5-HT (○) or 2-methyl-5-HT (△). The coefficient of linear regression did not differ significantly from 1.0 ($P > 0.05$) and so unity slope was imposed. The intercepts correspond to pA_2 ($= pK_B$) values of 7.62 and 7.64 respectively. Each point represents the mean from 4 (5-HT) or 5 (2-methyl-5-HT) determinations; s.e.mean shown by vertical bars.

($n = 12$) and 7.64 ± 0.09 against 2-methyl-5-HT ($n = 15$). Figure 5b shows the antagonist activity of ondansetron displayed in the conventional manner as Schild plots. Since there was no significant departure from unity slope, and the agonist and antagonist were presumed to be in an equilibrium state, the pA_2 intercept is taken to reflect the equilibrium dissociation constant K_B, expressed as a pK_B value.

Discussion

The results presented here show that in the guinea-pig ileum, under the conditions of this study, the contractile action of 5-HT, and in addition that of 2-methyl-5-HT and α-methyl-5-HT, are mediated predominantly through the activation of 5-HT_3 receptors. Thus, contractile responses to all three agonists were readily blocked by the selective 5-HT_3 receptor antagonist, ondansetron. In contrast, the selective 5-HT_2 receptor antagonist, ketanserin, was ineffective indicating that 5-HT_2 receptors have no significant involvement in responses to 5-HT and the two analogues. This conclusion is substantiated by the results from the ^{3}H-overflow studies, used as an estimation of ACh release, which show that the three agonists cause increases in spontaneous release in the same concentration-range as their contractile effects. Furthermore, the relative activities for 5-HT, 2-methyl-5-HT and α-methyl-5-HT in increasing ^{3}H-overflow are very similar to those for contraction, and this increase in release is sensitive to antagonism by ondansetron to a comparable extent for all three agonists. Taken together, three results are consistent with the notion that the contractile action of the three agonists can be accounted for largely in terms of 5-HT_3 receptor-mediated ACh release. In addition, they support the assumptions involved in estimating evoked ACh release in terms of increased ^{3}H-overflow.

These results also bear upon the suggested involvement of substance P (SP) in the contractile action of 5-HT in the guinea-pig ileum (Buchheit *et al.*, 1985). These authors proposed that 5-HT causes contraction through two different mechanisms. At low concentrations of 5-HT (<0.3 μM) it was proposed that activation of a high affinity, non-5-HT_3 receptor releases SP which in turn liberates ACh to cause contraction; at higher concentrations (>0.3 μM) 5-HT_3 receptor activation liberates SP which then causes direct contraction of the smooth muscle. It should be noted, however, that this work could not be repeated by other workers (Sanger & Nelson, 1989). Since we show that the action of 5-HT is mediated predominantly through ACh release this would seem to preclude a major direct action of SP. Furthermore, whilst from the results presented here we cannot rule out the proposal that SP, or some other neurokinin, mediates the ACh release measured following 5-HT application, our data do suggest that this is not likely under the conditions of our experiments.

In this regard, examination of the effects of ondansetron against the contractile activity of 5-HT, 2-methyl-5-HT and α-methyl-5-HT does indeed suggest the involvement of another, non-5-HT_3 receptor, component in the response to 5-HT. Thus, in the presence of ondansetron only the upper portions of the concentration-response curves to 5-HT and α-methyl-5-HT are shifted to the right, whereas the entire curve to 2-methyl-5-HT is shifted (see Figure 3). This effect of ondansetron on the 5-HT and 2-methyl-5-HT concentration-response curves confirms that reported by Butler *et al.* (1988), and the selective shift in the upper portion of the curve to 5-HT is seen also with other 5-HT_3 receptor antagonists such as BRL 43694 (Sanger & Nelson, 1989) and ICS 205-930 (Buchheit *et al.*, 1985). These results suggest that 2-methyl-5-HT contracts the ileum solely through 5-HT_3 receptor-mediated ACh release. It appears though, that some other receptor is involved in the response to 5-HT and also to α-methyl-5-HT, which produces the lower, ondansetron-insensitive, phase of their concentration-response curves. This receptor is likely to be the recently described excitatory neuronal non-5-HT_3 receptor (Clarke *et al.*, 1989; Hill *et al.*, 1990; Eglen *et al.*, 1990) which mediates ACh release and which corresponds to the high affinity receptor proposed by Buchheit *et al.* (1985; see above). However, we could not detect any increases in ^{3}H-overflow in response to 5-methyoxytryptamine, BRL 24924 or α-methyl-5-HT (each in the presence of ondansetron), all of which are reported to be agonists at this receptor (Eglen *et al.*, 1990; Hill *et al.*, 1990). This suggests that, under the conditions of our experiments, this non-5-HT_3 receptor plays at the most a minor role in the contractile action of 5-HT, and it is therefore unlikely that SP mediates the ACh release measured in this study in response to 5-HT.

As mentioned in the introduction, it has been suggested that the 5-HT_3 receptor of the ileum differs from those found in some other preparations. However, the use of contractility studies with the guinea-pig ileum for the study of 5-HT_3 receptors has been questioned since their activation is measured indirectly. For this reason 5-HT_3 receptors have recently been studied in the guinea-pig isolated vagus nerve, a new model for 5-HT_3 receptors in which their activation by 5-HT is measured directly. Here too, 5-HT_3 receptor antagonists produce lower pA_2 estimates than, for instance, in the rat vagus nerve (Burridge *et al.*, 1989; Lattimer *et al.*, 1989).

With this in mind we carried out experiments to obtain pA_2 estimates for ondansetron against 5-HT and 2-methyl-5-HT using direct estimates of ACh release through measurements of ^{3}H-overflow. The increases in release evoked by 5-HT and

2-methyl-5-HT were unaffected by any other of the antagonists tested (see Results) but were markedly inhibited by ondansetron. Figure 5 shows that pA_2 estimates can be taken to reflect true pK_B values and are indistinguishable when obtained for ondansetron against 5-HT or 2-methyl-5-HT, thereby indicating that both agonists are acting on the same receptor. This similarity in pK_B values also suggests that there is no significant activation of the excitatory neuronal non-5-HT_3 receptor by 5-HT since 2-methyl-5-HT is not an agonist at this site (Eglen *et al.*, 1990; Hill *et al.*, 1990). These pK_B values, both of 7.6, are slightly higher than previously reported pK_B (7.33; Butler *et al.*, 1988) or pA_2 (7.1; Lattimer *et al.*, 1989) values for ondansetron against 5-HT in the guinea-pig ileum which may reflect the complications seen with measurements made from contractility studies. The results presented here provide the first pK_B estimate for a 5-HT_3 receptor antagonist in the guinea-pig ileum obtained through direct estimates of neurotransmitter release. These estimates support the recent suggestion that 5-HT_3 receptors show considerable species variation, with that of the guinea-pig ileum being very similar to that of the guinea-pig vagus (Lattimer *et al.*, 1989).

Finally, it is clear from this study that α-methyl-5-HT, reported as a selective 5-HT_2 receptor agonist in preparations such as the rat uterus and rabbit aorta (see Bradley *et al.*, 1986) does not act in this way in the guinea-pig ileum. Given the fact that, under the conditions of these experiments, 5-HT_2 receptors are not involved in the response to 5-HT, α-methyl-5-HT would be expected to be inactive in this preparation. Instead, its general profile of activity is very similar to that of 5-HT and 2-methyl-5-HT, with it causing contraction and increases in [^{3}H]-ACh release via 5-HT_3 receptor activation, thereby throwing doubt on its use as a selective 5-HT_2 receptor agonist.

This work was supported by an SERC-CASE/Glaxo award to A.J.F. We thank Glaxo Group Research Ltd. for the gift of ondansetron and Dr G.J. Sanger for the gift of BRL 24924. We also thank Dr S.J. Ireland for his helpful criticism of the manuscript.

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(Received March 6, 1990
Revised July 6, 1990
Accepted July 10, 1990)

Br. J. Pharmacol. (1990), **101**, 559–562

Effect of intracerebroventricular administration of the $GABA_B$-receptor agonist baclofen on operant feeding in satiated pigs

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1 The present study investigated the effects of intracerebroventricular (i.c.v.) administration of the $GABA_B$-receptor agonist baclofen on food and water intake in satiated pigs previously trained to make operant responses for food and water, which were available *ad libitum*.

2 Baclofen (25–100 nmol) i.c.v. produced a dose-related increase in food intake. Baclofen (50 nmol) increased feeding during the first 15 min after administration ($P < 0.01$), while the 100 nmol dose increased feeding during the first 30 min ($P < 0.01$). None of these doses of baclofen had any affect on the daily (24 h) food intake.

3 The effect of baclofen (50 nmol) on feeding was prevented by pretreating the animals with the $GABA_B$ antagonist phaclofen (500 nmol, i.c.v.).

4 Baclofen (25–100 nmol) i.c.v. had no significant effects on water intake.

5 Intravenous administration of baclofen (100 nmol) had no effect on food intake, thus eliminating the possibility that i.c.v. baclofen might have stimulated feeding by a peripheral mode of action.

6 These results show that baclofen increases food intake in satiated pigs, and that this effect is mediated by the drug acting at central $GABA_B$-receptors.

Introduction

It is now widely accepted that γ-aminobutyric acid (GABA) is the major inhibitory neurotransmitter in mammalian brain (Cooper *et al.*, 1986), and in recent years considerable evidence has accumulated to support the view that a central GABAergic mechanism is involved in the control of food intake (Kelly *et al.*, 1979; Kelly & Grossman, 1980; Panksepp & Meeker, 1980). GABA exerts its effects by acting at two pharmacologically distinct receptor subtypes, namely $GABA_A$- and $GABA_B$-receptors (Bowery, 1989). Much of the work on food intake has focused on the $GABA_A$-receptor subtype, and it has been shown that intracerebroventricular (i.c.v.) injections of $GABA_A$-agonists, such as muscimol, increase meal size in a number of animal species, including the rat (Kelly *et al.*, 1979), the sheep (Seoane *et al.*, 1984), and the pig (Ebenezer & Baldwin, 1990). Moreover, microinjections of GABA and muscimol into the ventromedial nucleus or paraventricular nucleus of the hypothalamus also increase feeding (Kelly *et al.*, 1977; 1979; Grandison & Guiditti, 1977; Girard *et al.*, 1985), and these effects can be completely antagonized by $GABA_A$-receptor antagonists. The results of such studies thus indicate that a central $GABA_A$-receptor mechanism may be involved in the control of food intake. Further evidence to support this view comes from studies which show that drugs, such as the barbiturates and the benzodiazepines, which are believed to exert some of their pharmacological actions by acting on regulatory sites on the $GABA_A$-receptor to enhance the effects of endogenous GABA (Olsen, 1982; Turner & Whittle, 1983), also increase food intake.

Very little is known about the pharmacological and physiological actions of GABA that are mediated via $GABA_B$-receptors. We were therefore interested in finding out if pharmacological stimulation of central $GABA_B$-receptors would produce increased consumption of food and water. Thus, in the present study we investigated the effects of intracerebroventricular (i.c.v.) administration of the $GABA_B$-receptor agonist baclofen on food and water intake in pigs. The results show that baclofen causes a short-lasting dose-related increase in operant food intake, and that this effect can be antagonized by the competitive $GABA_B$-receptor antagonist phaclofen. Baclofen had no effect on fluid intake. A preliminary account of these results has been published in abstract form (Ebenezer & Baldwin, 1989).

Methods

Animals

Prepubertal large white pigs ($n = 18$; 10 male, 8 female) weighing between 30–60 kg, were used in these experiments. The pigs were housed individually in metabolism cages for the duration of the experiment. Each cage was equipped with two operant switch panels at the front end, which, when activated, delivered food (Right Panel) or water (Left Panel) into two bowls within the cage. The pigs were trained to press each panel with their snouts on a fixed ratio of 5 to obtain a single food reinforcement (10 g of pelleted pig food) or a single water reinforcement (10 ml of water).

Surgery

Each pig was implanted under halothane anaesthesia with an 18 gauge stainless steel guide tube aimed at the lateral cerebroventricle for subsequent i.c.v. injection of drugs. Details of the surgical procedures and the methods used for i.c.v. injections in the pig have been published previously (Baldwin & Thornton, 1986). In addition, two pigs were implanted under halothane anaesthesia with a catheter in the right external jugular vein for the intravenous (i.v.) administration of drugs. The catheter was exteriorized to the dorsal surface of the animal's neck.

Experimental procedure

Food and water were available *ad libitum*. Feeding and drinking were continously monitored for the duration of the experiment by means of a computer-based data logging system. In the first experiment we investigated the effects of baclofen on

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food and water intake. Injections of baclofen (25, 50 and 100 nmol) or vehicle (physiological saline solution) were administered i.c.v. between 10 h 00 min–11 h 00 min. The criterion for drug or saline administration was that pigs had not eaten for at least 30 min before injection. Drug sessions were usually conducted twice a week, and saline control sessions bracketed each drug session. The doses of baclofen were administered in random fashion. The logger recorded the amount of food (g) or water (ml) consumed by the pigs 0–15 min, 15–30 min, 30–60 min and 60–120 min after saline or baclofen. The total daily food and water intake was also recorded.

In the second experiment, we examined the effects of the $GABA_B$-antagonist phaclofen (50, 100 and 500 nmol i.c.v.) on food and water intake in the pigs. A similar protocol to that described for experiment 1 was used.

In the third experiment, the effects of pretreating the animals with phaclofen before the administration of baclofen were investigated. The pigs were injected with phaclofen (50, 100 or 500 nmol) or saline (control) i.c.v. 5 min before receiving a second i.c.v. injection of baclofen (50 nmol).

Statistical analysis

The effects of drug treatment on food and water intake for each pig was compared with the saline control values recorded on the day preceding the drug session by use of the two-tailed paired *t*-test. *P* values less than 0.05 were considered significant.

Drugs

The drugs used were (−)-baclofen (CIBA) and phaclofen (Cambridge Research Biochemicals). The drugs were dissolved in physiological saline solution (0.09% w/v). Physiological saline solution was used in control experiments.

Results

The pigs ate and drank *ad libitum*. They usually had a meal every 2–3 h, although considerable individual differences were noted among the animals as to the frequency and duration of their meals. The pigs normally drank water before they commenced eating, but also drank during and after the meal, and at various times in between bouts of eating. The metabolism cages were cleaned each morning between 9 h 00 min–9 h 30 min, and the pigs had a meal shortly thereafter. They were injected with saline or drug solutions about 30 min after their last meal, and were consequently not hungry at the time of injection. The animals were usually lying down in their cages before injection. Occasionally after i.c.v. administration of saline, the animals would get up and press the operant panels a few times for food and water, and this was thought to be due to the arousing effects of the injection procedure. Usually the pigs would continue to lie down in their metabolism cages after i.c.v. saline and not get up to eat or drink.

Effects of baclofen

Baclofen (25–100 nmol i.c.v.) caused a short-lasting dose-related increase in food intake (Figure 1). Baclofen (25 nmol) had no significant effect on feeding compared with control data. In contrast, baclofen (50 nmol) increased feeding ($P < 0.01$) during the first 15 min after injection, while the 100 nmol dose increased feeding ($P < 0.01$) during both the first and second 15 min periods after injection. The onset of eating usually began 2–5 min after administration. Administration of baclofen i.c.v. did not signficantly alter the total daily food intake of the animals, thus indicating that there was

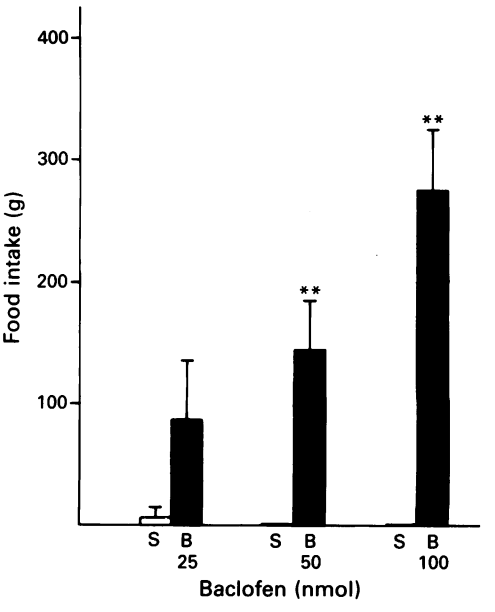

Figure 1 The dose-related effects of baclofen (25–100 nmol) on food intake (g) in pigs recorded in the first 15 min after i.c.v. administration. S = saline; B = baclofen. Vertical lines represent s.e.mean. ** $P < 0.01$. ($n = 9$ for 25 and 50 nmol doses; $n = 10$ for 100 nmol dose). Refer to text for further details.

no long-term effect of the drug on feeding (e.g. 2591 ± 322 g with saline controls and 2265 ± 340 g with 100 nmol baclofen treatment, $n = 10$). Baclofen had no significant effect on water intake compared with control data.

The 50 and 100 nmol doses of baclofen often caused the pigs to display signs of ataxia about 10–20 min after injection. The ataxia sometimes prevented the pigs pressing the food panel in a standing position, especially at the 100 nmol dose. However, they continued to eat and press the lever, sometimes from a semi-reclining position, for as long as they could. Eventually, the pigs succumbed to a state resembling deep sleep. They lay on their sides in the cages with their eyelids closed and were unresponsive to prodding in the ribs or to gentle shaking. These symptoms persisted in the majority of the pigs for between 2 and 3 h. After this time, the pigs displayed no signs of illness and began eating and drinking normally again.

Effects of phaclofen

The $GABA_B$-receptor antagonist phaclofen (50, 100 and 500 nmol i.c.v.) did not produce any significant short- or long-term effects on eating and drinking. Moreover, we did not observe any untoward behavioural side-effects with any of the doses of the drug.

Figure 2 shows the effects on food intake of pretreating pigs with phaclofen (50, 100 and 500 nmol i.c.v.) prior to administration of baclofen (50 nmol i.c.v.). Baclofen (50 nmol i.c.v.) given after saline caused a significant ($P < 0.05$) increase in food intake during the first 15 min after administration (Figure 2). Pretreatment with phaclofen (50 and 100 nmol) failed to reverse the effect of baclofen on feeding. In contrast, pretreating the animals with phaclofen (500 nmol i.c.v.) completely prevented the effects of baclofen (50 nmol) on food intake (Figure 2) and also abolished the motor and behavioural effects associated with i.c.v. administration of baclofen (50 nmol) (see above). The lower doses of phaclofen did not prevent or reduce the behavioural side-effects of baclofen. We observed no effects of phaclofen pretreatment on water intake.

Effects of intravenous administration of baclofen

In order to eliminate the possibility that i.c.v. baclofen produced its effects on food intake by a peripheral mode of action, the highest i.c.v. dose of the drug used in this study

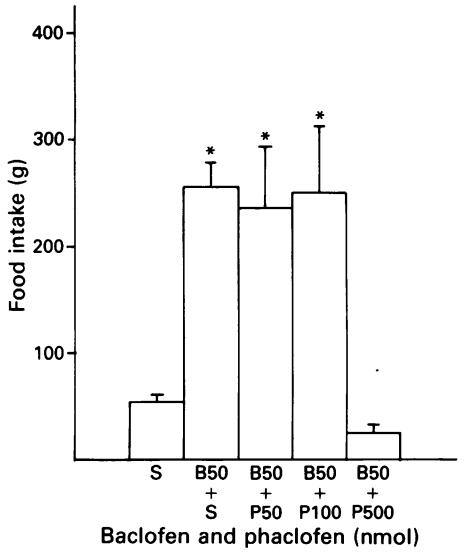

Figure 2 The effects of pretreating pigs with phaclofen (50, 100 and 500 nmol i.c.v.) on the feeding response elicited by baclofen (50 nmol, i.c.v.). S = saline; B = baclofen; P = phaclofen. Vertical lines represent s.e.mean. * $P < 0.05$, $n = 4$. Refer to text for further details.

was administered i.v. to 2 pigs. Systemic administration of baclofen (100 nmol) produced no increases in food or water intake, or any of the motor and behavioural side-effects associated with i.c.v. administration of this dose of the drug.

Discussion

The results of this study show that baclofen (25–100 nmol) i.c.v. causes a short-lasting dose-related increase in food intake in satiated pigs. Moreover, the observation that the highest dose of baclofen (i.e. 100 nmol) failed to elicit a feeding response when administered intravenously, suggests that this effect is centrally mediated. Operant methods were used in these experiments to eliminate the possibility that the increases in food intake were due to some non-specific effect, such as drug-induced chewing.

The mechanism(s) by which baclofen increases food intake is not known. However, it has been established that baclofen acts centrally on $GABA_B$-receptors to exert its pharmacological actions (Hill & Bowery, 1981; Bowery *et al.*, 1983). Thus, it is likely that baclofen enhances feeding by an action at the $GABA_B$-receptor subtype. This view is strengthened by the demonstration that the effect of baclofen on food intake is prevented by pretreating the pigs with the novel $GABA_B$-antagonist phaclofen (Kerr *et al.*, 1987). In this study a relatively high dose of phaclofen (i.e. 500 nmol, i.c.v.) was required to antagonize significantly the effects of baclofen (50 nmol) on feeding. However, these results are consistent with the observations of Kerr *et al.* (1987) who have demonstrated that phaclofen is a relatively weak antagonist at the $GABA_B$-receptor. In their experiments they found that the depression of guinea-pig ileal twitch responses produced by 8×10^{-6} M baclofen was only blocked by 2×10^{-4} M phaclofen.

Although the $GABA_A$-receptor is the most ubiquitious subtype in the CNS, $GABA_B$-receptors have been found throughout the CNS, in areas such as the cortex, hippocampus, cerebellum, hypothalamus, thalamus, striatum and spinal cord (Palacios *et al.*, 1981; Newberry & Nicoll, 1984; Bowery *et al.*, 1984; Gehlert *et al.*, 1985; Conzelmann *et al.*, 1986; Bonnano *et al.*, 1988). As there is strong evidence to support the view that the hypothalamus is involved in the control of food intake it is probable that i.c.v. baclofen acts in periventricular regions of this structure to stimulate feeding, although other brain areas may also be involved.

Recent experiments with GABA analogues and the $GABA_B$-antagonist phaclofen have suggested that $GABA_B$-receptors are not a homogeneous population (Scherer *et al.*, 1988), but there is more than one subset of $GABA_B$ receptors. The subtype of $GABA_B$-receptor on which baclofen acts to stimulate feeding in pigs has been termed 'phaclofen sensitive' (Kerr *et al.*, 1987). These 'phaclofen sensitive' receptors have been found at both pre- and post-synaptic sites (Pittaluga *et al.*, 1987; Dutar & Nicoll, 1988), and it is not possible at present to state whether baclofen is acting at a prejunctional, and/or postjunctional level to decrease eating.

Baclofen i.c.v. has no effect on water intake although normally, increases in food consumption in pigs are associated with increases in fluid intake (Bigelow & Houpt, 1988). The observation that there was no effect on the overall daily intake of food and water after baclofen (25–100 nmol) indicates that the animals accurately regulate their daily food and water consumption despite the acute actions of the drug.

Intracerebroventricular injections of the higher doses of baclofen (i.e. 50 and 100 nmol) caused the pigs to display signs of ataxia about 15–20 min after administration. However, despite the motor side-effects, the pigs continued to make operant responses for food, indicating a powerful drug-induced motivation to eat. A possible explanation of these motor disturbances is that they were due to the putative anaesthetic properties of baclofen. It has been demonstrated that both i.c.v. and systemic administration of baclofen can induce anaesthesia in rats and mice (Smith & Vestergaard, 1979; Sawynok & La Bella, 1981). Thus, it is possible that the i.c.v. doses of baclofen necessary to produce increases in feeding, eventually induce a state of anaesthesia. However, it is likely that the motor deficits and subsequent loss of consciousness are mediated by specific $GABA_B$-receptors as they were also abolished by phaclofen.

In conclusion, the results of this study indicate that stimulation of central $GABA_B$-receptors by baclofen will elicit feeding in satiated pigs. Further work is necessary to localize the site(s) of action of baclofen, and to establish whether a $GABA_B$-receptor-mediated mechanism plays a physiological role in the regulation of food intake.

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(Received March 12, 1990
Revised June 7, 1990
Accepted July 7, 1990)

Br. J. Pharmacol. (1990), **101**, 563–568

Spinal effects of four injectable anaesthetics on nociceptive reflexes in rats: a comparison of electrophysiological and behavioural measurements

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1 To assess the direct spinal contributions to the depression of reflexes caused by general anaesthetics, the intravenous potency of four injectable anaesthetics has been compared in two preparations: in decerebrate, spinalised rats, using a novel preparation requiring little surgical intervention, and in intact rats with chronically implanted i.v. cannulae.

2 Methohexitone (1–8 mg kg^{-1} i.v.), alphaxalone/alphadolone (0.5–8 mg kg^{-1} i.v.), alpha-chloralose (20–80 mg kg^{-1} i.v.) and ketamine (0.5–16 mg kg^{-1} i.v.) all produced a dose-dependent depression of single motor unit activity evoked by controlled noxious mechanical stimuli in decerebrate, spinalised animals.

3 The sedative and motor effects brought about by equivalent doses to those used in the electrophysiological experiments were assessed in intact rats. Methohexitone, alphaxalone/alphadolone and alpha-chloralose all caused similar levels of behavioural sedation at the doses that caused depression of spinal reflexes. Ketamine required relatively much higher doses to cause sedation.

4 To determine whether background anaesthesia modulated the potency with which these compounds affected spinal reflex activity, depressant effects in decerebrate, unanaesthetized rats were compared with those in animals maintained under anaesthesia with either alpha-chloralose or the steroid mixture of alphaxalone/alphadolone. The presence of either of these two agents as maintenance anaesthetics did not influence the effectiveness with which other compounds depressed nociceptive responses. However, additional doses of the maintenance anaesthetics were less effective than the same doses tested in decerebrate animals.

5 All the anaesthetics tested produced a significant depression of spinal reflex responses to noxious stimuli at doses well below those required for anaesthesia. Whilst the presence of maintenance anaesthetics appears not to distort pharmacological tests of other agents, there may nonetheless be a biasing of the samples of cells recorded.

Introduction

The group of compounds collectively known as general anaesthetics act to produce a widespread depression of the central nervous system involving hypnosis, analgesia, reflex suppression and relaxation of voluntary muscle. For each agent, the relative magnitudes of these effects are unique and vary with the level of anaesthesia.

It has been shown that anaesthetics exert direct influences on the spinal cord, on both dorsal and ventral horn neurones, so as to modulate spinal function in response to sensory stimuli (for reviews see Heavner, 1975; Davidoff & Hackman, 1983; see also Lodge & Anis, 1984). Although some similarities in modulation of transmitter action may be observed between compounds (e.g. alphaxalone/alphadolone, chloralose and barbiturates have all been shown to modulate the actions of γ-aminobutyric acid (Davidoff & Hackman, 1983; Lambert *et al.*, 1987)), clinical and behavioural observations of anaesthesia indicate that there are marked differences even within such groupings.

Despite the potential influences these compounds have on the central nervous system, the majority of electrophysiological studies concerned with spinal processing of sensory stimuli *in vivo* have been undertaken in the presence of one or several of a wide variety of gaseous and injectable anaesthetics. In view of this, it was the intention in this study to generate information relevant to three questions. Firstly, what are the relative potencies of various injectable anaesthetics within the spinal cord in depressing spinal nociceptive reflexes? Secondly, what is the relationship between behavioural sedation induced by injectable anaesthetics of various classes and spinal depression of nociceptive reflexes? Thirdly, to what extent are different maintenance anaesthetic regimes likely to affect the results obtained in unitary pharmacological tests of spinal function when performed *in vivo*?

A model was developed which requires little surgical intervention compared with more standard electrophysiological measurements of spinal function and which allowed continuous, stable recording of the reflex activity of single motoneurones to controlled noxious stimuli. This preparation reduces the influence that surgery has on the potencies with which anaesthetics depress spinal reflexes (Hartell *et al.*, 1990) and enabled comparisons to be made between different compounds in the same animal under the same conditions. Such results were compared with the behavioural effects produced by equivalent doses administered to conscious animals chronically prepared with intravenous cannulae.

Methods

Preparation of animals for electrophysiological recording

Surgery was performed, under halothane anaesthesia, on 62 male Wistar rats weighing between 250 and 400 g; respiration was spontaneous throughout and inspired air was supplemented with oxygen. Cannulae were inserted into the trachea,

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carotid artery and jugular vein. A small incision was made in the low thoracic region and the musculature covering the dorsal surface of the spinal cord between the thoracic 9–11 vertebrae retracted. One or two laminae were removed and the spinal cord transected at the level of thoracic 9–10 vertebrae. All wound surfaces were covered throughout with lignocaine (1%) plus adrenaline (1:200,000) and the skin incisions repaired with sutures.

Rectal temperature was monitored continuously and maintained at 37°C with the aid of a feedback controlled blanket and a dorsally positioned lamp. Blood pressure was also monitored and used as an indication of technical acceptability. Experiments were terminated if the systolic pressure fell below 100 mmHg for any sustained period.

Animals were then prepared according to one of three experimental protocols. One group was decerebrated under halothane anaesthesia by aspiration of all cranial contents rostral to a mid collicular level, and anaesthesia discontinued. A second group was given a $50\,mg\,kg^{-1}$ i.v. dose of alpha chloralose (dissolved in either a borax buffered solution or in isotonic saline) and the halothane discontinued. Supplementary doses of $20\,mg\,kg^{-1}$ i.v. were given at intervals of approximately one hour. Rats in the third group were anaesthetized with a continuous infusion of alphaxalone/alphadolone (6–$12\,mg\,kg^{-1}$ i.v.) and the halothane discontinued. The right hind limb was immobilised in a plaster of Paris cast and a period of at least one hour allowed before recording, so as to allow recovery from the effects of halothane and to permit recovery from the spinalisation. Fluid, either isotonic saline or Haemaccel (Hoechst), was given over the course of the experiment at a rate of approximately $80\,ml\,kg^{-1}\,24\,h^{-1}$. In some cases, particularly with decerebrate animals, 1–2 ml of whole blood was given postoperatively.

Electrical activity was recorded from hind limb flexor muscles by tungsten microwires embedded in a needle. The wires were 50 μm in diameter with a Teflon coating making the total diameter 75 μm. Electrodes were inserted through a small skin incision into either biceps femoris or, more usually, into flexor digitorum longus or flexor hallucis longus. Single motor unit responses were evoked by application of noxious pinch stimuli to the receptive field on the ipsilateral hind limb. Receptive fields were similar between animals and invariably encompassed the first and second most lateral toes and extended towards the hock. Stimuli were delivered by means of a pair of electronically controlled pneumatically driven Allis tissue forceps (Brown *et al.*, 1984) and were applied for a period of fifteen seconds at intervals of three minutes. Although the force of the stimulus applied to the limb was not measured directly, it was applied at similar pneumatic pressures between animals. Units were selected which responded with minimal adaptation both over the course of each stimulus and between successive stimuli.

Single motor units were discriminated according to spike height; each spike was delayed and displayed on an oscilloscope so that spike configuration could be monitored, thereby ensuring single unit recording over the entire experiment. Chart records were made of the number of spikes per second and of counts of the number of spikes during various parts of the stimuli. Spike counts over a period of 'early pinch', the first five seconds, were separated from those over the subsequent ten seconds ('late pinch'). It is presumed that the early phase of the pinch contains a considerably greater rapidly adapting, low threshold component. Most cells showed adaptation over this period and drugs affected this to a lesser extent. Therefore, quantitative analysis was restricted to the period of late pinch. A microcomputer was used for the online calculations of drug effects (Headley *et al.*, 1985); these were expressed as percentages of the mean of three stable control responses before drug administration. Results were only considered to be technically acceptable if the recovery from the maximum drug effect exceeded at least 50% of the reduction and the time course of recovery matched that expected for the known kinetics of the compound under study. The only exception to this was for alpha-chloralose which has too long-lasting an action for this criterion to be feasible.

Behavioural methods

Indwelling intravenous cannulae were implanted in sixteen male rats under halothane anaesthesia. Polythene tubing (i.d. 0.58, o.d. 0.96 mm), prefilled with sterile isotonic saline, was inserted aseptically through a small ventral incision, into the right jugular vein via a small side branch. The free end was passed under the skin and out through a dorsal incision at the back of the neck. Animals were allowed to recover from the surgery for at least 96 h before experimentation. They were housed individually and were given food and water *ad libitum*. To reduce the number of animals required, several drugs were tested on each animal. To minimize possible interactions between compounds tested, different drugs were tested on different days and the order of testing was rotated. All experiments were carried out in a quiet room free from interruption.

Following intravenous administration of the various compounds, several simple behavioural activities were assessed. These included the presence or absence of a righting reflex, and arbitrary scales of the degree of ataxia produced. From these observations, several estimations of drug-induced motor impairment were made. These were firstly, the dose required to produce a minimal ataxia in at least 75% of the animals (defined as the inability of the animal to walk on the rim of its cage), secondly the dose required to produce maximal ataxia (seen as an inability to walk on a flat surface), thirdly, the dose required to eliminate the righting reflex in at least 75% of the animals and, finally, the loss of spontaneous movements such as blinking and whisker twitch. Complete recovery of motor coordination was monitored.

Drugs and administration protocol

In order to allow comparisons between electrophysiological and behavioural experiments, the same drugs and administration procedures were used. The following compounds were selected for study: methohexitone (Eli Lilly), a short acting barbiturate; ketamine hydrochloride (Parke-Davis), a dissociative anaesthetic; the steroid mixture of alphaxalone/alphadolone (Saffan; Pitman-Moore) and alpha-chloralose (Sigma). All doses were administered in logarithmic (base 2) increments starting with doses found to be near threshold for effects on most cells in the electrophysiological tests. Those compounds with a short time course (methohexitone and alphaxalone/alphadolone) were administered as discrete doses at intervals appropriate for complete recovery judged according to both observation and their reported elimination kinetics. Ketamine and alpha-chloralose produce longer-lasting effects and so were given in a cumulative regime at intervals of 6 and 15 min, respectively (chloralose has been shown to have delayed onset: Collins *et al.*, 1983); this regime was halted once the evoked responses had been reduced to about 25% of control or less. The effects of alpha-chloralose last for hours, so in the acute experiments cumulative doses were given only as the last drug to be tested, and recovery was not followed. This regime of drug administration was necessary in the electrophysiological experiments to allow several drugs to be tested on each animal, thereby allowing both direct comparisons to be made between compounds and enabling numbers of experiments to be kept to a minimum. Since complete dose-response regimes were not performed on all units tested, the traditional mean dose-response analysis was not possible. As higher doses of drug were only tested on those units which were more resistant, the apparent dose-response curves for the collected data are more shallow than expected (see Parsons & Headley, 1989).

As it was the aim of the behavioural experiments to study the effects of doses which caused known degrees of depression

of spinal reflexes in the electrophysiological experiments, the same doses were used. As can be seen in the results section, this led to rather large steps in behavioural effects; in some cases a single dose increment caused effects that spanned 2–3 of the behaviourally defined states.

Results

Comparison of the depression of spinal reflexes caused by anaesthetics under decerebrate and different baseline anaesthetic protocols

The potencies of the compounds used in this investigation in suppressing spinal reflexes were compared in three groups of animals, namely those anaesthetized with either alphaxalone/alphadolone (23 rats) or with alpha-chloralose (21 rats), and a group of unanaesthetized decerebrate rats ($n = 18$). Dose-response relationships were obtained for alpha-chloralose (20–80 mg kg^{-1} i.v.), methohexitone (1–8 mg kg^{-1} i.v.), alphaxalone/alphadolone (0.5–8 mg kg^{-1} i.v.) and ketamine (0.5–16 mg kg^{-1} i.v.) in each of the experimental groups.

Figure 1 provides examples of the effects of two different anaesthetics on single motor unit responses to noxious pinch stimuli, recorded from two decerebrate, unanaesthetized animals. The top trace reveals that methohexitone depressed this 'naturally'-evoked response in a dose-dependent and rapidly reversible manner; the recovery seen between doses corresponds to that expected for this rapidly metabolised barbiturate. Alpha-chloralose also produced a dose-dependent depression of spinal reflex activity, but recovery was not followed because of the long acting nature of the drug.

In order to allow quantification of data and subsequent comparative analysis, the effects of each dose of drug were measured and the data subsequently pooled for the units in each of the three experimental groups: the calculation of reductions was the percentage of the control number of spikes evoked during late pinch. Wherever practicable, increasing doses of the drugs were administered until a dose was reached at which evoked activity was reduced to below 25% of control. However, as is often the case with this kind of *in vivo* pharmacological study, there was much variation in drug potency even between animals prepared in the same way. In order to illustrate the collected data between the three groups, the results are displayed in bar chart form. Figure 2 is an example showing the relative effectiveness of methohexitone and ketamine. Neither of these compounds displayed any preferential potency between the three experimental groups; this was confirmed statistically by use of both non-parametric (Mann-Whitney U-test) and parametric (Student's t) tests).

Such equipotency of drug action between decerebrate and anaesthetized animals was characteristic, except for those instances in which the drug under test was also being used as the maintenance anaesthetic. Figure 3 is a firing rate record of a single motor unit responding to pinch stimuli which were uniform throughout, and shows the depression of reflex activity caused by alphaxalone/alphadolone under two different baseline anaesthetic conditions. The same dose of alphaxalone/alphadolone had less effect on the reflex whilst the animal was maintained on alphaxalone/alphadolone than when the background anaesthetic was subsequently switched to alpha-chloralose. Note that the firing rates evoked during the stimuli before testing were similar in the two cases.

This phenomenon was also seen when the pooled data between the groups were compared. Figure 4 shows bar charts of the dose-dependent effects of alphaxalone/alphadolone and alpha-chloralose. Figure 4a shows that the same doses of alphaxalone/alphadolone (0.5–8 mg kg^{-1} i.v.) were significantly less effective when given to animals already anaesthetized with alphaxalone/alphadolone than when administered to chloralose anaesthetized or decerebrate animals. This suggests that the presence of chloralose as a

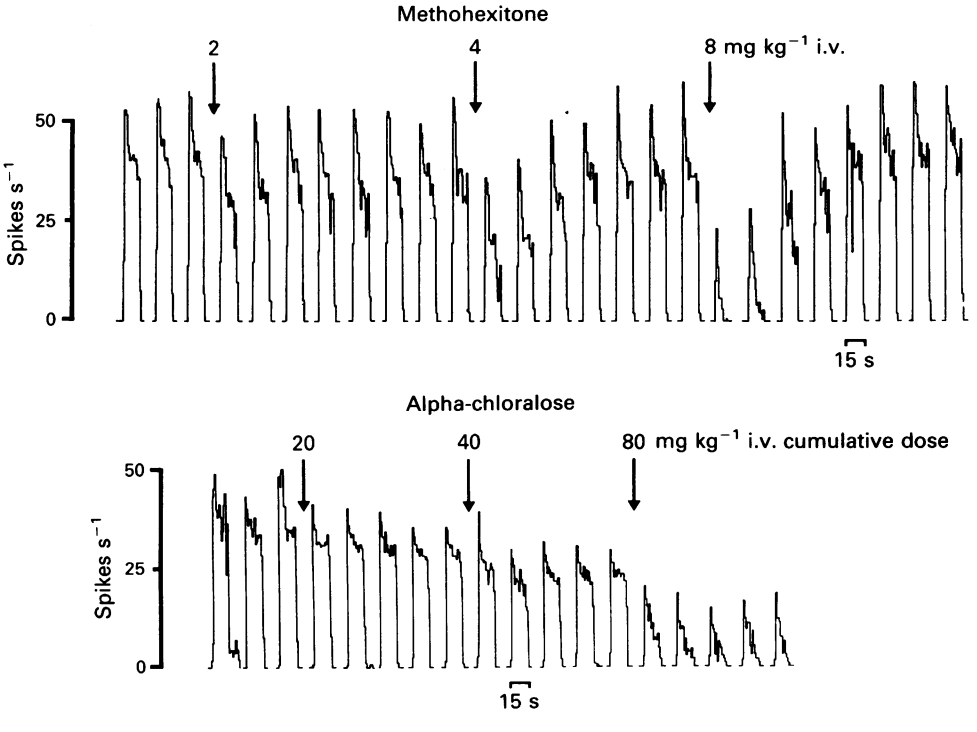

Figure 1 Chart records displaying the effects of two anaesthetics on stimulus-evoked firing of single motor units recorded from the flexor digitorum longus muscle in decerebrate, spinalised rats. Fifteen second pinch stimuli were appled to a single toe of the ipsilateral hind paw of the rats and were repeated at three minute intervals. The chart was halted between pinch stimuli. Methohexitone (top trace) caused a dose-dependent and rapidly reversible reflex depression over the range of 2–8 mg kg^{-1} i.v. In view of the short acting nature of this barbiturate, additional doses were given only following recovery from the previous dose to control levels. The lower trace displays the effect of alpha-chloralose (20–80 mg kg^{-1} i.v.) administered in a cumulative dose regime. The depressant effect of alpha-chloralose on these spinal reflexes is maintained for several hours, as is expected for this long lasting anaesthetic, and so recovery was not followed. The peak effect of alpha-chloralose did not occur, in some cases, for up to 15 min after administration.

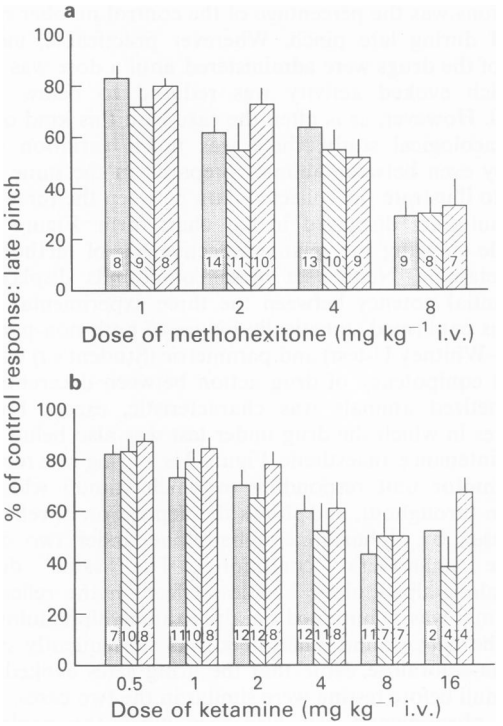

Figure 2 Dose-dependent effects of methohexitone (a) and ketamine (b) on noxious pinch evoked single motor unit activity. Responses, expressed as a percentage of the mean pre-test control values are compared at each dose between animals anaesthetized with alphaxalone/alphadolone (▩), those anaesthetized with alpha-chloralose (▧) and decerebrated, unanaesthetized rats (▨). Analysis was restricted here to counts of the spikes evoked during the late part of the pinch stimulus. Bars show s.e. and numbers within columns are the numbers of units recorded.

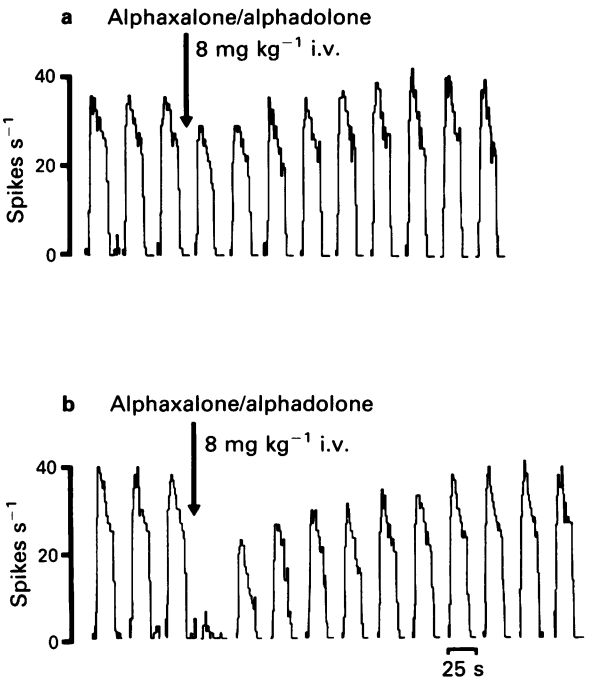

Figure 3 Chart record of single motor unit firing rate recorded from flexor digitorum longus, illustrating the different effects of a single dose of alphaxalone/alphadolone (8 mg kg^{-1} i.v.) when given under different maintenance anaesthetic protocols. The pinch stimuli were delivered at the same site (toe 5) with the same force for 15 s, repeated once every 3 min throughout the experiment. (a) Recorded whilst the animal was anaesthetized with alphaxalone/alphadolone infused at a rate of 12 mg kg^{-1}h^{-1}. Over a subsequent two hour period, the maintenance anaesthetic was changed to alpha-chloralose (60 mg kg^{-1} i.v. initially with 20 mg kg^{-1} i.v. supplementary) and the effects of the steroid infusion allowed to wear off. The same drug test was then repeated (b). The effects were completely reversible.

maintenance anaesthetic does not affect the potency with which alphaxalone/alphadolone depresses single motor unit response. The results shown in Figure 4b indicate that chloralose behaved in an equivalent manner.

It is possible that a significant difference in neuronal responsiveness, evident as disparate levels of evoked activity, might account for the difference between anaesthetic states. Although we were unable to measure directly the force with which the stimuli were applied, the same pinching device, operated at consistent pneumatic pressures, and always applied to one toe, was used in all animals. We were therefore able to estimate relative excitability by comparing the mean firing rates evoked by each stimulus during control periods (prior to drug administration) between the decerebrate and anaesthetized groups of animals. The following results were obtained and are expressed as mean firing rate during late pinch ± s.e.mean for n cells tested: decerebrate rats, 30.4 ± 1.8 spikes per second ($n = 9$); chloralose anaesthetized, 21.3 ± 3.1 ($n = 11$); alphaxalone/alphadolone anaesthetized 24.2 ± 1.8 ($n = 12$). The mean firing rates obtained in decerebrate animals were significantly higher ($P < 0.02$; unpaired Student's t test) than in chloralose or alphaxalone/alphadolone anaesthetized animals, but there was no significant difference between the two groups of anaesthetized animals.

Comparison of the depression of reflex activity caused by behaviourally similar doses of four anaesthetics

Sixteen conscious intact rats, previously implanted with intravenous cannulae, were used for the behavioural studies. Each anaesthetic was examined at the same doses as those tested in the electrophysiological experiments, and the behavioural effects (see Methods for criteria) and the time courses of recovery noted. The time courses of recovery from doses of

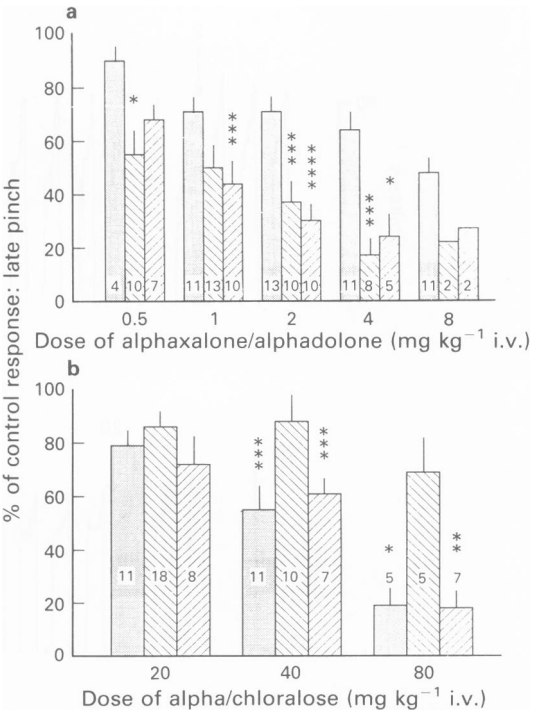

Figure 4 Dose-dependent effects of alphaxalone/alphadolone (a) and alpha-chloralose (b); presented in the same format as Figure 2, but with the addition of statistical data. Where indicated, significance was reached (Mann-Whitney U-test, * $P < 0.05$, ** $P < 0.025$, *** $P < 0.01$ and **** $P < 0.001$, 1 tailed). Data were compared between those obtained from animals anaesthetized with alphaxalone/alphadolone (a) and alpha-chloralose (b). For key to shading of columns see Figure 2 legend.

methohexitone and alphaxalone/alphadolone were similar to those observed in the electrophysiological experiments, having approximate half recovery times of 3–4 and 6 min respectively. The effects of ketamine and alpha-chloralose were more long lasting and these compounds were found to have full recovery times of about 2.5–3.5 h (from a total dose of ketamine of 128 mg kg^{-1} i.v.) and 5–6.5 h (from a total dose of alpha-chloralose of 80 mg kg^{-1} i.v.). Table 1 shows the behavioural effects produced by the four anaesthetics. Alongside these data are shown the effects on nociceptive spinal reflexes measured in spinalised, decerebrated animals.

By comparing our electrophysiological data with the behavioural observations, it is apparent that spinal reflex activity in decerebrate spinalised animals was significantly depressed by the doses of these compounds which produced ataxia in intact animals. However, if one compares different compounds, the ataxic effects occurred at very different proportions of the doses needed to cause immobility (i.e. the doses which approach those needed for adequate experimental anaesthesia). For example, while alphaxalone/alphadolone produced depression of reflexes and ataxic effects only at doses approaching those causing loss of consciousness, ketamine depressed motor coordination and spinal reflexes at a dose much smaller than that required to produce some degree of dissociative anaesthesia. These differences are highlighted by the ratio of the dose producing loss of spontaneous motor activity to that producing maximal ataxic effects. Within the limits of the dose regime utilised the following ratios were found, ketamine = 16; alphaxalone/alphadolone = 1; methohexitone = 1; chloralose = 2.

Discussion

The first question posed in this study, the relative potency within the spinal cord of these anaesthetics in depressing nociceptive reflex responses of spinal flexor motoneurones, is answered by the experiments on decerebrate spinalised rats, and is summarized in Figures 2 and 4.

In order to assess the relevance of these spinal actions of anaesthetics to those experiments in which anaesthetics need to be used, it is necessary to relate the doses causing depression of spinal reflexes to those causing the various stages of anaesthesia. For this reason the same intravenous doses as those examined in our current electrophysiological experiments were tested in chronically prepared conscious animals of the same strain.

One major problem of such comparisons is that because these compounds act in such different ways, it is difficult to select the criteria for determining equivalent levels of anaesthesia. We have approached the problem by observing arbitrarily defined dose-related degrees of behavioural sedation and by comparing these with the reflex depression caused by equivalent doses in spinalised decerebrate animals. Whilst the behavioural states we recorded do not necessarily equate to anaesthesia *per se*, they are, according to text book definitions, phases through which animals pass during induction and may be used to help define various planes of anaesthesia.

The spinal flexion reflex was depressed in decerebrate, spinalised rats at doses of each drug that produced a minimal ataxia in intact rats. The relative depression of the reflex from control levels was, however, different between drugs, methohexitone having the greatest effect (Table 1). Those doses that prevented the animals from walking ('maximal ataxia') in all cases had a greater direct effect on the spinal cord, causing a significantly greater depression of nociceptive reflexes. These data therefore indicate that anaesthetics have considerable depressant actions on the spinal cord at sub-anaesthetic doses. For alphaxalone/alphadolone and methohexitone, the dose-response relationship was sufficiently steep for maximal ataxia and loss of spontaneous activity to be encompassed by one dose step within our logarithmic administration protocol. The depression of reflexes caused by equivalent doses was significant.

Alpha-chloralose is often considered to protect reflex responses (Shimamura *et al.*, 1968) and alphaxalone/alphadolone has been shown to preserve spinal reflexes at doses which cause hypnosis (Lovick, 1986). In contrast, barbiturates are often considered to depress spinal activity relatively more. The present data, however, suggest little difference in the direct depressant effect of these three anaesthetics on spinal reflexes at the lowest dose required to cause loss of spontaneous activity.

The third aim of this set of experiments was to determine whether anaesthetics, when used to maintain animals in an anaesthetized condition suitable for experimental recording, are likely to affect the potency with which other drugs depress nociceptive spinal reflex transmission. Over the dose ranges indicated, neither ketamine nor methohexitone displayed any differential potency between tests performed in spinalised animals that were either unanaesthetized and decerebrate, or anaesthetized with either alpha-chloralose or alphaxalone/alphadolone. This suggests not only that there is no differential effect between these two maintenance anaesthetics, but also that the presence of either of these two anaesthetics does not interfere with the spinal reflex depressant actions of ketamine or methohexitone.

Work already carried out in this laboratory (Parsons & Headley, 1989) has shown that if two intensities of pinch are given so that the same motoneurone responds alternately to strong and weak pinch stimuli with high and low firing rates respectively, then the weaker of the responses is invariably reduced to a greater degree by the same dose of drug, whether opiate or anaesthetic. This highlights the importance of matching stimulus intensity if one is to compare the potencies of compounds. Although we have made a direct comparison between the three experimental groups, the question still arises as to whether the noxious stimuli administered in the present study may have been different between groups. Whilst we cannot state that the intensity of stimuli given to each of the groups was identical, we did use constant pneumatic pressures to drive the stimulator as well as similar placements of the pincher device on the toes, so that it is unlikely that there

Table 1 Comparison of the behavioural and the reflex depressant effects of four injectable anaesthetics

Anaesthetic	*Lowest dose causing minimal ataxia* (mg kg^{-1} i.v.)	*Effect on reflex responses at same dose* (% control)	*Lowest dose causing maximal ataxia* (mg kg^{-1} i.v.)	*Effect on reflex responses at same dose* (% control)	*Lowest dose causing loss of righting reflex* (mg kg^{-1} i.v.)	*Lowest dose causing loss of spontaneous activity* (mg kg^{-1} i.v.)
Ketamine	1 (n = 7)	84 ± 3.1 (n = 8)	8 (n = 7)	50 ± 12.3 (n = 7)	64 (n = 7)	128 (n = 7)
Alphaxalone/alphadolone	0.5 (n = 7)	68 ± 6.6 (n = 7)	4 (n = 8)	24 ± 10.4 (n = 5)	4 (n = 8)	4 (n = 8)
Methohexitone	4 (n = 8)	52 ± 7.5 (n = 9)	8 (n = 8)	33 ± 11.4 (n = 7)	8 (n = 8)	8 (n = 8)
Alpha-chloralose	20 (n = 16)	72 ± 9.8 (n = 8)	40 (n = 16)	61 ± 5.9 (n = 7)	80 (n = 16)	80 (n = 16)

See Methods for the interpretation of the behavioural terms used. The doses causing each level of behavioural depression were those having effects in >75% of the rats tested. For 'minimal ataxia' and 'maximal ataxia' the effect of this dose on the electrophysiologically recorded reflexes is also shown: values are % of pre-drug controls.

were consistent differences in stimulus intensity between the groups. Our examination of the firing rates of motor units prior to drug administration indicates that in the decerebrate preparations, the pinch stimuli elicited a slightly but significantly greater firing rate. This was not, however, associated with any corresponding decrease in the potency of methohexitone or ketamine, as might have been predicted from the previous study (Parsons & Headley, 1989).

Equipotency of drug action between the three experimental groups only occurred when the test compound differed from the maintenance anaesthetic. For example, alpha-chloralose displayed similar effects when administered to alphaxalone/alphadolone anaesthetized or decerebrate animals, but was significantly less potent when given as additional doses to chloralose maintained animals. Similar effects were seen for alphaxalone/alphadolone which was selectively less potent on animals already anaesthetized with this steroid anaesthetic. There are two possible explanations for these findings. If we consider a hypothetical dose-response curve for alpha-chloralose, for example, then an animal maintained on this compound would be expected to have a modified reflex response compared to a decerebrate, unanaesthetized animal. This is suggested by the data since the firing rates during pre-drug control responses were lower in anaesthetized than in decerebrate preparations. One might expect, therefore, that in anaesthetized animals, we are looking at drug effects over the upper part of the dose response curve and that, accordingly, the effects of subsequent additional doses are less than when administered to decerebrate animals. Scrutiny of the data suggests an alternative explanation, namely that there are different populations of units, some more resistant to particular drug action than others. Therefore, whilst searching for units under one particular maintenance anaesthetic, we automatically bias our sample of units to those which are resistant to that compound. This is suggested by the observation that the mean stimulus evoked firing rate in rats with an anaesthetic dose of alpha-chloralose (about $80\,mg\,kg^{-1}$ i.v. as indicated by the behavioural experiments) was 70% of that in decerebrate unanaesthetized animals. In contrast, Figure 4 suggests that such a dose would have reduced responses of the units recorded in decerebrate preparations to about 20% of control.

We can conclude, firstly that all the anaesthetics tested act in the spinal cord to produce a significant depression of spinal reflex responses to noxious stimuli at doses below those required for full anaesthesia. Methohexitone, alphaxalone/alphadolone and alpha-chloralose had similar direct actions on the cord to reduce spinal reflexes at doses causing similar levels of behavioural sedation. Secondly, it appears that neither the presence of the anaesthetic, nor the choice between alphaxalone/alphadolone and chloralose, influenced the effectiveness with which various other compounds depressed naturally evoked single motor unit responses evoked by noxious somatic stimuli. This is suggested both by the tests performed in this study with other anaesthetic agents, and by data with μ and κ opioids (Hartell & Headley, 1989 and unpublished observations). Whilst this finding implies that the use of anaesthetics may not be detrimental to pharmacological testing *in vivo*, the decreased potency of additional doses of the same anaesthetic may be explained by biasing the cells recorded.

This work was funded by the Medical Research Council.

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(Received March 15, 1990
Revised June 8, 1990
Accepted June 26, 1990)

Br. J. Pharmacol. (1990), **101**, 569–574

Evidence for the existence of 'atypical' β-adrenoceptors (β_3-adrenoceptors) mediating relaxation in the rat distal colon *in vitro*

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1 Experiments were carried out to characterize the adrenoceptors mediating relaxant responses in the rat distal colon. Three agonists were used: noradrenaline, isoprenaline and the β_3-adrenoceptor agonist BRL 37344. Phentolamine, propranolol and (±)-cyanopindolol were tested as antagonists. Tone in the rat distal colon was induced with KCl (30–40 mM) as a spasmogen, and relaxations of this KCl-induced tone produced by the agonists were measured.

2 Relaxant responses to noradrenaline that were obtained in the presence of propranolol (1 μM) were not antagonized by phentolamine (0.01 to 1 μM). Relaxant responses to isoprenaline that were obtained in the presence of phentolamine (1 μM) were antagonized in a concentration-dependent manner by propranolol (0.01 to 3 μM), although this antagonism was weak and non-competitive. Relaxant responses to BRL 37344 that were obtained in the presence of phentolamine (1 μM) were only weakly antagonized by high (1 μM) concentrations of propranolol.

3 Tachyphylaxis to BRL 37344 was observed, a second concentration-response curve being shifted to the right by 15 fold. Exposure of the tissues to BRL 37344 (1 μM) between concentration-response curves also caused rightward shifts in the responses to noradrenaline (18 fold) and isoprenaline (19 fold) but not to papaverine.

4 In the presence of phentolamine (1 μM) and propranolol (1 μM), the rank order of potency of the agonists was: (−)-isoprenaline (1.0) ≥ BRL 37344 (0.93) > (−)-noradrenaline (0.3).

5 Responses to BRL 37344 in the presence of phentolamine (1 μM) and propranolol (1 μM) were antagonized by (±)-cyanopindolol (1 μM), with an apparent pA_2 value of 6.67. Responses to isoprenaline, under the same conditions, were antagonized in a competitive manner by (±)-cyanopindolol (0.1 to 10 μM), with the slope of the Schild plot close to unity and a pA_2 value of 7.12.

6 The resistance of the relaxant responses to antagonism by phentolamine and propranolol, along with the relatively high potency of the β_3-adrenoceptor agonist BRL 37344 and the antagonism of 'resistant' responses by (±)-cyanopindolol would suggest that 'atypical' β-adrenoceptors, similar to the β_3-adrenoceptors of rat adipocytes and other tissues, exist in the rat distal colon.

Introduction

Responses to noradrenaline and isoprenaline which are resistant to blockade of classical α- and β-adrenoceptors have been reported in a number of animal isolated tissues, such as guinea-pig ileum (Wikberg, 1977; Bond *et al.*, 1986; Bond & Clarke, 1987); rabbit stomach (Bristow *et al.*, 1970); rabbit colon (Gillespie & Khoyi, 1977); dog colon (Grivegnee *et al.*, 1984); rat gastric fundus (Dettmar *et al.*, 1986b; Kelly & MacDonald, 1990) and guinea-pig gastric fundus (Coleman *et al.*, 1987). So-called 'atypical' β-adrenoceptors mediating lipolysis in rat adipocytes are also resistant to blockade by classical β-adrenoceptor antagonists (Stanton, 1972; Harms *et al.*, 1977) and in addition are characterized by the high selectivity and potency of a novel group of agonists (Arch *et al.*, 1984; Wilson *et al.*, 1984). It has been proposed that adipocyte β-adrenoceptors should be termed 'β_3-adrenoceptors' (Tan & Curtis-Prior, 1983; Arch, 1989). The finding that selective β_3-adrenoceptor agonists also have high potency at 'atypical' adrenoceptors in the guinea-pig ileum (Bond & Clarke, 1988) and the guinea-pig gastric fundus (Coleman *et al.*, 1987) suggests that β_3-adrenoceptors similar to those present in rat adipocytes may exist in gastrointestinal tissues.

The present study investigated responses to the catecholamines, noradrenaline and isoprenaline and the selective β_3-adrenoceptor agonist, BRL 37344 (Arch *et al.*, 1984) in the rat distal colon. A preliminary account of these findings has been presented to the British Pharmacological Society (McLaughlin & MacDonald, 1989).

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Methods

Tissue preparation

Male Wistar rats (200–300 g), fed *ad libitum*, were killed by a blow to the head and cervical dislocation. The distal 6 cm of the colon was removed and cleared of any faecal material by gentle squeezing of the colon with the fingers. Portions of colon were then immediately placed in cool Krebs physiological saline solution (PSS). Segments (3 cm) of colon were suspended in organ baths containing 30 ml Krebs solution, at 37°C, bubbled continuously with 95% O_2 and 5% CO_2, under an initial tension of 1 g. The composition of the Krebs solution was as follows (mM): NaCl 118, $CaCl_2$ 2.5, KCl 4.7, $NaHCO_3$ 25, KH_2PO_4 1.2, $MgSO_4$ 1.2 and glucose 11.1. The Krebs solution contained cocaine (3 μM) to block neuronal uptake of the catecholamines and ascorbic acid (30 μM) and EDTA (30 μM) to prevent oxidation of the catecholamines. In some experiments, hydrocortisone (30 μM) was also included in the Krebs solution, to block extra-neuronal uptake of isoprenaline. Tissues were allowed to equilibrate for at least 30 min before experimental procedures were begun.

Concentration-response curves to noradrenaline were constructed in the presence of propranolol (1 μM) to allow investigation of the contribution of α-adrenoceptors to responses.

Concentration-response curves to isoprenaline were carried out in the presence of phentolamine (1 μM) to remove any possible contribution from α-adrenoceptors.

Concentration-response curves (CRCs)

The relaxant action of agonists was determined by measuring relaxations of KCl-induced tone evoked by addition of the agonists. Initially a CRC to KCl was constructed for each tissue, to determine a concentration of KCl which gave approximately 75% of maximal tone. Concentrations of 30–40 mM were added hypertonically to the organ baths by adding 0.3–0.5 ml of a 2 M KCl stock solution.

CRCs to adrenoceptor agonists were constructed by cumulative addition (0.5 log unit increments) to KCl-contracted strips at 1–2 min intervals until a stable response was observed. CRCs were constructed at 1 h intervals, allowing 45 min equilibration time for antagonists, which were added at the end of each CRC.

In experiments testing the effect of antagonists on the relaxations evoked by BRL 37344, only one CRC to BRL 37344 was constructed in any one tissue. In these cases paired experiments were carried out. If CRCs to isoprenaline were not significantly different in the paired tissues then one tissue was subsequently exposed to BRL 37344 in the presence of antagonist and a paired tissue exposed to BRL 37344 in the absence of antagonist. The antagonist was present for 45 min before construction of CRCs to BRL 37344.

Schild plots

Agonist concentration-ratios (CRs) were determined from the IC_{50} point on the CRC, with or without antagonist. The plot of log (agonist concentration ratio − 1.0) versus log [antagonist] (Arunlakshana & Schild, 1959) was analysed by linear regression. Antagonism was considered to be competitive if the slope of the regression line was not significantly different from unity. In these cases a mean pA_2 value was obtained from individual estimates using the equation

$$pA_2 = \log(\text{agonist concentration ratio} - 1) - \log[\text{antagonist}]$$

after first verifying that there was no significant regression of pA_2 on antagonist concentration (MacKay, 1978).

In cases where the slope of the Schild plot was significantly different from unity, or in cases where only one concentration of antagonist was tested, 'apparent' pA_2 values were estimated from a single antagonist concentration and the above equation.

Drugs used

The following were dissolved in distilled water, with the exception of hydrocortisone and (±)-cyanopindolol which were dissolved in absolute ethanol and 0.1 M tartaric acid, respectively: BRL 37344 (sodium-4[2[2-hydroxy-2(3-chlorophenyl)ethylamino]propyl]phenoxyacetate) (Beecham Research Laboratories, Great Burgh, Epsom); cirazoline hydrochloride (Synthelabo); cocaine hydrochloride (Thornton and Ross Ltd.); (±)-cyanopindolol (Sandoz); hydrocortisone-21-acetate (Sigma); (−)-isoprenaline (+)-bitartrate (Sigma); (−)-noradrenaline (+)-bitartrate (arterenol) (Sigma); papaverine hydrochloride (Sigma); phentolamine mesylate (Ciba-Geigy); (±)-propranolol hydrochloride (Sigma); UK 14,304 (5-bromo-6[2-imazolin-2-yl-amino]quinoxaline) bitartrate (Pfizer).

Statistical analysis

Results are expressed as mean ± s.e.mean with the number of determinations, n, given in parentheses with the exception of regression line slopes which are expressed as slope ± 95% confidence limits. Statistical significance between two data sets was tested by either Student's t test or a paired t test. A probability level of $P < 0.05$ was considered statistically significant.

Results

Responses to noradrenaline

In the presence of propranolol (1 μM), noradrenaline relaxed KCl-induced tone in the rat distal colon, with a pIC_{50} of 6.3 ± 0.18 ($n = 4$). These relaxant responses to noradrenaline were highly reproducible, CRCs being unchanged when constructed at hourly intervals over a period of 3 h (Table 1).

The CRCs to noradrenaline were unaffected by phentolamine (0.01 to 1 μM, Table 1). Also, in three experiments in the presence of 1 μM propranolol, neither cirazoline nor UK 14,304 relaxed KCl-induced tone in the rat distal colon (in concentrations up to 10 μM).

Responses to isoprenaline

In the presence of phentolamine (1 μM), isoprenaline relaxed KCl-induced tone in the rat distal colon, with a pIC_{50} of 7.66 ± 0.06 ($n = 6$). The relaxant responses to isoprenaline were highly reproducible, CRCs being unchanged when constructed at hourly intervals over a period of 3 h (Figure 1a). The responses to isoprenaline were weakly antagonized in a concentration-dependent manner by propranolol (0.01 to 3 μM, Figure 1b). A Schild plot (Figure 1c) revealed that this antagonism was non-competitive in nature, as demonstrated by the slope of the plot, which was significantly less than unity (0.44 ± 0.18, 95% CL, $n = 36$). An apparent 'pA_2' value for propranolol, calculated on the basis of the shift produced by 1 μM propranolol, gave a value of 6.57 ± 0.18 ($n = 6$).

Hydrocortisone (30 μM) had no effect on the potency of isoprenaline (pIC_{50} of 7.65 ± 0.1, $n = 8$) or on the antagonism by propranolol (apparent pA_2 of 6.48 ± 0.09, $n = 8$).

Responses to BRL 37344

In the presence of phentolamine (1 μM) the β_3-adrenoceptor agonist BRL 37344 relaxed KCl-induced tone in the rat distal colon, with a pIC_{50} of 7.33 ± 0.12 ($n = 4$). Tachyphylaxis to BRL 37344 was observed, a second CRC being shifted to the right by 15 fold, when compared to the control CRC (Figure 2a). The responses to BRL 37344 were not antagonized by low concentrations of propranolol (0.01 to 0.1 μM), but were antagonized to some extent by 1 μM propanolol (mean 8 fold shift of IC_{50} values, $P < 0.05$, Figure 2b). An apparent pA_2 value for propranolol, calculated on the basis of the shift produced by 1 μM propranolol, gave a value of 6.39.

In the presence of phentolamine (1 μM) and propranolol (1 μM), exposure of the tissues to BRL 37344 (1 μM) for 3 min approximately mid-way between CRCs to noradrenaline and isoprenaline produced significant (paired t test, $P < 0.01$) mean rightward 18 and 19 fold shifts of the CRCs to noradrenaline and isoprenaline, respectively (Figures 3a and 3b).

Table 1 Effect of phentolamine on noradrenaline-induced relaxations in rat distal colon in the presence of propranolol (1 μM)

	Phentolamine (μM)		*pIC_{50} of noradrenaline*	
	time		*time*	
CRC No.	*controls*	*treated*	*controls*	*treated*
1	0	0	6.55 ± 0.02	6.30 ± 0.18
2	0	0.01	6.43 ± 0.08	6.35 ± 0.19
3	0	0.1	6.51 ± 0.10	6.37 ± 0.15
4	0	1.0	6.50 ± 0.03	6.33 ± 0.08

Four concentration-response curves (CRCs) to noradrenaline were constructed at hourly intervals, allowing 45 min equilibration with each of the concentrations of phentolamine in the treated tissues. Values shown are means ± s.e.mean for 6 tissues in each case.

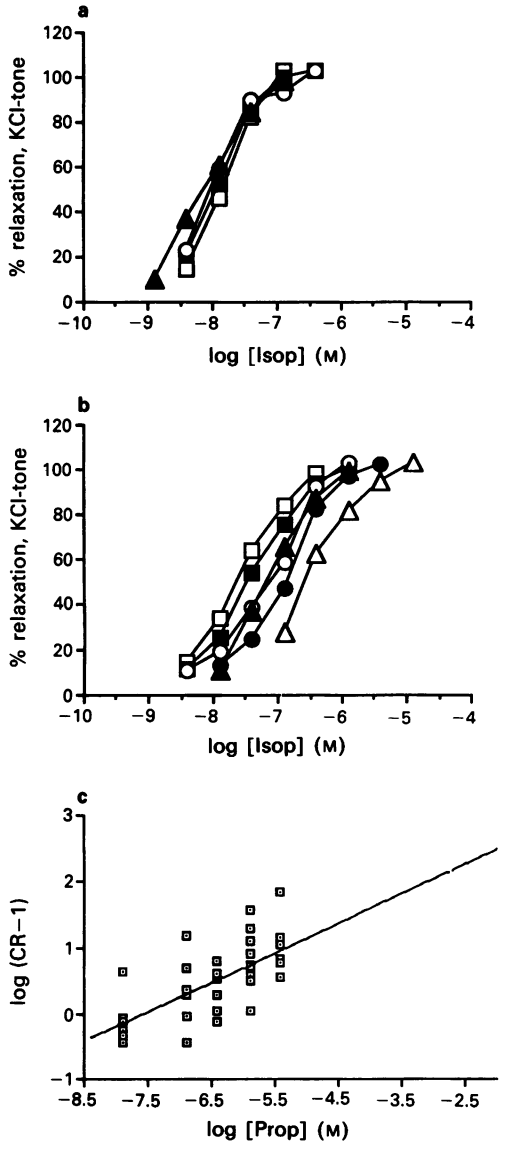

Figure 1 (a) Time control concentration-response curves to isoprenaline (Isop) in the rat distal colon in the presence of 1 μM phentolamine. Tone was raised by the addition of KCl (30–40 mM) to the organ baths. Each of the concentration-response curves was constructed at hourly intervals: the first (□, $n = 6$), second (■, $n = 6$), third (○, $n = 6$) and fourth curves (▲, $n = 6$) are shown. (b) The effect of propranolol, in concentrations 0.01 μM (■, $n = 6$), 0.1 μM (○, $n = 6$), 0.3 μM (▲, $n = 6$), 1 μM (●, $n = 12$) and 3 μM (△, $n = 6$) on control (□, $n = 12$) concentration-response curves to isoprenaline in the rat distal colon is shown. Error bars have been omitted for clarity. Standard errors were less than 15% in each case. (c) Schild plot of the antagonism of isoprenaline action by propranolol (Prop) in the rat distal colon.

The same experimental procedure, however, had no effect on the relaxant effect of papaverine on KCl-induced tone in the rat distal colon (Figure 3c).

Order of potency of agonists under conditions of classical α- and β-adrenoceptor blockade

In the presence of phentolamine (1 μM) and propranolol (1 μM), the pIC_{50}s of isoprenaline, BRL 37344 and noradrenaline were, respectively, 6.85 ± 0.18 (12), 6.82 ± 0.16 (4) and 6.33 ± 0.08 (6), giving a rank order of potency of: isoprenaline (1.0) ⩾ BRL 37344 (0.93) > noradrenaline (0.3).

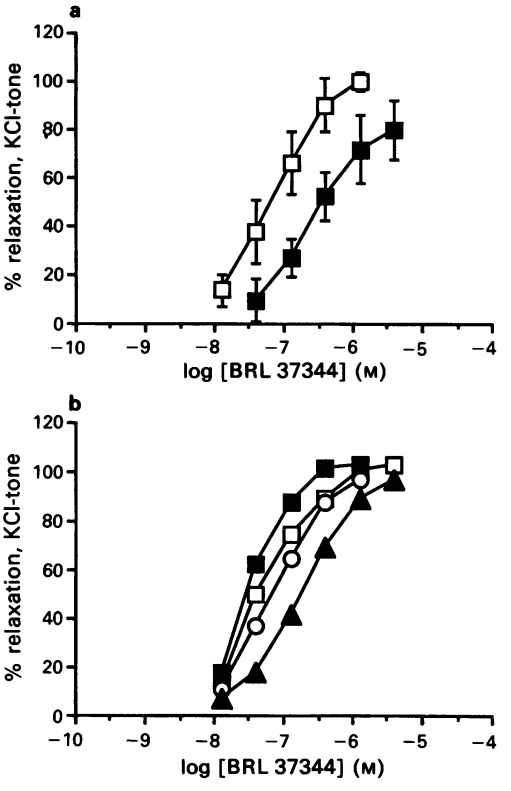

Figure 2 Effect of BRL 37344 on KCl-induced tone in the rat distal colon in the presence of 1 μM phentolamine. (a) Time controls for responses to BRL 37344. Curves represent first (□, $n = 4$) and second (■, $n = 4$) concentration-response curves, constructed 1 h apart. (b) The effect of propranolol, 0.01 μM (■, $n = 4$), 0.1 μM (○, $n = 4$) and 1 μM (▲, $n = 4$) on control (□, $n = 4$) concentration-response curves to BRL 37344. Tone was raised by the addition of KCl (30–40 mM) to the organ baths. Error bars have been omitted from the figure for clarity. In each case, standard errors were less than 15%.

Effect of (±)-cyanopindolol on responses to isoprenaline and BRL 37344 under conditions of α- and β-adrenoceptor blockade

In the presence of phentolamine (1 μM) and propranolol (1 μM), responses to BRL 37344 were antagonized by the β-adrenoceptor antagonist (±)-cyanopindolol (1 μM), with an apparent pA_2 value calculated from the single concentration of cyanopindolol of 6.67 ± 0.25 ($n = 7$). Also, cyanopindolol (0.1 to 10 μM) antagonized responses to isoprenaline in a concentration-dependent manner (Figure 4a). The antagonism of the action of isoprenaline was competitive in nature, since the slope of the Schild plot was not significantly different from unity (1.03 ± 0.17, 95% CL, $n = 12$, Figure 4b). The pA_2 value for cyanopindolol was 7.12 ± 0.06 ($n = 12$). The difference between the pA_2 values for cyanopindolol against BRL 37344 and isoprenaline was statistically significant ($P < 0.05$, Student's t test). Cyanopindolol itself had no effect on the degree of tone induced by KCl (tone in cyanopindolol-treated strips as % of control, $n = 4$; 0.1 μM, 89.7 ± 7; 1 μM, 105 ± 10; 10 μM, 109 ± 7).

Discussion

The action of catecholamines on the rat distal colon was not mediated by α-adrenoceptors, since (a) the responses to noradrenaline were resistant to the classical α-adrenoceptor antagonist phentolamine and (b) the selective α_1- and α_2-adrenoceptor agonists cirazoline (van Meel *et al.*, 1981) and UK 14,304 (Cambridge, 1981) were devoid of any relaxant

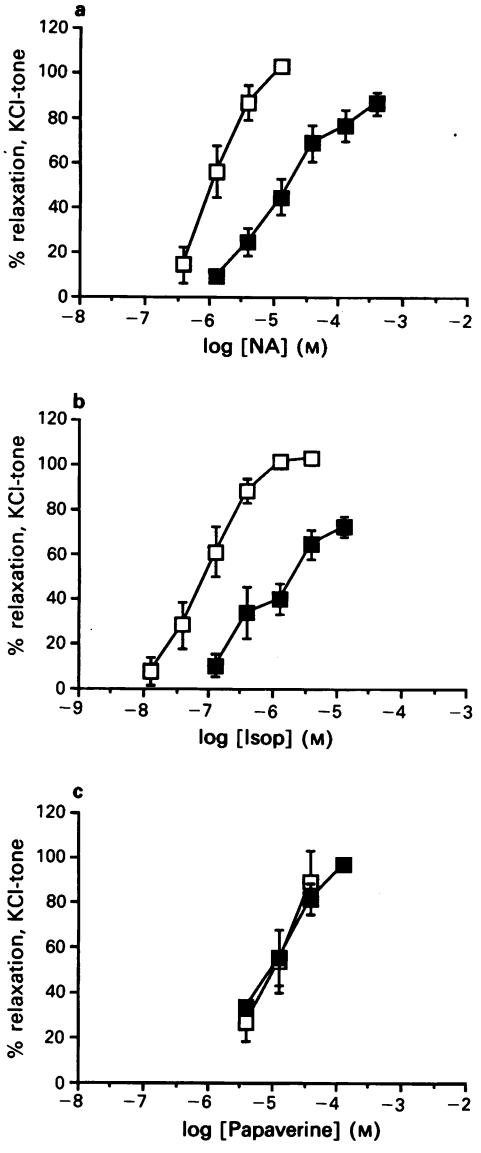

Figure 3 The effect of exposure of the tissues to 1 μM BRL 37344 for a period of 3 min between concentration-response curves to agonists. In each case, first (□, $n = 6$) and second (■, $n = 6$) concentration-response curves are shown. Tone was raised by the addition of KCl (30–40 mM) to the organ baths and phentolamine (1 μM) and propranolol (1 μM) were present throughout. (a) Noradrenaline (NA), (b) Isoprenaline (Isop) and (c) papaverine.

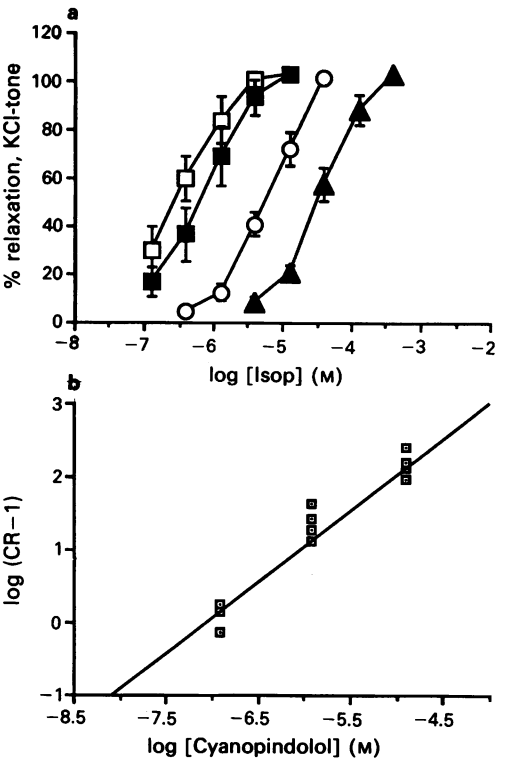

Figure 4 (a) The effect of cyanopindolol, in concentrations 0.1 μM (■, $n = 4$), 1 μM (○, $n = 4$) and 10 μM (▲, $n = 4$) on control concentration-response curves (□, $n = 4$) to isoprenaline in the rat distal colon in the presence of 1 μM phentolamine and 1 μM propranolol. Tone was raised by the addition of KCl (30–40 mM) to the organ baths. (b) Schild plot of the antagonism of isoprenaline action by cyanopindolol.

action. Inhibitory postjunctional α_1-adrenoceptors have previously been shown to exist in the rat distal colon (Dettmar *et al.*, 1986a) and the failure to show their presence in the present study is probably related to the KCl-induced depolarization, since α-adrenoceptor-mediated inhibitory effects in intestinal smooth muscle involve abolition of spontaneous spike discharge and hyperpolarization (Bülbring, 1954; 1957) and are not seen if the tissue is depolarized sufficiently to block spike generation (Magaribuchi & Kuriyama, 1972). Thus the present study does not rule out the presence of inhibitory α-adrenoceptors in this preparation, but their effects are not seen in the presence of KCl-induced tone.

The relaxations to the catecholamines were also elicited in the presence of propranolol (1 μM), conditions under which one would assume that classical β-adrenoceptors had been blocked. Propranolol did produce a shift of the isoprenaline CRC but the antagonism was non-competitive (slope of Schild plot less than unity) and weak with an apparent pA_2 of 6.57 compared with 8.2–8.8 for classical β_1-adrenoceptors mediating atrial stimulation and 8.3–8.6 for β_2-adrenoceptors mediating tracheal relaxation (Wilson *et al.*, 1984). However a similarly low pA_2 value (6.6) for propranolol against the lipolytic effect of isoprenaline was reported (Wilson *et al.*, 1984) and therefore it seems possible that the receptors mediating relaxation of the colon and lipolysis are similar.

Non-competitive antagonism of responses to catecholamines may result from failure to block extraneuronal uptake processes for the catecholamines (Furchgott, 1967). This possibility was ruled out in the present study since both the responses to isoprenaline and the antagonism by propranolol were similar in the presence and absence of the extraneuronal uptake blocker hydrocortisone (Iversen & Salt, 1970).

Relaxation of the rat distal colon mediated by the selective β_3-adrenoceptor agonist BRL 37344 was only weakly antagonized by propranolol, the apparent 'pA_2' value of 6.39 being similar to the reported pA_2 values for propranolol against β_3-adrenoceptor agonists stimulating lipolysis of rat white adipocytes (Wilson *et al.*, 1984). This again suggests that the receptors mediating relaxation in the rat distal colon and the receptors mediating lipolysis in adipocytes may be similar.

The relative potency of BRL 37344 under conditions of α- and β-adrenoceptor blockade, isoprenaline (1.0) $\geq$ BRL 37344 (0.93) > noradrenaline (0.3), compares with rat adipocyte β_3-adrenoceptors where BRL 37344, or its methyl ester BRL 35135, were equipotent with or more potent than isoprenaline (Arch *et al.*, 1984; Wilson *et al.*, 1984). A similar high potency of selective β_3-adrenoceptor agonists was found in guinea-pig gastric fundus (Coleman *et al.*, 1987) and guinea-pig ileum (Bond & Clarke, 1988).

In the present study BRL 37344 caused tachyphylaxis to itself and also to noradrenaline and isoprenaline. This was not simply a non-specific effect on all relaxant responses in the rat distal colon since responses to papaverine, which acts by inhibition of phosphodiesterase (Bar, 1974), were unaffected. Tachyphylaxis to BRL 35135 has also been reported in guinea-pig gastric fundus (Coleman *et al.*, 1987). The mechanism of the tachyphylaxis in the present study and in the

study by Coleman *et al.* (1987) is unclear but the cross-desensitization may be evidence that BRL 37344 and the catecholamines are acting at the same site.

Responses to isoprenaline in the presence of α- and β-adrenoceptor blockade with phentolamine and propranolol were antagonized in a competitive manner by cyanopindolol. Cyanopindolol has previously been shown to be a potent competitive antagonist at propranolol-resistant 'atypical' β-adrenoceptors in guinea-pig ileum (Blue *et al.*, 1989) and the reported pA_2 of 7.63 is similar to that obtained in the present study (7.12). Thus the receptor mediating responses to isoprenaline in rat distal colon appears to be similar to the 'atypical' β-adrenoceptor present in guinea-pig ileum. A Schild regression was not carried out for cyanopindolol against BRL 37344 but the single concentration of cyanopindolol tested (1 μM) gave an apparent pA_2 of 6.67 which was significantly lower than the value obtained with isoprenaline as agonist. Lower pA_2 values for β-adrenoceptor antagonists when BRL 37344 or analogues were the agonists than when isoprenaline was the agonist have previously been found (Stock & Sudera, 1988; Jones *et al.*, 1989; Arch, 1989), suggesting that the interaction of the BRL compounds with the β_3-adrenoceptor may differ from that of isoprenaline. It has been suggested that the bulky N-substituent of the BRL compounds may bind to an accessory site and reduce the affinity of antagonists for the main binding site (Arch, 1989).

There is evidence that some partial adrenoceptor agonists acting on human myocardial tissue, designated unconventional and including (−)-pindolol, cause part of their effects through heart adrenoceptors which are neither β_1- nor β_2-adrenoceptors (Walter *et al.*, 1984; Kaumann, 1989). These agonist effects are seen at concentrations greater than are necessary to produce blockade of β_1- and β_2-adrenoceptors, are resistant to conventional β-blockade and have been attributed to an action at a third heart β-adrenoceptor, termed β_3 (Kaumann, 1989). It may be that the cardiac β_3-adrenoceptors are similar to the β-adrenoceptors of the rat distal colon and other tissues and that there is some relationship between the partial agonist effect of pindolol at β_3-adrenoceptors and the antagonistic effect of cyanopindolol at 'atypical' β-adrenoceptors in the gut. Pindolol analogues may also act as partial agonists at rodent β_3-adrenoceptors (Engel *et al.*, 1981). However, there was no evidence in the present study for a partial agonist effect of cyanopindolol at the concentrations used (up to 10 μM, approximately 100 × pK_a at the 'atypical' β-adrenoceptors in this study).

Recently, a human gene has been isolated that encodes a product referred to as the "β_3-adrenoceptor" (Emorine *et al.*, 1989). Investigation of the properties of this receptor in Chinese Hamster ovary cells transfected with the gene showed low affinities for classical β-adrenoceptor antagonists and a high potency of BRL 37344. It is likely, therefore, that this receptor represents a separate class of β-adrenoceptor which includes the β_3-adrenoceptors of adipocytes, myocardium and gastrointestinal smooth muscle. It has been suggested that these receptors may represent intermediate stages in evolution towards classical β_1- and β_2-adrenoceptors (Bond & Clarke, 1988; Zaagsma & Nahorski, 1990).

In conclusion, the resistance of catecholamine-induced relaxations to propranolol, the relatively high potency of the β_3-adrenoceptor agonist, BRL 37344, and the competitive antagonism of propranolol-resistant responses by cyanopindolol suggest that the rat distal colon contains 'atypical' β-adrenoceptors similar to the β_3-adrenoceptors in rat adipocytes and other tissues. The physiological significance of such receptors in the gastrointestinal tract is at present unknown, although it has been shown that their activation by selective agonists can affect the motility of rat proximal colon *in vivo* (Manara *et al.*, 1989).

UK14,304 bitartrate and cirazoline hydrochloride were gifts from Dr P.W. Dettmar of Reckitt and Colman plc, Dansom Lane, Hull. BRL 37344 was kindly supplied by Dr M.A. Cawthorne, Beecham Pharmaceuticals Research Division, Great Burgh, Epsom. D.P.McL. was supported by a Glasgow College Research Studentship. The authors are also grateful to the College for financial support.

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(Received March 6, 1990
Revised June 18, 1990
Accepted July 10, 1990)

Br. J. Pharmacol. (1990), **101**, 575–580

A novel series of non-quaternary oxadiazoles acting as full agonists at muscarinic receptors

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1 A novel series of non-quaternary oxadiazole-based muscarinic agonists demonstrated high affinity for muscarinic receptors.

2 These agonists possessed high efficacy in the nanomolar range at muscarinic receptors in the superior cervical ganglion, atrium and ileum but did not show selectivity across the tissue preparations.

3 Two amino oxadiazoles, one from a quinuclidine series (L-660,863) and one from a 1-azanorbornane series (L-670,207) possessed a high ratio of potency for displacing the binding of [^{3}H]-N-methyl-scopolamine ([^{3}H]-NMS) to potency for displacing the agonist [^{3}H]-oxotremorine-M ([^{3}H]-oxo-M) (NMS/oxo-M ratio) predictive of high efficacy in the cortex.

4 The two azanorbornane derivatives L-670,548 and L-670,207 stimulated the turnover of phosphatidyl-inositol in the cortex with a potency higher than that obtained with any other known muscarinic agonist (ED_{50} 0.26 and 0.18 μM respectively).

5 The maximum response obtained with L-670,207 was greater than that observed for carbachol but was comparable to that of the natural ligand acetylcholine.

6 These oxadiazole muscarinic agonists are among the most potent and efficacious non-quaternary muscarinic agonists ever described.

Introduction

Muscarinic cholinoceptors are present throughout the body in many peripheral organs, and are also found throughout the central nervous system (CNS) (Eglen & Whiting, 1986; Goyal, 1989). Interest in muscarinic pharmacology has been stimulated recently by the reports of specific cholinergic deficits in brain at autopsy from patients diagnosed as having Alzheimer's disease (Perry, 1986). This has led to the suggestion that enhancement of cholinergic neurotransmission would alleviate the symptoms of the disease, including the deficits observed in cognition and memory.

One approach to therapy in Alzheimer's disease has been the development and subsequent clinical trials of a number of directly acting muscarinic receptor agonists. These include such natural agents as arecoline (Christie *et al.*, 1981) and pilocarpine (Caine, 1980), and synthetic compounds such as RS-86 (Mouradian *et al.*, 1988). Clinical trials with such agents have generally been disappointing, at least partly due to the side effects associated with muscarinic receptor stimulation. Recent attention has focused on investigating the pharmacology of muscarinic receptor subtypes as a means of overcoming these problems. At least three distinct muscarinic receptors (M_1, M_2 and M_3) have been distinguished from both functional studies and binding studies (Mutschler *et al.*, 1989; Goyal, 1989). This work was made possible by the development of selective antagonists such as pirenzepine (Hammer *et al.*, 1980), AF-DX 116 (Hammer *et al.*, 1986), methoctramine (Melchiorre *et al.*, 1987) and hexahydrosiladiphenidol (Lambrecht *et al.*, 1984) and the identification of tissues containing a response mediated predominantly by a single receptor subtype. In complementary studies using a molecular biological approach, evidence has accumulated that at least five distinct muscarinic receptors can be identified (Bonner *et al.*, 1987; 1988). Using *in situ* hybridization it has been possible to identify all of these subtypes within the CNS and to identify a selective regional localisation (Buckley *et al.*, 1988).

The coupling of muscarinic receptors has been shown to vary between regions showing linkage to a variety of secondary messenger systems: adenylate cyclase, phosphatidyl-inositol (PI) turnover and ion channels (Brown *et al.*, 1984; Ehlert, 1985). There are also considerable differences in the efficiency of the coupling. The latter phenomenon is important since even in the absence of different receptors or selective agents, a functional selectivity that is based on differences in receptor reserve in different organs may be achieved by agents that are partial agonists (Kenakin, 1986).

In the cerebral cortex, the muscarinic receptors that are coupled to phosphatidyl-inositol turnover appear to lack an effective receptor reserve. Thus full agonists such as carbachol and muscarine have relatively low potency compared with that seen in parotid gland, and partial agonists such as pilocarpine and RS-86 produce a smaller maximal response relative to carbachol (Fisher *et al.*, 1983; Freedman, 1986; Freedman *et al.*, 1988). To date the only compounds with sufficient efficacy to stimulate phosphatidyl-inositol turnover in cerebral cortex have been quaternary agonists such as carbachol, muscarine, oxotremorine-M and acetylcholine itself. Although these compounds are highly efficacious at cortical muscarinic receptors they would be expected to have only limited ability to pass through the blood brain barrier and hence would not be able to activate effectively these cortical receptors in the whole animal.

In the present study we describe a novel series of non-quaternary muscarinic agonists that includes the most efficacious and potent muscarinic agonists known. These compounds possess the ability to activate fully muscarinic receptors in a range of pharmacological preparations, and in one particular example produce a maximal stimulation of cortical PI turnover comparable to that obtained with acetylcholine itself.

Portions of this work were presented at the subtypes of muscarinic receptor meeting (IV) at Wiesbaden, 20-22 July 1989 (Saunders, J. & Freedman, S.B., 1989).

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Methods

Brain membrane preparation

Crude synaptosomal-mitochondrial membranes were prepared by homogenizing cerebral cortex from rat (250–300 *g*) in 0.32 M ice-cold sucrose (1/10, w/v) in a motor-driven teflon/glass homogenizer at 500 r.p.m. (10 strokes). The homogenate was centrifuged at 1000 *g* for 15 min and the resulting supernatant centrifuged at 17,000 *g* for 20 min. This yielded the crude synaptosomal mitochondrial pellet (P_2), which was used fresh or stored at −20°C before use.

Receptor binding studies

[^{3}H]-N-methylscopolamine binding ([^{3}H]-NMS) P_2 fractions were homogenized and resuspended at a final dilution of 1/600 (wet w/v) in ice-cold Krebs-HEPES buffer pH 7.4 (composition, mM: NaCl 118, KCl 4.7, $MgSO_4$ 1.2, $NaHCO_3$ 5, KH_2PO_4 1.2, $CaCl_2$ 2.5, glucose 11 and HEPES 20). Binding of [^{3}H]-NMS was determined with 0.01–1.0 nM ligand and non-specific binding defined with 2 μM atropine. Displacing drugs were added in a volume of 10 μl to give a final assay volume of 1.0 ml. Incubations were initiated by adding 750 μl of membrane solution and were allowed to proceed for 60 min at 30°C. Assays were terminated by filtration using a Brandell Cell Harvester and Whatman GF/B filters with 2 × 10 ml rinses in ice cold saline (0.9% NaCl, w/v). Filters were then placed in 10 ml of scintillation fluid (Hydrofluor, National Diagnostics, New Jersey) and radioactivity estimated by liquid scintillation spectrometry. Kinetic analysis has been previously reported (Freedman *et al.*, 1988) with a dissociation constant (K_D) of 0.14 ± 0.02 nM and a maximum binding capacity (B_{max}) of 1400 ± 340 fmol mg^{-1} protein ($n = 5$).

[^{3}H]-oxotremorine-M binding ([^{3}H]-oxo-M) P_2 fractions were washed by resuspending in 10 ml of 20 mM HEPES buffer pH 7.4 and centrifuged at 17,000 *g* for 20 min. The washed membranes were homogenized and resuspended at a final dilution of 1/100 (wet w/v) in ice-cold 20 mM HEPES buffer pH 7.4. Binding of [^{3}H]-oxo-M was determined by use of 0.2–5 nM of ligand and non-specific binding defined with 2 μM atropine. Displacing compounds were added in a volume of 10 μl to give a final assay volume of 1.0 ml. Incubations were initiated by adding 750 μl of membrane solution and were allowed to proceed for 40 min at 30°C. Assays were terminated by filtration over Whatman GF/C filters presoaked in 0.05% polyethyleneimine, using a Brandell Cell Harvester. Samples were washed with 10 ml of ice-cold saline and filters placed in 10 ml of scintillation fluid (Hydrofluor, National Diagnostics, New Jersey, U.S.A.) and radioactivity estimated by liquid scintillation spectrometry. Scatchard analysis indicated a dissociation constant (K_D) of 0.68 ± 0.12 nM and a maximum binding capacity of 520 ± 160 fmol mg^{-1} protein.

Binding parameters were determined by non-linear, least-squares regression analysis using RS1 (BBN Research Systems, Cambridge, Mass.) and a computerised iterative procedure written by Dr A. Richardson, NRC Terlings Park.

Phosphatidyl-inositol turnover

Tissue slices of rat cerebral cortex (350 × 350 μm) were prepared with a McIlwain tissue chopper and were washed three times in Krebs bicarbonate buffer, followed by a 30 min preincubation in the presence of [^{3}H]-myo-2-inositol, 2 μCi, (Amersham International, TRK.807 13.8 Ci mmol^{-1}) and 10 mM lithium. Tissue slices were subsequently incubated in the presence of muscarinic agonists for 45 min in a volume of 250 μl. The reaction was terminated by addition of 940 μl of chloroform/methanol (1/2 v/v) and water-soluble inositol monophosphates were isolated by ion exchange chromatography. The methods have previously been described in detail by Brown and colleagues (1984). For studies with muscarinic antagonists, tissue slices were incubated with antagonists for 15 min before addition of agonist. All test compounds were added in a volume of 10 μl. Curves were fitted to data analysed by non-linear regression using the Allfit, four parameter logistic curve fitting programme (Delean *et al.*, 1978).

Rat superior cervical ganglion

Superior cervical ganglia from male Sprague-Dawley rats were superfused *in vitro* as previously described (Newberry & Priestley, 1987). The d.c. potential between the ganglion body and the internal carotid nerve was recorded across a greased gap with Ag/AgCl electrodes. Agonists were superfused with increasing concentrations for a 1 min period at 10 min intervals. Since the response did not return to baseline during the 9 min wash period, calculations were made from the extrapolated baseline. Before determining the dose-response relationship, a reproducible response to 1 μM (±)-muscarine chloride was obtained on each ganglion. Subsequent responses were all related to that depolarizing response, given an arbitrary value of 1.0. The maximum response and the concentration required to evoke half of that response (EC_{50}) were determined on a number of ganglia, each from a different rat. It should be noted that the dose-response relationship was determined by increasing the agonist concentration until the response levelled off. However, it has recently been reported (Newberry & Gilbert, 1989) that the dose-response curve to muscarine is biphasic with higher concentrations of muscarine (>3 μM) causing the dose-response curve to rise to a second peak. We have preliminary evidence indicating that the dose-response curves of these novel compounds may also be biphasic. Given that the reasons for this phenomenon are not totally clear, the relative maximum values quoted in Table 3 correspond to the point where the dose-response curve first levelled off.

Guinea-pig isolated atria

Paired atria were removed from male guinea-pigs (300–400 g weight) and suspended under 1 g tension in Krebs bicarbonate solution containing 22 mM glucose. Preparations were paced by electrical field stimulation (3–4 Hz, 2–3 ms) with platinum electrodes. Following a 60 min equilibration period, non-cumulative dose-response curves to muscarinic agonists were constructed, allowing exposure to any one application of test compound until a maximum negative chronotropic effect was obtained. A period of at least 45 min was allowed between each dose-response curve. Agonist potency (EC_{50}) and the maximum response relative to the maximum response to carbachol or muscarine were determined using RS1 (BBN Research Systems, Cambridge, Mass.) and the computerised procedure described above.

Guinea-pig ileum, longitudinal muscle – myenteric plexus preparation

Preparations of longitudinal muscle with the myenteric plexus from the distal ileum of 300–400 g male guinea-pigs were obtained as described by Rang (1964). Preparations were washed and suspended under 1 g tension in glass organ baths containing 3 ml Krebs bicarbonate solution at 37°C and allowed to equilibriate for at least 60 min. Isometric contractions to muscarinic agents were measured for at least 30 s until a clear peak of response was obtained. A period of at least 45 min was allowed between each test compound. Potency and maximum response relative to that for carbachol or muscarine were determined as for the atrium. Tissue responses were measured as changes in isometric tension in the ileum.

The responses were then calculated as a percentage of the maximum response obtained relative to a dose of 1 μM carbachol. Agonist potency (EC_{50}) was determined by a non-linear iterative curve fitting procedure.

Materials

Compounds and reagents used in this study were obtained from the following sources. RS-86 (2 ethyl 8 methyl-2, 8 diazaspiro[4,5]decan-1,3-dion hydrobromide), Sandoz Ltd. Radioligands were purchased through New England Nuclear ([^{3}H]-N-methylscopolamine, NET 636, 85–90 Ci mmol^{-1}; [^{3}H]-oxotremorine-M, NET 671, 84.9 Ci mmol^{-1}). All other compounds were obtained from Sigma (Dorset). The detailed synthesis of the novel compounds reported here is published elsewhere (Saunders *et al.*, 1990; Street *et al.*, 1990).

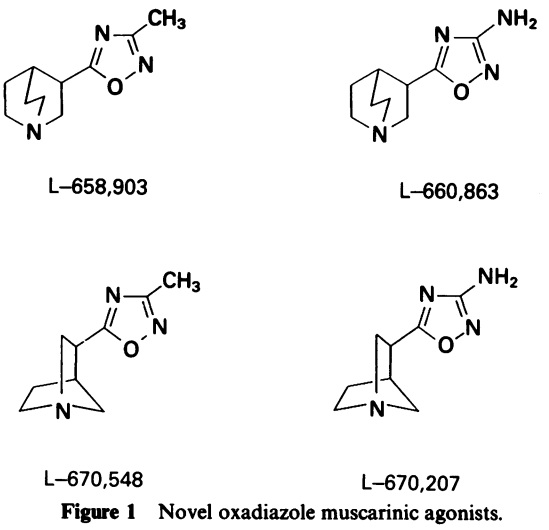

Figure 1 Novel oxadiazole muscarinic agonists.

Results

Receptor binding studies

We recently described (Freedman *et al.*, 1988) a receptor binding paradigm that can be used to measure affinity of compounds for the cortical muscarinic receptor population and also to predict their relative efficacy at cortical muscarinic receptors. This assay measures the ability of compounds to displace low concentrations of the potent muscarinic agonist [^{3}H]-oxo-M from the high affinity state of the receptor. In parallel studies the ability of these compounds to displace the non-selective antagonist [^{3}H]-NMS from the predominately low affinity states is measured. The ratio of affinities of compounds in these assays appears to correlate with the ability of compounds to stimulate the hydrolysis of phosphatidyl-inositol turnover. The results shown in Table 1 show that four broad categories of compounds can be identified. These range from the high affinity muscarinic antagonist atropine (NMS/oxo-M ratio of 2.1), the weak partial agonist pilocarpine (NMS/oxo-M ratio 100), the more efficacious partial agonist arecoline (NMS/oxo-M ratio of 560), to the full agonist carbachol (NMS/oxo-M ratio 4500).

It was previously shown in our laboratories that the ester functionality in benzodiazepines could be replaced by an oxadiazole group (Watjen *et al.*, 1989). This replacement appeared to produce an increase in the relative efficacy of benzodiazepines as measured by the relative shift in binding affinity observed in the presence and absence of GABA. We have recently shown that similarly for muscarinic agents the ester functionality of arecoline can be replaced by the oxadiazole moiety (Saunders *et al.*, 1990). In the present study four of the oxadiazole compounds have been extensively characterized. These include methyl oxadiazoles from the quinuclidine and 1-azanorbornane series, and two corresponding amino oxadiazoles (Figure 1).

Compared with the original natural agonist, arecoline, the methyl oxadiazole from the quinuclidine series, L-658,903, showed more than a 10 fold increase in affinity in the [^{3}H]-NMS assay and had a comparable NMS/oxo-M ratio (Table 1). The amino oxadiazole in this series, L-660,863, possessed similar affinity to the methyl oxadiazole but had a higher NMS/oxo-M binding ratio of 1300.

Replacement of the quinuclidine base by a 1-azanorbornane ring system produced a further 3 to 10 fold increase in binding affinity (Table 1) for the [^{3}H]-NMS binding site. Thus for the methyloxadiazole L-670,548 the apparent affinity in the [^{3}H]-NMS assay was 60 fold greater than that for arecoline, while for the amino oxadiazole (L-670,207) the affinity was 200 times that of arecoline. These compounds possess the highest binding affinity of any known muscarinic agonists; in addition both of the 1-azanorbornane derivatives display NMS/oxo-M binding ratios greater than either arecoline or pilocarpine with values of 720 and 1100 for the amino and methyl oxadiazole respectively.

Phosphatidyl-inositol turnover

Muscarinic agonists appear to show large differences in their efficacy for producing a stimulation of cortical phosphatidyl-inositol turnover (Table 2). The methyl oxadiazole L-658,903

Table 1 N-methylscopolamine/oxotremorine-M ratio in rat cerebral cortex

Compound	*[^{3}H]-NMS (K_{app} nM)*	*[^{3}H]-Oxo-M (K_{app} nM)*	*NMS/Oxo-M ratio*
Atropine	1.0 (0.94;1.1)	0.48 (0.40;0.57)	2.1
Pilocarpine	4000 (3000;5100)	40 (36;44)	100
Arecoline	6200 (4900;7800)	11 (9.7;11)	560
Carbachol	22000 (20000;24000)	4.9 (3.6;6.1)	4500
L-658,903	440 (420;450)	0.96 (0.82;1.1)	460
L-660,863	600 (550;660)	0.47 (0.40;0.54)	1300
L-670,548	100 (90;120)	0.090 (0.07;0.11)	1100
L-670,207	31 (27;36)	0.043 (0.039;0.049)	720*

Results are expressed as an apparent affinity constant (K_{app}) which has been corrected for ligand occupancy by use of the Cheng-Prussoff equation (1973). Each curve is typically 6–10 concentrations performed in triplicate. The values above are geometric means of at least 3 determinations performed on separate occasions. Numbers in parentheses indicate the range low and high error values of the geometric mean. Inhibition studies were performed with 0.1 nM [^{3}H]-N-methylscopolamine ([^{3}H]-NMS) and 3 nM [^{3}H]-oxotremorine-M ([^{3}H]-Oxo-M).

* Evidence of depletion was obtained for this compound resulting in an underestimation of the affinity of this compound (see Discussion).

Table 2 Stimulation of phosphatidyl-inositol turnover in rat cerebral cortex

	Phosphatidyl-inositol turnover	
Compound	EC_{50} (μM)	% maximum response
Pilocarpine	13 (6.6,25)	12 (11,14)
Arecoline	16 (9.4,29)	19 (6,25)
Carbachol	220 (150,330)	130 (110–170)
L-658,903	4.3 (2.4,7.6)	20 (17–21)
L-660,863	2.5 (2.1,3.1)	58 (51–66)
L-670,548	0.26 (0.21,0.32)	93 (69–120)
L-670,207	0.18 (0.15–0.22)	160 (150–170)

EC_{50}: Potency of compounds in eliciting breakdown of labelled inositol-phospholipids.
% maximum response: results have been expressed as a % of the maximum response to 1 mM carbachol included in all experiments. The maximum response was equivalent to approximately an 8–10 fold increase over unstimulated controls.
Each experimental value is calculated from between 2–5 independent determinations. Curves were fitted to data and analysed by non-linear regression using the Allfit, four parameter logistic curve fitting programme (DeLean *et al.*, 1978). The EC_{50} value was calculated from the individual maximum response of each compound. Results are expressed as the geometric mean with low and high error margin. The % maximum response is the median value (low, high range).

showed only a limited ability to stimulate phosphatidyl-inositol turnover in rat cerebral cortex and like arecoline produced a maximum response of 20% of that for carbachol. The amino oxadiazole L-660,863 which had a higher binding ratio, stimulated PI turnover dose-dependently (EC_{50} 2.5 μM) with a maximum response 58% that of carbachol (Figure 2a).

The two 1-azanorbornane derivatives were potent agonists for producing a stimulation of cortical PI turnover with EC_{50} values of 0.26 μM for the methyl oxadiazole L-670,548 and 0.18 μM for the amino oxadiazole L-670,207. These two compounds are the most potent muscarinic agonists that we have tested in this assay. The maximum response for L-670,548 was 93% of that observed with carbachol (1 mM) while the amino oxadiazole L-670,207 produced an increased response greater than that of carbachol (160%) (Figure 2b). These effects were comparable with those observed with acetylcholine, the natural ligand for the muscarinic receptor. However the potency of acetylcholine was over 100 fold weaker with an EC_{50} value of 30 μM.

All of the responses to the novel muscarinic agonists were dose-dependently blocked by atropine (1 μM) which was added 15 min before addition of the agonist. Very high concentrations of L-660,863 (1000 to 30,000 μM) produced an additional stimulation (250%) which was not completely reversed by atropine. Solubility of L-660,863 prevented detailed study of this additional component.

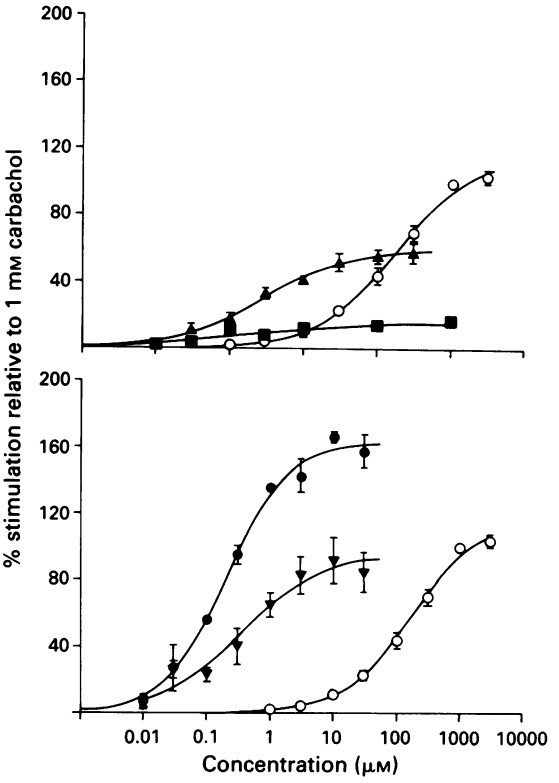

Figure 2 Effect of novel muscarinic agents upon phosphatidyl-inositol turnover in rat cerebral cortex. Methods were as described in Methods section. (a) Effects of quinuclidine derivatives L-658,903 (■) and L-660,863 (▲). (b) Effects of 1-azanorbornane derivatives L-670,548 (▼) and L-670,207 (●). Each curve is the cumulative results of three to five individual experiments, each of which was performed in triplicate. Each curve is the mean with s.e.mean shown by vertical bars. Carbachol (○) is included as a reference compound. Curves were fitted by Allfit as described previously (see Methods section).

Evaluation in pharmacological preparations

The identification of muscarinic receptor subtypes has been possible by the characterization of tissue preparations containing responses mediated by a single receptor subtype. These include the depolarizing response on the rat superior

Table 3 Pharmacological evaluation of novel oxadiazole muscarinic agonists

	Rat superior cervical ganglion M_1			*Guinea-pig atrium* M_2			*Guinea-pig ileum* M_3		
Compound	EC_{50} (nM)	RM	*n*	EC_{50} (nM)	RM	*n*	EC_{50} (nM)	RM	*n*
Muscarine	90 (70;100)	1.2 (1.0–1.8)	10	200 (150;230)	1.0 (0.9–1.1)	8	120 (110;140)	1.0 (0.9–1.1)	18
Carbachol	—	—	—	400 (280;450)	1.0 (0.9–1.1)	7	130 (110;150)	1.0 (0.9–1.1)	10
L-658,903	20 (10;30)	1.2 (0.9–1.2)	4	40 (30;50)	0.95 (0.9–1.0)	5	16 (13;19)	1.0 (0.9–1.1)	8
L-660,863	10 (9.9;2.0)	0.7 (0.5–1.1)	6	17 (14;20)	1.0 (0.9–1.1)	6	10 (80;11)	1.0 (0.9–1.1)	6
L-670,548	2.0 (1.0;3.0)	0.9 (0.9–1.2)	6	3.0 (2.0;3.4)	0.95 (0.9–1.0)	6	1.2 (1.0;1.5)	0.95 (0.9–1.0)	5
L-670,207	10 (7.0;20)	1.3 (0.6–1.6)	5	2.0 (1.0;3.0)	0.8 (0.6–0.9)	4	0.9 (0.8;1.1)	0.8 (0.75–0.85)	3

Detailed methods are described in the Methods section.
EC_{50}: EC_{50} is the concentration required to produce half of RM. Geometric mean (−s.e.mean, +s.e.mean)
RM: relative maximum i.e. amplitude of the response at which the dose-response curves exhibit a plateau relative to the amplitude of a response to 1 μM muscarine (see Methods) or to the maximum response to muscarine or carbachol (atrium and ileum) on the same preparation. Results are expressed as median (range).

cervical ganglion (mediated by M_1 receptors, Brown *et al.*, 1980; Newberry & Priestley, 1987), the negative chronotropic effect on electrically driven guinea-pig atria (mediated by M_2 receptors) and the contraction of the guinea-pig myenteric plexus preparation (mediated by M_3 receptors). The four oxadiazole derivatives were assessed for their relative potencies and efficacies at the muscarinic receptor in these preparations in order to identify any possible selectivity between the M_1, M_2 and M_3 subtypes. The results are shown in Table 3. All four compounds were similarly potent muscarinic agonists in the three pharmacological preparations with a relative maximum response that was indistinguishable from muscarine. The two quinuclidine compounds were up to 10 fold more potent than muscarine whereas the two 1-azanorbornane derivatives were up to 100 fold more potent than muscarine. None of the compounds exhibited any appreciable degree of selectivity between the pharmacological preparations.

Discussion

In cerebral cortex the muscarinic receptors coupled to PI turnover appear to lack an effective receptor reserve. Before the start of these studies it appeared that only quaternary agonists could possess the necessary efficacy to stimulate maximally cortical PI turnover. We now report on the design of non-quaternary compounds that have higher efficacy than natural non-quaternary agonists. In recent studies we have reported that the distribution of the electrostatic charge on a quaternary methylammonium group could be closely mimicked by a protonated 1-azabicyclic ring system exemplified by a quinuclidine ring system (Saunders *et al.*, 1990). This approach led to the development of L-658,903 in which affinity and potency at muscarinic receptors in cortex were increased 10 fold compared with arecoline. Removing a carbon atom from one of the bridges of the quinuclidine ring gave the target compound L-670,548 in which the correct 3 dimensional topography was retained. This derivative showed a further 3 fold increase in affinity and in potency at muscarinic receptors. These results suggested that removal of one methylene group from a bridge of the quinuclidine ring was associated with an increased ability to interact within the binding pocket in the muscarinic receptor pharmacophore.

Both of these methyl-oxadiazole analogues were potent and efficacious agonists on the pharmacological preparations with no evidence of selectivity for any of the three muscarinic receptor subtypes in the pharmacological models. These compounds are therefore amongst the most potent muscarinic agonists known and support the results from the NMS/oxo-M binding assays that suggested that the compounds were potent agonists at the muscarinic receptor in cortex. The findings from the peripheral tissues also support the view that in all three of the pharmacological preparations there is a relatively high receptor reserve for muscarinic receptor stimulation since all of the compounds had a similar maximum response relative to carbachol or muscarine. In contrast when the compounds were examined on the cortical PI model, L-658,903 was a partial agonist with a response similar to that of arecoline though with a substantial increase in potency. Replacement of the methyl oxadiazole in both series by amino oxadiazole resulted in large increases in the efficacy in both chemical series. This can be correlated with the electron donating properties of the amino group resulting in enhancement of the hydrogen bond acceptor properties of the oxadiazole ring system (Saunders *et al.*, 1990). These compounds are some of the most efficacious compounds known and are the first examples to be reported of non-quaternary muscarinic agonists able to stimulate PI turnover in rat cerebral cortex to a level seen with carbachol.

The amino oxadiazole in the 1-azanorbornane series, L-670,207, displayed significantly greater efficacy than either carbachol or muscarine in the cortical slice assay. This response to L-670,207 was entirely sensitive to antagonism by atropine and was the same as that seen with the natural ligand acetylcholine (170% relative to 1 mM carbachol). Acetylcholine was however considerably less potent with an EC_{50} value of 30 μM, even when tested in the presence of the cholinesterase inhibitor, physostigmine (1 μM). This greater relative efficacy was somewhat different from that seen in the results obtained in both the binding studies and the results in the functional studies. The very high affinity of L-670,207 for muscarinic receptors posed a number of technical problems in studying its effects. In the binding studies evidence of significant ligand depletion was observed, an effect that was more pronounced at low concentrations in the agonist binding assay because of the higher protein concentrations that are routinely used for this assay. In order to compensate for this depletion, corrections were made to the displacement curves for L-670,207 by approximating the amount of free drug remaining after depletion by the receptor content of the assay. When this correction was made, the K_{app} was found to be 0.035 (0.030, 0.040) nM which corresponded to an NMS/Oxo-M ratio of 890. In other experiments, lowering the protein content of this assay did also result in higher binding affinity and correspondingly higher ratios; however, there were practical limits in how far this could be followed since lowering the protein concentration reduced the numbers of counts in the assays.

In the functional assays it seems likely that there was a much larger receptor reserve than that present in the cortical PI assay. Under these circumstances even compounds with relatively low NMS/oxo-M ratios, such as RS-86, can be shown to be relatively efficacious agonists. Correspondingly the maximum responses observed for the more efficacious agonists were all similar.

These results describe four oxadiazole based muscarinic agonists that are amongst the most potent muscarinic agonists known. These compounds are highly efficacious agonists at all three of the muscarinic receptor subtypes examined and are the first reports of non quaternary agonists with sufficient intrinsic efficacy to stimulate maximally cortical PI turnover. These compounds will provide useful tools in the study of muscarinic receptors and our understanding of their involvement in central processes.

The authors would like to thank Mrs E. Brawn and Mrs S. Burton for manuscript preparation.

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(Received March 23, 1990
Revised June 15, 1990
Accepted July 3, 1990)

Br. J. Pharmacol. (1990), 101, 581–586

Pinacidil inhibits neuromuscular transmission indirectly in the guinea-pig and rabbit mesenteric arteries

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1 Effects of pinacidil were investigated on neuromuscular transmission in smooth muscle tissues of the rabbit and guinea-pig mesenteric arteries by both electrophysiological procedures and a bioassay of noradrenaline (NA) outflows.

2 Pinacidil (over 1 μM) hyperpolarized smooth muscle cell membranes in both tissues, in a concentration dependent manner. Pinacidil hyperpolarized and increased the ionic conductance of smooth muscle membrane more markedly in the rabbit mesenteric artery than in the guinea-pig. The hyperpolarization induced by pinacidil occurred in the presence or absence of endothelial cells and was blocked by glibenclamide.

3 Perivascular adrenergic nerve stimulation produced excitatory junction potentials (e.j.ps) and repetitive stimulation produced a facilitation of e.j.ps in both tissues. Pinacidil (over 1 μM) reduced the amplitude and the decay time of e.j.ps to a consistently greater extent in the rabbit mesenteric artery than in the guinea-pig. However, the facilitation process of e.j.ps was not modified following application of pinacidil (1 μM). The pinacidil-induced inhibition of e.j.ps was prevented by pretreatment with glibenclamide.

4 Pinacidil (30 μM) marginally increased the overflows of NA and its metabolite, 3,4-dihydroxyphenylglycol (DOPEG) released following repetitive perivascular nerve stimulations.

5 Pinacidil (10 μM) partly inhibited the voltage-dependent Ca channel, as estimated from the recovery process following removal of pinacidil, of action potentials evoked on e.j.ps.

6 It is concluded that pinacidil increases ionic conductance and hyperpolarizes smooth muscle cell membranes of the guinea-pig and rabbit mesenteric arteries and as a consequence, inhibits the neuromuscular transmission process occurring on adrenergic nerve stimulation with no reduction in the amount of released transmitter.

Introduction

Pinacidil is a vasodilator currently under clinical investigation as a potential anti-hypertensive agent (Petersen *et al.*, 1978; Arrigoni-Martelli *et al.*, 1980; Carlsen *et al.*, 1981; Bray *et al.*, 1987; Cook *et al.*, 1988). The main mechanism by which this drug relaxes vascular smooth muscle tissues is thought to be activation of a K channel which may be Ca-dependent (Bray *et al.*, 1987; Cook *et al.*, 1988; Hermsmeyer, 1988; Videbaek *et al.*, 1988). Thus, in vascular smooth muscle tissues, pinacidil consistently hyperpolarized the membrane (rat portal vein: Bray *et al.*, 1987; rat aorta: Southerton *et al.*, 1987; rat mesenteric artery: Videbaek *et al.*, 1988). In addition, pinacidil also exhibits a bronchodilator action and the mechanism underlying this effect is thought to be the same as that observed in vascular tissues (Karlsson & Persson, 1981; Nielsen-Kudsk *et al.*, 1988; Mellemkjaer *et al.* 1989).

The contraction-relaxation cycle in vascular tissues is regulated by many endothelium-derived factors such as endothelium-derived relaxing (Furchgott & Zawadzki, 1980) and hyperpolarizing factors (Chen *et al.*, 1988), prostacyclin (Moncada & Vane, 1979) and endothelin (Yanagisawa *et al.*, 1988). In addition, the above cycle in vascular tissues is also regulated by transmitters released from adrenergic nerves such as noradrenaline (NA) and ATP (Burnstock, 1981; Hirst & Edward, 1989; Stjärne, 1989; Starke *et al.*, 1989). Therefore, in studies of any vasoactive drug action, whether *in vivo* or *in vitro*, the possible effects of the drug on such factors needs to be clarified.

In the present experiments, the effects of pinacidil on neuromuscular transmission were investigated by stimulating the nerves supplying rabbit and guinea-pig mesenteric arteries. Changes in the membrane potential and in the excitatory junction potentials (e.j.ps) of the smooth muscle cells were measured in the presence or absence of endothelial cells. The effects of pinacidil on neuromuscular transmission were also observed by measuring the overflows of noradrenaline (NA) and 3,4-dihydroxyphenylglycol (DOPEG) with bioassay techniques.

Methods

Guinea-pigs of either sex, weighing 250–300 g, were stunned and bled. Albino rabbits (Nippon White; 1.8–2.2 kg) of either sex were anaesthetized with sodium pentobarbitone (Pitman & Moor Inc., Washington Cross NJ, U.S.A. 40 mg kg^{-1} i.v.) and exsanguinated. From the mesenteric vascular bed of the jejunum of each animal, an artery together with vein and lymph vessels running parallel to it was excised. The artery was dissected free from the other tissues under a binocular microscope, and pieces with an external diameter of 120–200 μm and a length of about 5 mm were mounted in an organ bath (capacity 2 ml).

Electrical response recording procedures

To stimulate perivascular nerves (0.05–0.1 ms pulse duration), the partition stimulating method was used (Abe & Tomita, 1968). To prevent excess release of transmitters from nerve terminals, the stimulus frequency used was below 1.0 Hz. E.j.ps were also evoked by the $AgCl_2$ wire stimulating method (Kuriyama & Suzuki, 1981). The electrical responses generated (membrane potential, e.j.ps and spike potentials) from single smooth muscle cells were recorded by use of a glass capillary micro-electrode filled with 3 M KCl, the resistance of the electrodes being 40–80 MΩ. A microelectrode was inserted into the muscle cell from the outer surface through the surrounding connective tissue. The organ bath was superfused with Krebs solution at 35–36°C at a flow rate of 2 ml min^{-1}.

To measure the membrane resistance by the partition stimulating method, constant intensities of rectangular inward and outward current pulses (2 s in duration) were applied alternately before and during application of pinacidil. The

recording microelectrode was inserted into cells less than 100 μm from the stimulating electrode.

Assay procedures for NA and DOPEG

Spontaneous and evoked release of NA and DOPEG from the isolated mesenteric artery were measured by the method described by Mishima *et al.* (1984). Briefly, two parallel Ag-AgCl wires (0.5 mm in diameter and 10 mm in length) separated by about 1 mm were fixed in a vertical plane and the isolated mesenteric artery was attached between the two wires by means of fine threads. Krebs solution (35°C) was dripped onto the tissue at a constant flow rate of 1 ml min^{-1}, so that the tissue was perfused evenly. Electric current pulses (1 ms pulse duration and 100 V intensity, square pulses) were applied for 1 min at 10 Hz, through the two silver wires. Preliminary experiments confirmed that in the presence of 0.3 μM tetrodotoxin, the outflows (release) of NA and DOPEG were not increased by these stimuli. The perfusates were collected into conical 10 ml test tubes placed under the tissue, for 5 min both before and after the period of nerve stimulation. It was confirmed that this time period was sufficient to collect nearly all the catecholamine coming out into the perfusate during nerve stimulation (Mishima *et al.*, 1984). Collected solutions were added to 50 ml perchloric acid (60%), and assayed for their NA and DOPEG content by the modified alumina adsorption method (Oishi *et al.*, 1982). The extracted samples were subjected to high performance liquid chromatography (Yanagimoto MFC, L-200L, Tokyo, Japan). After the experiments, the tissue was blotted on filter paper and weighed. The outflows of NA and DOPEG were expressed both as ng g^{-1} wet weight of tissue and as relative values.

Solutions and drugs

Modified Krebs solution contained (mM): Na^+ 137.4, K^+ 5.9, Ca^{2+} 2.5, Mg^{2+} 1.2, HCO_3^- 15.5, $H_2PO_4^-$ 1.2, Cl^- 134.0 and glucose, 11.5. The solution was bubbled with 97% O_2 and 3% CO_2 and the pH was kept at 7.2–7.3. Solutions containing excess concentrations of KCl (up to 90 mM) were prepared by replacing NaCl isotonically. The drugs used were pinacidil (Shionogi), noradrenaline HCl (NA; Sigma), tetrodotoxin (Sigma) and glibenclamide (Sigma).

Statistics

The values of the measured parameters from the muscle membrane and the amplitude of the e.j.ps were expressed as the mean value with s.d. Statistical significance was determined by Student's *t* test. A value of *P* less than 0.05 was considered to be significant.

Results

Effects of pinacidil on the membrane potential of smooth muscle cells from the guinea-pig and rabbit mesenteric arteries

The resting membrane potential of smooth muscle cells of the guinea-pig mesenteric artery (endothelial cells present; 'intact tissue') was -70.5 ± 2.1 mV ($n = 20$, 3 preparations) and -69.8 ± 1.8 mV ($n = 35$; 3 preparations) in the rabbit mesenteric artery. The muscle cells of both tissues were electrically quiescent. Following removal of endothelial cells ('endothelial-denuded tissue') from both tissues, the membrane potentials of smooth muscle cells remained unchanged (-69.7 ± 1.6 mV, $n = 15$ in the guinea-pig and -69.2 ± 1.7 mV, $n = 15$ in the rabbit; 3 preparations). When pinacidil was added to the superfusate, the membranes of both tissues were hyperpolarized in a concentration-dependent manner. For example, in the guinea-pig, 1 μM pinacidil only marginally hyperpolarized the membrane (not significant, $P > 0.05$), but with application

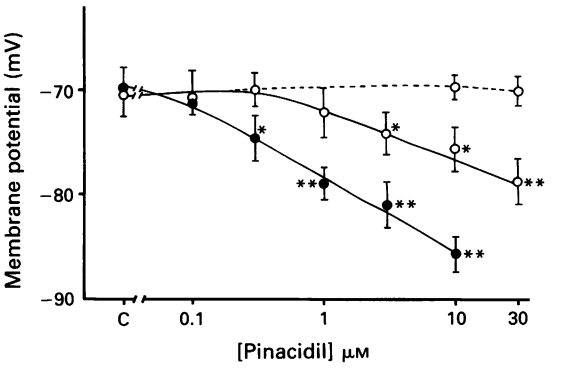

Figure 1 Effects of pinacidil on the membrane potential recorded from smooth muscle cells of the guinea-pig and rabbit mesenteric arteries: (○) guinea-pig mesenteric artery; (●) rabbit. Dashed line indicates pretreatment with glibenclamide (10 μM). Vertical bars: s.d., $n = 15$–35, 2–5 preparations. * $P < 0.05$, and ** $P < 0.01$.

of 30 μM pinacidil, the membrane was hyperpolarized by 7.1 ± 2.0 mV ($P < 0.01$, $n = 20$, 5 preparations; Figure 1). Greater concentrations of pinacidil (up to 400 μM) did not further hyperpolarize the membrane. On the other hand, in the rabbit mesenteric artery, 0.1 μM pinacidil marginally hyperpolarized the membrane ($P > 0.05$). When 10 μM pinacidil was superfused, the membrane was markedly hyperpolarized (17.2 ± 2.1 mV; $P < 0.01$, $n = 25$, 5 preparations; Figure 1) and when the effects of pinacidil on the two tissues were compared, the minimum concentration required to modify the membrane potential was 10 times higher in the guinea-pig mesenteric artery than in the rabbit mesenteric artery.

In the endothelium-denuded tissues, pinacidil (10 μM) hyperpolarized the membrane to the same extent as in intact tissues (16.4 ± 2.1 mV, $n = 15$; 3 preparations in the rabbit, and 3.6 ± 0.7 mV, $n = 7$; 3 preparations in the guinea-pig). Figure 1 also shows that glibenclamide (10 μM) completely prevented the hyperpolarization normally induced by pinacidil (up to 30 μM) in the guinea-pig mesenteric artery.

Figure 2 shows the effects of pinacidil (10 μM) on the membrane potential of smooth muscle cells of the guinea-pig mesenteric artery under pretreatment with various concentrations of NA (3 μM to 100 μM). As described previously (Kuriyama & Makita, 1983), a much higher concentration of NA (10 μM) was required to depolarize the muscle membrane of the guinea-pig mesenteric artery than to depolarize that of the rabbit mesenteric artery (0.1 μM). As shown in Figure 2a and b, when 10 μM NA was applied, the smooth muscle membrane of the guinea-pig mesenteric artery was depolarized from -70.2 ± 2.8 mV to -67.7 ± 2.4 mV ($n = 10$, 2 preparations, $P < 0.05$). When 10 μM pinacidil was given during exposure to NA (30 μM; Figure 2b), the membrane potential returned to the control value (NA; -65.2 ± 2.1 mV vs pinacidil with NA; -71.8 ± 2.2 mV, $n = 15$, 2 preparations). With a higher concentration of NA (100 μM), the membrane was more strongly depolarized (-57.1 ± 2.9 mV, $n = 12$, 2 preparations), and on treatment with 10 mM pinacidil, the membrane was repolarized almost to the control value; -70.2 ± 1.8 mV, $n = 10$, 2 preparations).

The effects of pinacidil on the KCl (1–90 mM)-induced membrane potential change were observed. The membrane potential of smooth muscle cells of the guinea-pig mesenteric artery was -70.5 ± 2.8 mV ($n = 6$, one preparation; 5.9 mM KCl). With application of high concentrations of KCl, the membrane was depolarized in a concentration-dependent manner (the maximum slope of membrane potential changes produced by 10 fold changes in KCl concentrations plotted on the logarithmic scale was 50 mV). When the [KCl] in the Krebs solution was reduced below 5.9 mM, the membrane was slightly hyperpolarized, but when [KCl] in the bath was reduced below 1 mM, the membrane became depolarized relative to the membrane potential in 5.9 mM KCl (Figure 3). When pinacidil

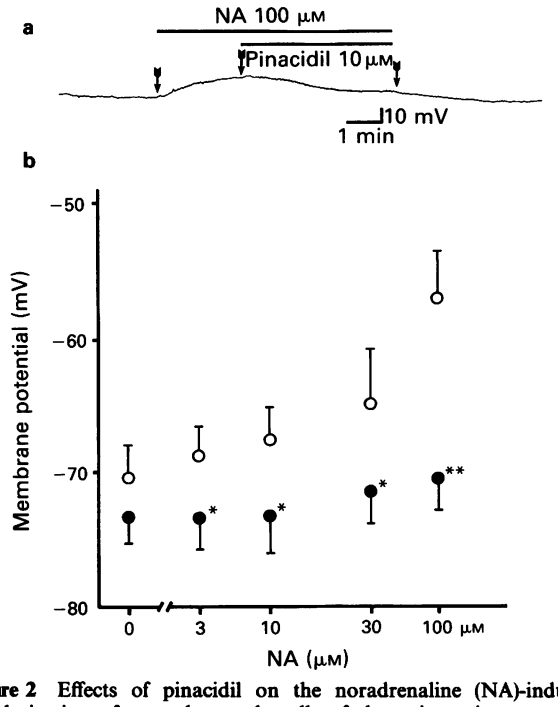

Figure 2 Effects of pinacidil on the noradrenaline (NA)-induced depolarization of smooth muscle cells of the guinea-pig mesenteric artery. (a) Effects of NA (100 μM) and of NA with 10 μM pinacidil (arrows and horizontal bars). (b) Effects of 10 μM pinacidil on the NA-induced depolarization: (○) NA alone; (●) NA with pinacidil. Vertical bars: s.d., n = 13–28, 2–5 preparations. * $P < 0.05$ and ** $P < 0.01$.

(10 μM) was applied, the muscle membrane in the presence of below 10 mM KCl was consistently hyperpolarized ($P < 0.05$, Figure 3), while in the presence of 20 or 40 mM KCl, the membrane was only marginally hyperpolarized (Figure 3). The maximum hyperpolarization observed with application of 10 μM pinacidil was -85.0 ± 3 mV ($n = 5$) in 2.4 mM KCl. The maximum slope of membrane depolarization against 10 fold changes in KCl was increased to 58 mV in smooth muscle cells of the guinea-pig mesenteric artery. This value is close to

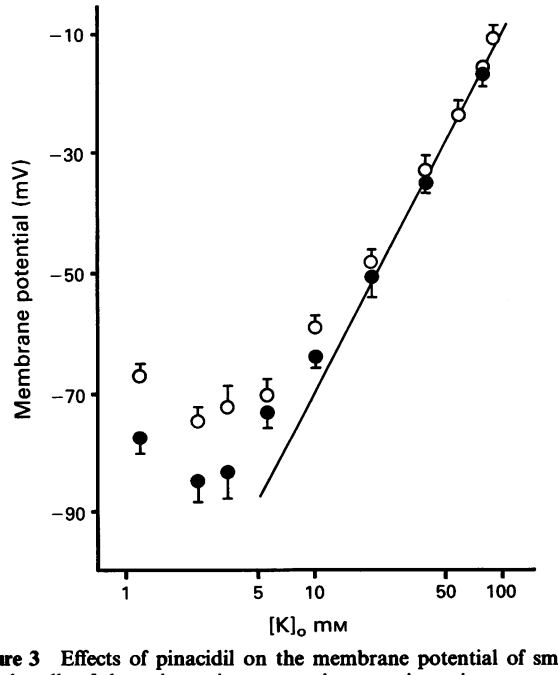

Figure 3 Effects of pinacidil on the membrane potential of smooth muscle cells of the guinea-pig mesenteric artery in various concentrations of KCl: (○) control; (●) in the presence of pinacidil (10 μM). Vertical bars: s.d., n = 6–15, 2–3 preparations. The line indicates the maximum slope of the mean membrane potential changes induced by various concentrations of KCl.

the value for the K electrode as estimated from the Nernst equation (Figure 3).

To elucidate the mechanism underlying the hyperpolarization induced by pinacidil, changes in the membrane input resistance of smooth muscle cells of the rabbit mesenteric artery were estimated from the amplitude of electrotonic potentials recorded before and after application of pinacidil (the recording electrode was inserted within 100 μm of the stimulating electrode, the length constant of smooth muscle tissues of the rabbit mesenteric artery being 1.2 mm; Suzuki, 1985). When pinacidil (1 or 10 μM) was applied, electrotonic potentials evoked were consistently reduced during the hyperpolarization (0.62 ± 0.04 times and 0.36 ± 0.03 times the control at the maximum hyperpolarization, respectively, $n = 4$). In the guinea-pig mesenteric artery, the input resistances were reduced to 0.84 ± 0.06 times and 0.68 ± 0.04 times the control, respectively, with the same doses of pinacidil ($n = 3$). This means that the hyperpolarization induced by pinacidil is due to an increase in the K permeability of the membrane, as has been reported previously (Bray *et al.*, 1987; Southerton *et al.*, 1987; Videbaek *et al.*, 1988).

Effects of pinacidil on e.j.ps recorded from the guinea-pig and rabbit mesenteric arteries

Perivascular nerve stimulation produced an e.j.p. in smooth muscle tissues of the guinea-pig and rabbit mesenteric arteries. Repetitive stimulation at frequencies over 0.1 Hz produced an enhancement (facilitation) of e.j.p. amplitude which reached a steady level after several stimulations and then was either sustained or slightly reduced below the peak value (Figure 4a and b). The amplitude of e.j.ps after completion of the facilitation process was frequency-dependent. These e.j.ps were abolished by 1 μM tetrodotoxin. The amplitude of e.j.ps evoked by perivascular nerve stimulation in the rabbit mesenteric artery was almost the same whether in the presence or absence of endothelial cells (2–5 mV evoked by single stimuli in intact tissues, and 2–6 mV in endothelium-denuded tissues).

Differences were observed in the time course of the falling phase of the e.j.ps recorded from the rabbit and guinea-pig mesenteric arteries. E.j.ps in both tissues showed a single exponential decay measured after the peak and when the time constant of the falling phase was calculated from e^{-1}, the e.j.p. decay period was longer in smooth muscle cells of the rabbit mesenteric artery than in those of the guinea-pig mesenteric artery (mean value of the time constant being 205 ± 16 ms, $n = 8$, 3 preparations in the guinea-pig, and 303 ± 31 ms, $n = 9$, 3 preparations in the rabbit; Figure 4c vs Figure 4d). In the rabbit mesenteric artery, the growth in the e.j.p. amplitude during the facilitation was more marked than in the guinea-pig. Thus, the steady amplitude of e.j.ps following completion of the facilitation was more than three times control in the rabbit but only about twice in the guinea-pig following a train stimulation of 1.0 Hz (Figures 4 and 6). In addition, after repetitive stimulation, a slow depolarization often occurred in the rabbit mesenteric artery but not in the guinea-pig mesenteric artery (Kuriyama & Suzuki, 1981; Kuriyama & Makita, 1983; Suzuki, 1985 and the present experiments).

Pinacidil (over 1 μM) consistently reduced the amplitude of e.j.ps in both tissues (Figure 5). In the guinea-pig, 0.3 μM pinacidil marginally reduced the e.j.p. amplitude (0.93 times control; $n = 16$, 4 preparations; $P > 0.05$) which was further reduced as the pinacidil concentration was increased. However, the reduction was greater in the rabbit (guinea-pig: pinacidil, 1 μM and 10 μM; 0.88 ± 0.03 times and 0.79 ± 0.41 times the control, respectively, $n = 16$, 4 preparations; $P < 0.05$; rabbit: pinacidil, 1 μM and 10 μM; 0.65 ± 0.05 and 0.31 ± 0.03 times the control, respectively, $n = 10$, 3 preparations). When glibenclamide (20 μM) was given as a pretreatment, pinacidil (up to 10 μM) did not inhibit e.j.p. amplitude (in the guinea-pig, in 20 μM glibenclamide with 10 μM pinacidil; 1.01 ± 0.02 times the control, $n = 9$, 7 prep-

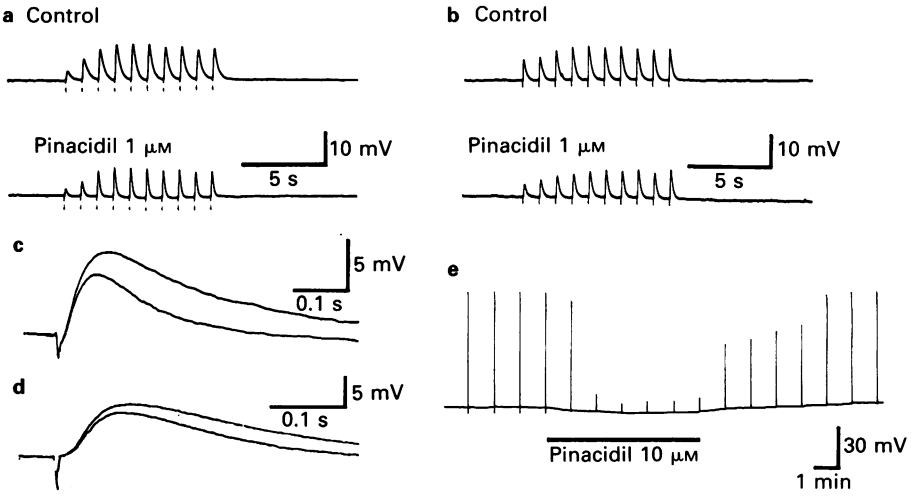

Figure 4 Effects of pinacidil on the excitatory junction potentials (e.j.ps) recorded from smooth muscle cells of the guinea-pig and rabbit mesenteric arteries. (a and b) Rabbit and guinea-pig mesenteric arteries, respectively. E.j.ps were evoked by 1 Hz stimulation before and after treatment with 1 μM pinacidil. (c and d) Superimposed e.j.ps evoked by single stimuli before and after application of 1 μM pinacidil in the rabbit and guinea-pig mesenteric arteries, respectively. (e) Effect of 10 μM pinacidil on the action potentials evoked by perivascular nerve stimulation (50 V, 1 ms pulse duration).

arations; Figure 5). Glibenclamide also prevented the pinacidil-induced inhibition of e.j.ps in rabbit mesenteric arteries. The time constant of e.j.ps was consistently reduced following application of 1 μM pinacidil from 303 ± 31 ms to 138 ± 94 ms in the rabbit mesenteric artery (n = 10, 3 preparations) and from 205 ± 16 ms to 177 ± 9 ms in the guinea-pig mesenteric artery (n = 10, 3 preparations; Figure 4c and d).

When the effects of pinacidil on e.j.ps were observed in endothelium-denuded segments of guinea-pig mesenteric artery, 10 μM pinacidil inhibited the e.j.p. amplitude to 0.822 ± 0.02 times the control (n = 4, 3 preparations). This inhibition was similar to that observed in the presence of endothelial cells.

Pinacidil (1 μM) consistently inhibited the amplitude of e.j.ps in both tissues at any number and frequency of stimulations (measured frequencies of below 1.0 Hz; Figures 4 and 6). However, as shown in Figure 6, if the amplitudes of the first e.j.p. evoked by the first pulse in a stimulation train before and after application of pinacidil were normalized, the facilitation curves obtained using 1.0 Hz stimulation overlapped in both tissues (n = 4 preparations). This means that the facilitation process is not modified by pinacidil in either tissue.

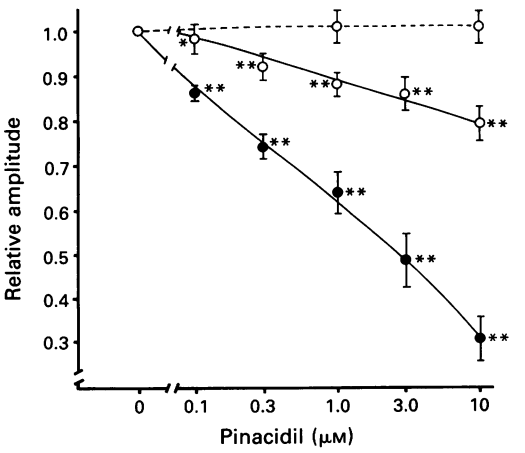

Figure 5 Effects of various concentrations of pinacidil on the amplitude of e.j.ps evoked in the guinea-pig (○) and rabbit (●) mesenteric arteries. Dashed lines indicate the effects of pinacidil following application of glibenclamide (10 μM). Symbols are as in Figure 1, n = 15–23, 2–4 preparations.

When strong intensities of perivascular nerve stimulation were applied, an e.j.p. was evoked which triggered spike generation in both the rabbit and guinea-pig mesenteric arteries. When pinacidil (10 μM) was applied to guinea-pig mesenteric artery during repetitive stimulations of strong intensity and at long intervals, the membrane was slightly hyperpolarized and the spike amplitude reduced (Figure 4e). Removal of pinacidil depolarized the membrane within a few min to the level before application, but the amplitude of the spike superimposed on the e.j.p. returned to the control level with a much longer time course than that associated with the recovery of the membrane potential.

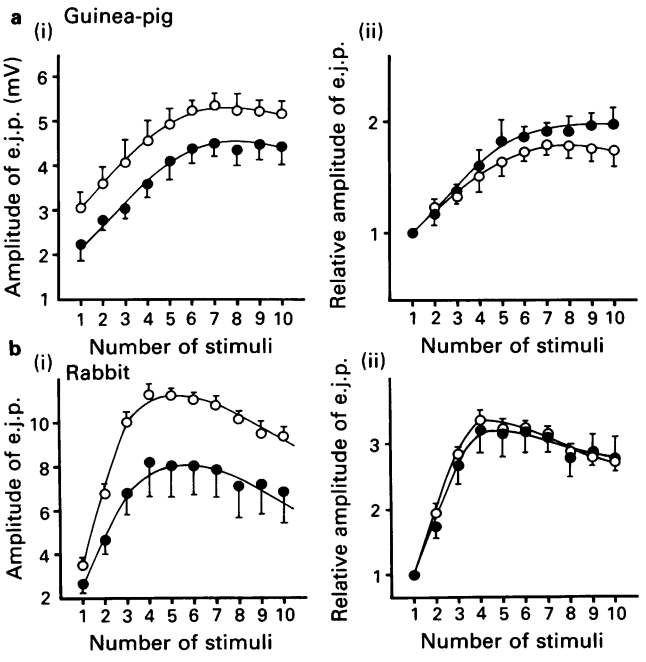

Figure 6 Effects of pinacidil on the facilitation of e.j.ps recorded from the guinea-pig (a) and rabbit (b) mesenteric arteries. (ai) and (bi): Actual e.j.p. amplitudes recorded by perivascular nerve stimulation (1 Hz) before and after application of 1 μM pinacidil. (○) Control; (●) after pinacidil application. (aii) and (bii) The amplitude of the first e.j.p. was normalised as a relative amplitude of 1.0. Data from the same samples were used to derive the points in (i) and (ii). Vertical bars; s.d., n = 4 preparations.

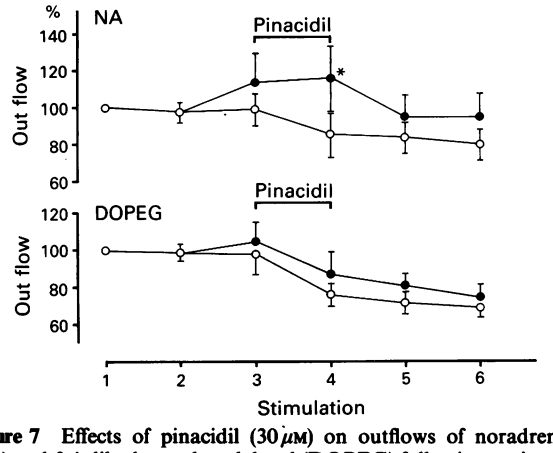

Figure 7 Effects of pinacidil (30 μM) on outflows of noradrenaline (NA) and 3,4-dihydroxyphenylglycol (DOPEG) following perivascular nerve stimulation (see Methods). Pinacidil was applied during 3rd and 4th stimulations. The outflows of NA and DOPEG measured on the first stimulation was normalized as 100% ($n = 9$, 5 preparations). (○) Control (after the 3rd stimulation, $n = 5$, 3 preparations); (●) pinacidil treatment (after the 3rd stimulation, $n = 4$, 2 preparations). Absolute values of NA and DOPEG measured during the first stimulation are given in the text). * $P < 0.05$.

Effects of pinacidil on the outflows of NA and DOPEG

In the absence of perivascular nerve stimulation, the resting outflows of NA and DOPEG were 9.8 ± 1.1 and $10.4 \pm 1.0\,ng\,g^{-1}$ wet weight of tissue, ($n = 12$, 7 preparations). During any first period of perivascular nerve stimulation, the outflows of NA and DOPEG were increased to 36.0 ± 15.1 and $40.3 \pm 8.4\,ng\,g^{-1}$ wet weight of tissue, respectively ($n = 9$, 5 preparations). With repeated stimulation for periods of 1 min at intervals of 30 min, the evoked outflows of both substances gradually decreased. In the absence of pinacidil, application of perivascular nerve stimulation six times at 30 min intervals resulted in a reduction of the outflows of both NA and DOPEG (at the 6th stimulation, the amounts of NA and DOPEG were reduced respectively, to 0.79 ± 0.06 times and 0.70 ± 0.05 times those evoked by the first stimulation). When pinacidil (30 μM) was applied from the 3rd to the 4th stimulation periods, the outflows of NA, but not of DOPEG, were marginally increased but this was not statistically significant, except for the 4th stimulation for the NA outflow in the presence of pinacidil ($P < 0.05$; Figure 7).

Discussion

Pinacidil hyperpolarized smooth muscle cell membranes of the rabbit and guinea-pig mesenteric arteries, in a concentration-dependent manner, as observed in other vascular tissues (Bray *et al.*, 1987; Cook *et al.*, 1988; Hermsmeyer, 1988; Southerton *et al.*, 1988; Videbaek *et al.*, 1988). The pinacidil-induced hyperpolarization was completely inhibited by glibenclamide. Therefore, this hyperpolarization may be induced by the activation of a K channel modulated by intracellular [ATP] as observed with other K channel openers (nicorandil or cromakalim) in cardiac and vascular smooth muscles (Standen *et al.*, 1989; Buckingham *et al.*, 1989; Hiraoka & Fan, 1989; Kajioka *et al.*, 1990). The resting membrane potential of smooth muscle cells in both tissues was almost the same. However, in the rabbit mesenteric artery, the membrane was more hyperpolarized by pinacidil and the associated increase in ionic conductance, as estimated from the amplitude of electrotonic potential was greater than in the guinea-pig. Therefore, these differences may be due to variations in the density of the K channels responsible for producing the hyperpolarization, or to differences between the K equilibrium potentials in the two tissues. Such differences were also observed in the actions of nicorandil on the rat and rabbit portal veins; i.e. the smooth muscle cells of both tissues showed almost the same membrane potential in the resting state but nicorandil, another K channel opener, more markedly increased the open probability and open time of the Ca-dependent, ATP- and glibenclamide-sensitive K channel in the rabbit portal vein than those in the rat (Nakao *et al.*, 1988; Kajioka *et al.*, 1990). Presumably, species differences in channel density may occur and underlie the different magnitudes of response to pinacidil.

The pinacidil-induced hyperpolarization and inhibition of e.j.ps were not modified following removal of endothelial cells from the vessels and thus pinacidil probably does not modify the actions of EDHF or endothelin in the tissues.

E.j.ps are thought to be generated by activation of P_2-receptors (Burnstock, 1980; 1981; Sneddon & Burnstock, 1985) or γ-adrenoceptors (Hirst & Neild, 1980; Hirst *et al.*, 1982; Hirst & Edward, 1989) distributed on the post-junctional smooth muscle membrane. However, under physiological conditions the electrical response (e.j.p.) occurring following perivascular nerve stimulation is a good indicator of the amount of NA released, as estimated from direct measurements of the amount of released NA by assay procedures and from the amplitude of e.j.ps (Suzuki, 1989).

In the present study pinacidil (30 μM) increased the mean outflow of NA but not that of DOPEG in the rabbit mesenteric artery, although the values did not reach statistical significance, except during a single measurement period. Recently, Nedergaard (1989) reported that pinacidil, in concentrations over 10 μM, increased the outflow of [^{3}H]-NA in rabbit pulmonary artery. If pinacidil possesses the ability to hyperpolarize nerve terminals, an enhanced outflow of NA might result from the greater excitation at the hyperpolarized nerve terminals. However, we observed that 10 μM pinacidil markedly inhibited e.j.p. amplitude (0.3 times the control in the rabbit and 0.8 times the control in the guinea-pig) in spite of the marginal increase in the NA outflow.

The amplitude of e.j.ps varies with tissues and species (Kuriyama *et al.*, 1982) and facilitation following repetitive perivascular nerve stimulation was observed in both the rabbit and guinea-pig mesenteric arteries presumably due to an increase in Ca mobilisation in the nerve terminals (Kuriyama & Makita, 1983). The falling phase of the e.j.p. decayed in an exponential manner, thus indicating that the time constant of the decay ($\tau_{e.j.p.}$) is dependent on the time constant of the passive membrane characteristics (τ_m) expressed as the product of membrane resistance (r_m) and membrane capacitance (c_m), that is $\tau_m = c_m \times r_m$. When the reduction in e.j.p. amplitude observed on application of 10 μM pinacidil was compared with the parallel reductions in $\tau_{e.j.p.}$ and in r_m, it appeared that the reduction in e.j.p. amplitude could be explained by an increased ionic conductance of the smooth muscle cell membrane. Thus, in the guinea-pig, the reductions in r_m, $\tau_{e.j.p.}$ and in e.j.p. amplitude in the presence of 1 μM pinacidil were 0.84 times, 0.86 times and 0.88 times the control, respectively. In the rabbit, they were 0.62 times, 0.45 times and 0.66 times the control, respectively and in the presence of 10 μM pinacidil, the reductions in r_m and e.j.p. amplitude were 0.36 times and 0.31 times the control, respectively. In contrast, facilitation itself was not modified by pinacidil suggesting that this process is not influenced by this agent.

Pinacidil also blocked the spikes produced on some e.j.ps. However following removal of pinacidil, the muscle membrane became repolarized to the resting membrane potential before complete restoration of the spike amplitude. This suggests that pinacidil may partly inhibit the voltage-dependent Ca channels, a phenomenon also observed with the K-channel opener, cromakalim (Nakao *et al.*, 1988; Okabe *et al.*, 1990).

In conclusion, pinacidil produced hyperpolarization, increased the ionic conductance of the smooth muscle cell membrane and reduced the amplitude of e.j.ps, effects which were blocked by glibenclamide. The reduction in e.j.p. amplitude was mainly due to an increase in the membrane K-conductance. In addition, pinacidil may partly inhibit the

voltage-dependent Ca channel in vascular smooth muscle. Thus, pinacidil indirectly inhibits neuromuscular transmission in mesenteric arteries in the rabbit and guinea-pig via changes in the membrane properties of the smooth muscle cells.

We are grateful to Dr R.J. Timms, Birmingham University for critical reading of the manuscript. This work was supported by Grants in Aid for Scientific Research from the Ministry of Education, Science and Culture in Japan.

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(Received April 4, 1990
Revised June 8, 1990
Accepted June 28, 1990)

Br. J. Pharmacol. (1990), **101**, 587–590

Alterations in histamine receptors of guinea-pig ileal smooth muscle produced during agonist-induced desensitization

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1 The effects of prolonged treatment with histamine (10^{-4} M, 30 min) on desensitization at histamine H_1-receptors of guinea-pig ileal longitudinal muscle were investigated.

2 This treatment did not change either the maximum amount or dissociation constant (K_d) of [^{3}H]-mepyramine binding to membranes of guinea-pig ileal muscle.

3 In contrast, this treatment shifted the histamine inhibition curves of [^{3}H]-mepyramine binding to the right both in the presence and absence of 0.5 mM guanosine-5'-triphosphate (GTP). This rightward shift of the curves occurred rapidly in the first 10 min of exposure to histamine.

4 The histamine inhibition curves were analyzed with a two binding sites model. It was shown that the histamine-induced affinity change of the receptor for the agonist occurred with the high affinity binding component (which comprise about 80% of the total), whereas no significant change occurred with the low affinity component. The GTP-dependent decrease in the affinity of the receptor for the agonist also occurred with the high affinity component both in control and histamine-treated preparations.

5 These studies suggest that histamine-induced desensitization was caused by alteration in the affinity of the receptor for the agonist rather than reduction in the number of the receptors and that the interaction of the receptor with a guanine nucleotide regulatory protein was retained in the desensitized state.

Introduction

The administration of a large dose of histamine to guinea-pig ileal smooth muscle is followed by a period of desensitization, during which the responses to histamine are reduced. This desensitization phenomenon has been shown to be non-specific, that is, the responses to other agonists such as acetylcholine may also be reduced (Paton, 1961; Gosselin & Gosselin, 1973; Aboulafia *et al.*, 1987). This situation is currently explained by assuming that steps common to the excitation-contraction coupling pathway used by these agonists have been modified in the desensitization process (Siegel *et al.*, 1984). However, a specific component of desensitization is also proposed as one of the mechanisms of histamine-induced desensitization (Kenakin & Cook, 1980; Bielkiewicz & Cook, 1984). These authors suggested that histamine-induced desensitization involved changes at the receptor level. Our previous study (Horio *et al.*, 1990) confirmed this proposition. At present, however, there is no direct evidence which indicates the involvement of the receptor molecule in the histamine-induced desensitization processes. Thus, in the present study, we used the method of receptor binding assay using [^{3}H]-mepyramine as a selective ligand to determine the changes in the histamine H_1-receptor which might occur in the process of desensitization of guinea-pig ileum.

Methods

Preparation of the membrane fraction

Guinea-pigs of either sex, weighing 300–500 g, were killed by a blow on the head and cutting the throat. The ileum was removed and strips of longitudinal muscle were obtained according to the method of Rang (1964). The strips were suspended in Tyrode solution at 31°C and bubbled with air under a resting tension of about 0.5 g. The Tyrode solution had the following composition (mM): NaCl 136.9, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1.05, NaH_2PO_4 0.42, $NaHCO_3$ 11.9 and glucose 5.6. The strips were then treated with 10^{-4} M histamine for 30 min in Tyrode solution, washed for 5 min with Tyrode solution, and transferred to ice-cold 50 mM sodium-potassium phosphate buffer (pH 7.4). Strips were cut into small pieces with scissors, and homogenized in 10 volumes of 50 mM phosphate buffer with a Polytron blender (setting 6) for three periods of 15 s at 1 min intervals. In control experiments, the strips were incubated for 35 min in Tyrode solution without histamine, and transferred to ice-cold 50 mM phosphate buffer and treated by the same procedure as above. In the experiments on time course, strips were treated with 10^{-4} M histamine for 5, 10, and 30 min, washed for 5 min with Tyrode solution and transferred to ice-cold 50 mM phosphate buffer and then treated by the same procedure as above. The homogenate was centrifuged at 50,000 *g* for 30 min; the pellet was resuspended in the same buffer and then centrifuged at 50,000 *g* for 30 min. The final pellet was resuspended in 5 mM phosphate buffer. The suspension was used immediately.

Measurements of [^{3}H]-mepyramine binding

Aliquots of the membrane suspension (450 μl, 0.4 mg of protein in final concentrations of 50 mM Tris-HCl, pH 7.4) were incubated with 50 μl of a [^{3}H]-mepyramine solution. After 30 min at 25°C, the reaction was stopped by dilution with 4 ml of ice-cold buffer (50 mM Tris-HCl, pH 7.4) containing 10 μM mepyramine, followed by rapid filtration over Whatman GF/B filters under vacuum (according to Daum *et al.*, 1982). Filters were washed once more with 4 ml of the same buffer containing 10 μM mepyramine, and twice with 5 ml of buffer. Radioactivity trapped on the filters was measured by liquid scintillation spectrophotometry in a toluene-based scintillation cocktail. Specific binding was evaluated as the difference between radioactivity bound in the absence and in the presence of 2 μM promethazine. For experiments on the inhibition of [^{3}H]-mepyramine binding by histamine, the concentration of [^{3}H]-mepyramine was fixed at 2 nM and the concentration of histamine varied. In this case, non-specific binding was evaluated as the binding in the presence of 2 μM promethazine and varying concentrations of histamine, specific binding representing about 80% of the total. Protein was determined by the method of Lowry *et al.* (1951) with bovine

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serum albumin as a standard. All binding measurements were done in duplicate or triplicate.

Data analysis

Values for maximal binding (B_{max}) and dissociation constants (K_d) of [^{3}H]-mepyramine were obtained by Scatchard analysis (Scatchard, 1952) by linear regression of least squares. The inhibition curves of [^{3}H]-mepyramine binding by histamine were analyzed with a model for either one or two binding sites by a nonlinear least squares curve-fitting procedure – the programme was implemented on the microcomputer system PC-9800 (NEC, Japan) using the SIMPLEX method (Nelder & Need, 1965) as previously described (Zhou *et al.*, 1988). The equations used for curve fitting were

$$\frac{B}{B_{max}} = \frac{L^n}{L^n + IC_{50}{}^n}$$

for one-site model, and

$$\frac{B}{B_{max}} = \frac{N_1 \times L}{L + (IC_{50})_1} + \frac{N_2 \times L}{L + (IC_{50})_2}$$

for two-site model, where B represents specific [^{3}H]-mepyramine binding, B_{max} maximum binding of [^{3}H]-mepyramine, L the concentration of histamine, IC_{50} the histamine concentration giving half maximal inhibition of [^{3}H]-mepyramine binding, n the Hill coefficient, N_1 and N_2 the respective contributions of the two components expressed as percentages of the total population, $(IC_{50})_1$ and $(IC_{50})_2$ the respective IC_{50} values of the two components. The equilibrium dissociation constants (K_d) were calculated using the relationship

$$K_d = \frac{IC_{50}}{C_{mep}/K_{mep} + 1}$$

where C_{mep} is the concentration of mepyramine and K_{mep} its dissociation constant. Statistical evaluation of significant differences was performed with Student's *t* test.

Drugs

[^{3}H]-mepyramine (28 and 32 Ci mmol^{-1}) was obtained from New England Nuclear. Mepyramine maleate was purchased from Sigma, and histamine dihydrochloride and promethazine hydrochloride were from Wako Chemical Industry. Guanosine-5'-triphosphate (GTP) was from Boehringer Mannheim. All other chemicals were from Wako Chemical Industry.

Results

Effects of agonist exposure on [^{3}H]-mepyramine binding

We previously demonstrated (Horio *et al.*, 1990) that a 30-min exposure to 10^{-4}–10^{-1} M histamine caused substantial desensitization of guinea-pig ileal smooth muscle. In the present study, we examined whether such agonist treatment which led to desensitization of the histamine response also caused alterations in the histamine receptors. First, we measured the binding of the histamine H_1-antagonist, [^{3}H]-mepyramine, to membranes prepared either from control or histamine-pretreated guinea-pig ileal muscle. In control membranes, the maximum binding (B_{max}) of [^{3}H]-mepyramine varied between membrane preparations ranging from 102 to 335 fmol mg^{-1} protein as was pointed out by Hill & Young (1981). Therefore, in the present study, we used the membranes prepared from the same animal for both control and histamine-pretreated experiments. Figure 1 shows the result obtained from one of three such experiments. Scatchard analysis of the data gave a single affinity of [^{3}H]-mepyramine binding both for control and histamine-pretreated membranes. The dissociation constant (K_d) was 0.91 ± 0.03 nM for control membranes and 0.81 ± 0.04 nM for histamine-pretreated membranes (n = 3). The maximum binding (B_{max}) was 240 ± 71 fmol mg^{-1} protein for control membranes and 297 ± 29 fmol mg^{-1} protein for histamine-pretreated membranes (n = 3). Both the K_d values and the B_{max} values were not significantly different in control and pretreated membranes. Thus neither the total number nor the affinity of antagonist binding to histamine receptors was changed by histamine treatment.

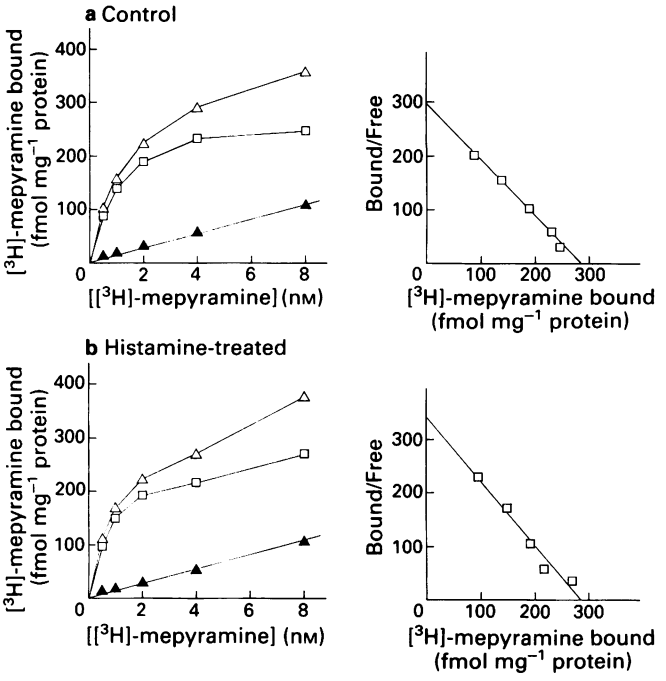

Figure 1 Saturation curves and Scatchard plots for [^{3}H]-mepyramine binding to membranes from (a) control and (b) histamine-treated preparations. Saturation curves for [^{3}H]-mepyramine binding were examined in 50 mM Tris-Cl (pH 7.4) as described under Methods. Specific binding (□) was obtained by subtracting the binding in the presence of 2 μM promethazine (▲) from binding with no inhibitor present (△). Scatchard plots were derived from these specific [^{3}H]-mepyramine binding data. The results shown are from a representative experiment. Mean K_d and B_{max} values are given in the text.

Effects of agonist exposure on the inhibition of [^{3}H]-mepyramine binding by histamine

Next, we examined the affinity of the receptor for agonist in control and histamine-treated preparations by studying the inhibition curves of [^{3}H]-mepyramine binding by histamine. These assays were performed both in the presence and the absence of 0.5 mM GTP. The experiments summarized in Figure 2 demonstrated that histamine receptors in membranes from histamine-pretreated preparations had a decreased affinity for the agonist. This was shown by increases in the IC_{50} values and rightward shifts in the histamine inhibition curves. The IC_{50} values were increased from 16.0 ± 2.1 μM to 26.6 ± 2.8 μM (significantly different, $P < 0.05$). When assays were conducted in the presence of GTP, the inhibition curves were also shifted rightward both in control and histamine-treated preparations, that is, the IC_{50} values were increased from 16.0 ± 2.1 μM to 25.7 ± 1.4 μM in control preparations, and 26.6 ± 2.8 μM to 45.4 ± 5.6 μM in histamine-treated preparations. These changes of the IC_{50} values were significantly different ($P < 0.05$). Hill coefficients of the inhibition curves for control and histamine-treated membranes were 0.73 ± 0.06 and 0.79 ± 0.04 in the absence of GTP, and 0.78 ± 0.04 and 0.92 ± 0.03 in the presence of GTP. These values were significantly less than unity ($P < 0.05$) except for the last one. Each inhibition curve was analyzed on the assumption that histamine binding occurred at two independent sites (Hill & Young, 1981) using nonlinear least square regression. The best-fit values of dissociation constant (K_d) and the percentage of each site are shown in Table 1. These data indicate that histamine receptors are separated into two groups, a high-

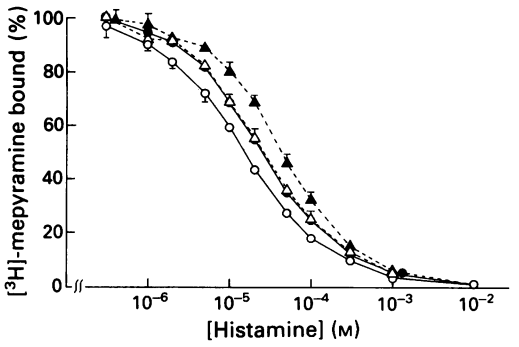

Figure 2 Curves for inhibition by histamine of [^{3}H]-mepyramine binding to membranes prepared from control and histamine-treated preparations. The inhibition of the binding of 2 nM [^{3}H]-mepyramine by histamine in 50 mM Tris-Cl (pH 7.4) was measured both in the presence and the absence of 0.5 mM guanosine 5'-triphosphate (GTP) as described in Methods. (○) Control; (△) histamine-treated; (●) control, with GTP; (▲) histamine-treated, with GTP. Points are the mean calculated from six (control) or four (histamine-treated) experiments. Bars represent s.e.mean. Where no error bars are shown the error was within the size of the symbol.

affinity component and a low-affinity component, the former amounts to about 80%, the latter about 20%. The proportions of the two components were not changed by the prior treatment with histamine or in the presence of GTP. Rather, the K_d values for the high affinity component were changed by these treatments but not the K_d values for the low affinity component.

To examine whether changes in the affinity of the receptor for agonist may be related to histamine-induced desensitization of the histamine response, we determined the time course for histamine to cause a decrease in the receptor affinity. As shown in Figure 3, a decrease in the receptor affinity occurred within 10 min and remained at the maximal level for the next 20 min.

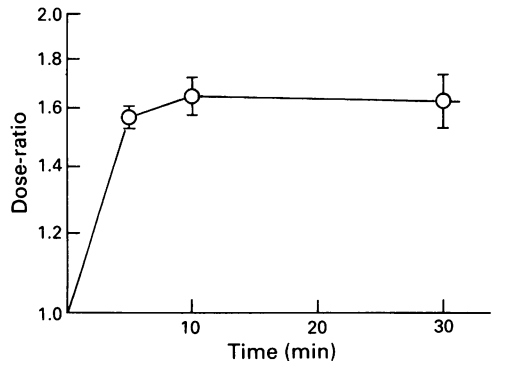

Figure 3 Time course of histamine-induced decrease in receptor affinity for agonist. Guinea-pig ileal strips were exposed to 10^{-4} M histamine for the times indicated on the abscissa scale, and membranes were prepared for binding assays as described under Methods. Dose-ratios on the ordinate scale were determined from the histamine inhibition curves of [^{3}H]-mepyramine binding to assess the extent of affinity change: the dose-ratio was calculated as the ratio of the IC_{50} value for histamine-treated membranes to that for control membranes. Each point is the mean of four experiments and the s.e.means are indicated by vertical lines.

Discussion

It has been proposed that histamine-induced desensitization of guinea-pig ileal smooth muscle occurs at the receptor level (Kenakin & Cook, 1980; Bielkwiewicz & Cook, 1984), and our previous study (Horio *et al.*, 1990) supported this view. The aim of the present study was, then, to determine if agonist treatments which led to desensitization of the histamine response were accompanied by alterations in histamine receptors. Our data showed that histamine pretreatment of the tissue did not change the total number of histamine receptors, but altered the affinity of the histamine receptor for the agonist. The affinity change observed in the desensitized state is not due to the effects of any residual histamine, since residual histamine in the histamine-treated preparations would produce a leftward shift in the histamine inhibition curves and in fact a rightward shift was observed in the present study. The receptor affinity for agonist was analyzed by the use of a two binding sites model as previously employed by Hill & Young (1981). This analysis showed that histamine treatment did not change the proportion of high and low affinity components but caused a decrease in the affinity of the receptor of the high affinity component. The high affinity component of the receptor comprised about 80% of the total and can be considered to take a physiologically significant role. It is not certain whether any change occurred in the receptors of the low affinity component because of the large standard errors of the parameter estimates in the case of the low affinity component (see Table 1). Previous studies (Quach *et al.*, 1981; Mitsuhashi & Payan, 1988) of histamine H_1-receptor desensitization in slices from mouse cerebral cortex or cultured smooth muscle cell line have revealed a decrease in the number of [^{3}H]-mepyramine binding sites. These results differ from our results in that our data show that histamine treatment leads to a decrease in the affinity, but not in the numbers of the receptor. These differences probably come from differences in the preparations used, in which different mechanisms of desensitization may be operating. The decrease in the receptor affinity for the agonist should reflect a conformational change which occurred at the receptor molecules (Hoyer *et al.*, 1984). It is possible that such receptors are functionally impaired in their coupling to the effector component leading to a decreased response to the agonist, that is, desensitization. The time course of the affinity change of the receptor (see Figure 3) was similar to that of histamine-induced desensitization which was observed previously (Horio *et al.*, 1990), further supporting the view that the alteration in histamine receptors is the cause of desensitization.

There have been no previous reports indicating the alteration of the receptor molecule in the case of histamine-induced desensitization. The results of the present study

Table 1 Analysis of histamine inhibition curves as binding to two independent sites

Histamine pretreatment	*Addition of GTP*	*High affinity component* N (%)	K_d (μM)	*Low affinity component* N (%)	K_d (μM)
−	−	84.9 ± 3.4	3.62 ± 0.43	14.6 ± 3.8	205 ± 49
+	−	77.2 ± 5.2	6.84 ± 1.18*	24.1 ± 3.6	225 ± 164
−	+	84.1 ± 3.4	6.05 ± 0.66*	16.9 ± 3.4	200 ± 56
+	+	89.5 ± 6.3	15.33 ± 2.76**	8.1 ± 5.3	261 ± 213

The histamine inhibition curves of [^{3}H]-mepyramine binding to membranes from control and histamine-pretreated preparations were analysed by nonlinear least squares regression with two binding sites model as described in Methods. K_d is a dissociation constant and N is the percentage of receptors in each affinity state. Values are means ± s.e. (n = 4–6). * Significantly different from the value for control preparations ($P < 0.05$). ** Significantly different from the value for histamine-pretreated preparations and the value for control preparations examined in the presence of guanosine-5'-triphosphate (GTP) ($P < 0.05$).

concerning histamine receptors can be compared to results obtained with other receptors. A similar decrease in the affinity of the receptor for the agonist has been reported to occur in the process of desensitization in β-adrenoceptors (Harden *et al.*, 1979; Hoyer *et al.*, 1984) and in muscarinic receptors (Galper & Smith, 1980; Kagiya *et al.*, 1986). In these cases, phosphorylation of the receptor proteins correlated well with the process of desensitization (Sibley *et al.*, 1984; Kwatra *et al.*, 1987), leading to the proposition that the phosphorylation results in a functional modification of the receptor protein such that it is less efficacious in coupling to the effector system. It has not yet been shown that histamine receptors are phosphorylated in the process of desensitization but our data suggest it may be the case.

It has been considered that histamine H_1-receptors interact with guanine nucleotide regulatory protein, since guanine nucleotide decreases the affinity of the receptors for the agonist (Chang & Snyder, 1980). Then the question was posed whether the interaction between histamine H_1-receptors and a guanine nucleotide regulatory protein was altered by the desensitizing treatment. Our results showed that agonist binding to histamine H_1-receptors was still regulated by guanine nucleotide in the desensitized membranes (Figure 2). The analysis by a two binding sites model showed that the guanine nucleotide-dependent decrease in receptor affinity for the agonist occurred in the high affinity component of the receptors both in control and desensitized membranes. These results suggest that the high affinity component of histamine H_1-receptors is responsible for both alterations induced by desensitizing treatment and guanine nucleotide treatment. The present results are in good agreement with results obtained with muscarinic receptors, where the interaction of receptors with a guanine nucleotide regulatory protein is retained in the desensitized state (Harden *et al.*, 1985; Kagiya *et al.*, 1986), but contrast with the results obtained with β-adrenoceptors, where a loss of interaction of receptors with a guanine nucleotide regulatory protein is observed after desensitizing treatment (Harden *et al.*, 1979; Hoyer *et al.*, 1984).

We are indebted to Miss Yasuyo Kamekawa for technical assistance and to Dr Hideki Moritoki for critical reading of the manuscript. We wish to thank Dr Atsushi Mizushima and Dr Shuji Uchida, Osaka University School of Medicine, Japan, for kindly supplying the computer programme used in this study. This work was supported, in part, by a Grant-in-Aid for Scientific Research (No. 62771906) from the Ministry of Education, Science and Culture of Japan.

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(Received April 9, 1990
Revised June 14, 1990
Accepted June 27, 1990)

Br. J. Pharmacol. (1990), **101**, 591–598

The pharmacological characterization of 5-HT_3 receptors in three isolated preparations derived from guinea-pig tissues

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1 The pharmacological characterization of the 5-HT_3 receptors in guinea-pig isolated tissues is described. The tissues used were ileum (longitudinal muscle-myenteric plexus), colon and vagus nerve. The guinea-pig isolated colon is a novel preparation.

2 In the guinea-pig isolated ileum, 5-hydroxytryptamine (5-HT, 1×10^{-8}–3×10^{-5} M) and the selective 5-HT_3 receptor agonist 2-methyl-5-HT (3×10^{-7}–1×10^{-4} M) caused concentration-related contractions. The 5-HT concentration-response curve was biphasic whilst the 2-methyl-5-HT curve was monophasic. The EC_{50} value for the low potency portion of the 5-HT curve was 4.1×10^{-6} M. The EC_{50} for 2-methyl-5-HT was 1.23×10^{-5} M. Selective 5-HT_3 receptor antagonists caused rightward shifts of the 2-methyl-5-HT curve and the lower potency portion of the 5-HT curve. Neither ketanserin (1×10^{-6} M) nor methysergide (1×10^{-5} M) antagonized the responses to 5-HT or 2-methyl-5-HT.

3 In the guinea-pig isolated colon, 5-HT (3×10^{-7}–3×10^{-5} M; EC_{50} 2.4×10^{-6} M) caused contractions which were mimicked by 2-methyl-5-HT (1×10^{-6}–1×10^{-4} M; EC_{50} 7.2×10^{-6} M). Selective 5-HT_3 receptor antagonists caused rightward displacements of the 5-HT concentration-response curves. Neither ketanserin (1×10^{-6} M) nor methysergide (1×10^{-5} M) had any effect on responses to 5-HT or 2-methyl-5-HT.

4 In the guinea-pig isolated vagus nerve, 5-HT (1×10^{-6}–3×10^{-4} M) and 2-methyl-5-HT (1×10^{-5}–1×10^{-3} M; EC_{50} 7.6×10^{-5} M) caused depolarizations; at higher concentrations there were after-hyperpolarizations. The maximum response to 2-methyl-5-HT was less than half that to 5-HT. Selective 5-HT_3 receptor antagonists caused rightward displacements of the 5-HT concentration-response curves. Antagonists at other 5-HT receptors (ketanserin, 1×10^{-5} M and methysergide, 1×10^{-6} M) had no effect.

5 The estimated affinity values of 5-HT_3 receptor antagonists correlated well between the three models. Phenylbiguanide was inactive as an agonist or antagonist (up to 1×10^{-4} M) in each preparation.

6 Comparisons with antagonist affinity values obtained in the rat isolated vagus nerve revealed marked differences. Antagonists were generally more potent on the rat isolated vagus nerve, although the differences varied considerably between antagonists.

7 The results are discussed in terms of species-related receptor differences.

Introduction

5-Hydroxytryptamine (5-HT) receptors of the 5-HT_3 type are thought to be located exclusively on neuronal tissue. These receptors appear to be directly linked to an integral monovalent cation channel (Higashi & Nishi, 1982; Neijt *et al.*, 1986; Surprenant & Crist, 1988; Derkach *et al.*, 1989). Agonists for the receptor include 5-HT and the selective compounds, 2-methyl-5-HT (Richardson *et al.*, 1985) phenylbiguanide (Fastier *et al.*, 1959; Ireland & Tyers, 1987) and *m*-chlorophenylbiguanide (Kilpatrick *et al.*, 1990b). Antagonists include the selective compounds MDL 72222 (Fozard, 1984), ICS 205–930 (Richardson *et al.*, 1985), ondansetron (GR38032; Butler *et al.*, 1988) and granisetron (BRL 43694; Sanger & Nelson, 1989).

Drugs which act as antagonists at 5-HT_3 receptors have been shown to have differing affinities in various isolated tissue models used to examine functional 5-HT_3 receptors (Richardson *et al.*, 1985; Butler *et al.*, 1988). Such observations led Richardson & Engel (1986) to propose the presence of 5-HT_3 receptor subtypes based upon the type of neurone on which each receptor was found. However, in this latter study, tissues from different species were used so that results obtained in guinea-pig ileum were compared with those obtained in rat vagus nerve and rabbit heart. Furthermore, many of the tissues used responded to 5-HT through an indirect mechanism. For example, in guinea-pig ileum, 5-HT-induced contractions may result from the release of both acetylcholine and substance P (Buchheit *et al.*, 1985). Neurotransmitter depletion and other factors complicate interpretation of such data (Kenakin, 1984). Because of these interpretation difficulties the Richardson & Engel classification has not been widely accepted (see Bradley *et al.*, 1986).

In order to remove one of these variables, we have investigated the responses to 5-HT in three tissues obtained from the same species. The guinea-pig isolated ileum longitudinal muscle-myenteric plexus preparation is now routinely used as a bioassay for substances acting on 5-HT_3 receptors but involves an indirect response to 5-HT which can be blocked by atropine. This preparation also contains other 5-HT receptors which are not of the 5-HT_3 type (Buchheit *et al.*, 1985; Craig & Clarke, 1989). The guinea-pig vagus nerve preparation has been described previously (Burridge *et al.*, 1989; Lattimer *et al.*, 1989). Depolarizations evoked by 5-HT in the preparation are believed to be direct responses mediated through 5-HT_3 receptors.

The guinea-pig isolated descending colon preparation has been described in a communication report (Grossman *et al.*, 1989) to the British Pharmacological Society. In this tissue responses to 5-HT are believed to be largely indirect.

Methods

Guinea-pig ileum longitudinal muscle-myenteric plexus

Male, Dunkin-Hartley guinea-pigs (*Porcellus*), weighing 250–300 g, were killed by cervical dislocation. A 3 cm portion of

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ileum was excised about 1 cm from the ileo-caecal junction. The longitudinal muscle layer was removed as described previously (Butler et al., 1988). Strips approximately 1.5 cm in length were placed in 5 ml organ baths containing Krebs-Henseleit solution gassed with 95% O_2/5% CO_2 and maintained at 37°C. Tissues were placed under an initial tension of 0.5–1 g. Agonists were applied directly to the bath (volume 50 μl–150 μl) and contractions were recorded isometrically. Non-cumulative concentration-response curves were constructed for agonists with a 15 min dosing cycle to prevent desensitization. Measurements were made at the highest point of the contraction and agonists were washed out as soon as the peak responses were reached.

Antagonists were added to the reservoir containing the bathing medium and were allowed to equilibrate with the ileum preparations for 1 h before the concentration-response curves were repeated.

Guinea-pig isolated colon

Female, Dunkin-Hartley guinea-pigs (*Porcellus*), weighing 250–300 g, were killed by cervical dislocation. A segment of the descending colon was removed and cut into 2–3 cm long sections. These sections were then cut longitudinally to reveal the mucosal surface, which was removed by careful excision and discarded. The muscle strips were placed in 20 ml organ baths containing modified Krebs-Henseleit solution, gassed with 95% O_2/5% CO_2 and maintained at 37°C. Each tissue was placed under an initial tension of 1 g and allowed to equilibrate for 40 min before the experiment was started. Agonists were applied directly to the baths and contractions were recorded isometrically. Non-cumulative concentration-response curves were constructed for agonists with a 10 min dosing-cycle. Measurements were made at the highest point of the contraction and agonists were washed out as soon as the peak responses were reached.

Antagonists were added to the reservoir containing bathing medium and were allowed to equilibrate with the colon preparations for 30 min before the concentration-response curves were repeated.

Guinea-pig isolated vagus nerve

Male, Dunkin-Hartley guinea-pigs (*Porcellus*), weighing 250–350 g, were stunned by a blow to the back of the head and killed by cardiac puncture. Segments of cervical vagus nerve were excised as rapidly as possible, desheathed and mounted in two-compartment Perspex baths. Each nerve was positioned so that 50% lay in the first compartment and the other 50% projected through a greased slot into the second. A Perspex barrier was carefully inserted to separate the two compartments. The d.c. potential between the two compartments was measured with silver/silver-chloride electrodes. The preparations were maintained at 27°C since Ireland et al. (1987) and Ireland & Tyers (1987) have shown that this increases recording stability. Both compartments were perfused continuously with Krebs-Henseleit medium dripped directly onto the tissue. Drugs were added via the superfusion stream to one compartment only.

Responses were recorded on a Servogor 220 pen recorder. Non-cumulative concentration-response curves to agonists were constructed with a 3 min contact time. Nerves were allowed to repolarize completely or settle to a new resting potential before application of the next concentration of agonist. This gave a dose-cycle of 15–45 min, depending on the size of depolarization. Antagonists were applied via the superfusion stream. An initial incubation period of 30 min was allowed after which time, repeated applications of agonist at a concentration inducing approximately 50% of the maximum response were given at approximately 15 min intervals until the response was constant, indicating that equilibration of the antagonist had been achieved. The agonist concentration-response curve was then repeated.

Rat vagus nerve

The method used for the measurement of 5-HT-induced depolarizations in the rat vagus nerve (Ireland & Tyers, 1987) was similar to that used for guinea-pig vagus nerve. Male, hooded rats (Glaxo), weighing 200–250 g, were used.

[^3H]-GR67330 binding

Male, Dunkin-Hartley guinea-pigs (*Porcellus*), weighing 200–250 g were killed and brain and ileum removed. The longitudinal muscle-myenteric plexus of the guinea-pig ileum was used and 16 regions of the brain dissected (see Kilpatrick et al., 1989). Pooled tissue from 5 animals was homogenized (Ultra Turrax) in 30 vol of HEPES buffer (50 mM, pH 7.4, 4°C) and centrifuged at 4°C and 48,000 g for 10 min. The supernatant was discarded and the process repeated for the pellet. The final pellet was suspended in 30 vol of HEPES buffer.

For binding, assay tubes contained 400 μl [^{3}H]-GR67330 (synthesized by the Radiochemistry Group, Glaxo Group Research; 85 Ci mmol^{-1}) in HEPES buffer. For inhibition studies a final concentration of 0.1 nM [^{3}H]-GR67330 was used, whilst for saturation analyses 10–20 concentrations between 0.01 and 2.0 nM were used. Tubes also contained 200 μl of competing drug or its vehicle (HEPES buffer), 300 μl of HEPES buffer and 100 μl of the tissue preparation (~0.2 mg protein). Tubes were incubated at 37°C for 30 min. The incubation was terminated by rapid vacuum filtration through Whatman GF/B filters using a Brandel Cell Harvester. Filters were washed immediately with 5 × 4 ml HEPES buffer (room temperature). Filters were placed in 10 ml of Picofluor 30 scintillation fluid and left overnight before radioactivity was assessed by liquid scintillation counting. All individual assays were carried out in replicates of three. Non-specific binding was determined by the addition of metoclopramide (30 μM).

Data analysis

EC_{50} values for agonists are means ± s.e.mean of results estimated from individual concentration-response curves. Estimation was performed graphically, correction being made when curves appeared multiphasic.

For antagonists pK_B values were estimated using the equation:

$$pK_B = \log(\text{concentration-ratio} - 1) - \log(\text{concentration of antagonist}).$$

Concentration ratios were measured at the level of approximately 50% of the maximum observed response. Only one concentration of antagonist was tested on each individual tissue preparation. For most antagonists a single concentration only was tested (Table 1). In these cases, the pK_B value is the mean of single determinations made on at least four individual preparations. For some antagonists, the effects of several concentrations were tested (Table 1). For these, a mean pK_B value (± s.e.mean) was calculated providing the plot of antagonism data according to Arunlakshana & Schild (1959) had a gradient not significantly different from unity.

In the rat vagus nerve, some antagonists caused reductions in the amplitude of the maximum response to 5-HT. In quantifying the effects of such antagonists, two assumptions have been made. The first is that 5-HT has low efficacy at 5-HT_3 receptors on rat vagus nerve, the second that the antagonists, although competitive, do not dissociate appreciably from the receptor during the period of 5-HT application. To estimate the affinity of antagonists under these conditions, the method of Paton & Ward (1967) (see also Kenakin, 1984) was used, although initial quantification of experimental data was performed according to Kennedy & Roberts (1985). Estimates of pK_B are quoted as the mean ± s.e.mean of determinations in at least three separate vagus nerves.

Table 1 Concentrations of 5-HT$_3$ receptor antagonists used

	Concentration(s) tested (M)			
Antagonist	*Guinea-pig ileum*	*Guinea-pig colon*	*Guinea-pig vagus nerve*	*Rat vagus nerve*
Metoclopramide	1×10^{-5}	1×10^{-5}	1×10^{-5}–1×10^{-4}	3×10^{-6}–10^{-4}†
MDL 72222	1×10^{-6}	1×10^{-6}	1×10^{-6}	1×10^{-8}–3×10^{-6}†
Ondansetron	1×10^{-7}–1×10^{-6}	1×10^{-6}–1×10^{-5}	3×10^{-7}–1×10^{-5}	1×10^{-8}–3×10^{-7}
R-GR38032	1×10^{-6}	NT	1×10^{-6}	1×10^{-8}–3×10^{-7}
S-GR38032	1×10^{-5}	NT	1×10^{-5}	1×10^{-8}–3×10^{-7}
GR80284	3×10^{-7}	3×10^{-7}	3×10^{-7}	1×10^{-7}
GR65630	1×10^{-6}	1×10^{-6}	1×10^{-6}	3×10^{-9}–3×10^{-7}
ICS 205-930	1×10^{-7}	1×10^{-7}	3×10^{-8}–1×10^{-6}	1×10^{-10}–3×10^{-9}†
Granisetron	1×10^{-7}	1×10^{-7}	3×10^{-8}–1×10^{-6}	1×10^{-9}
Zacopride	1×10^{-7}	1×10^{-7}	1×10^{-7}	1×10^{-8}
GR67330	1×10^{-7}	1×10^{-7}	1×10^{-7}	3×10^{-9}–1×10^{-6}

NT = Not tested.
† From Ireland & Tyers (1987).

Drugs and solutions

The composition of Krebs-Henseleit medium used was (mM): NaCl 118, $NaHCO_3$ 25, KCl 4.7, KH_2PO_4 1.18, $CaCl_2$ 2.5, $MgSO_4 \cdot 7H_2O$ 1.18 and glucose 11. The bathing medium for colon experiments was the same except for the concentrations of $CaCl_2$ and $MgSO_4 \cdot 7H_2O$ which were 1.3 mM and 0.6 mM respectively.

The following drugs (sources) were used: ondansetron (RS-GR38032), (R)- and (S)-GR38032 (1,2,3,9-tetrahydro-9-methyl-3-[(2-methyl-1H-imidazol-1-yl)methyl]-4H-carbazol-4-one, HCl · $2H_2O$; Glaxo), GR65630 (3-(5-methyl-1H-imidazol-4-yl)-1-(1-methyl-1H-indol-3-yl)-1-propanone maleate; Glaxo), GR67330 (1,2,3,9-tetrahydro-9-methyl-3-[(5-methyl-1H-imidazol-4-yl) methyl]-4H-carbazol-4-one maleate; Glaxo), GR80284 (endo-2,3-dihydro-N-(9-methyl-9-azabicyclo[3.3.1.]non-3-yl)-1H-indole-1-carboxamide maleate; Glaxo), BRL 24924 ([(±)-(endo)]-4-amino-N-1-azabicyclo[3.3.1]-non-4-yl-5-chloro-2-methoxybenzamide; Glaxo), granisetron (BRL 43694) (endo-1-methyl-N-(9-methyl-9-azabicyclo[3.3.1]non-3-yl-1H-indazole-3-carboxamide HCl; Glaxo), ICS 205-930 (endo-8-methyl-8-azabicyclo[3.2.1]oct-3-yl-1H-indole-3-carboxylate; Research Biochemicals Inc.), MDL 72222 (endo-8-methyl-8-azabicyclo [3.2.1]oct-3-yl-3,5-dichlorobenzoate HCl; Research Biochemicals Inc.), SDZ 206-830 (endo-9-methyl-9-azabicyclo [3.3.1]non-3-yl 5-fluoro-1-methyl-1H-indole-3-carboxylate HCl; Glaxo), 5-carboxamidotryptamine maleate (5-CT; Glaxo), haloperidol (Janssen), (±)-8-hydroxy-2-(di-N-propylamino)tetralin (8-OHDPAT; Research Biochemicals Inc.), 5-hydroxytryptamine creatinine sulphate (Sigma), ketanserin maleate (Salford Fine Chemicals), (±)-α-methyl-5-hydroxytryptamine maleate (α-methyl-5-HT; Glaxo), 2-methyl-5-hydroxytryptamine HCl · H_2O (2-methyl-5-HT; Glaxo), methysergide hydrogen maleate (Sandoz), metoclopramide HCl (Beecham), paroxetine HCl (Ferrosan), phentolamine mesylate (CIBA), 1-phenylbiguanide (Aldrich), zacopride HCl (A.H. Robins), 1,1-dimethyl-4-phenyl piperazinium iodide (DMPP, Sigma).

Drugs were dissolved in water and diluted in Krebs-Henseleit medium.

Results

Guinea-pig ileum longitudinal muscle myenteric plexus (LMMP) preparation

As described previously (Butler *et al.*, 1988), 5-HT (1×10^{-8}–3×10^{-5} M) and 2-methyl-5-HT (3×10^{-7}–1×10^{-4} M) caused concentration-related contractions of the guinea-pig isolated ileum LMMP preparation. The 5-HT concentration-response curve was biphasic whilst the 2-methyl-5-HT curve was monophasic (Figure 1a)). The EC_{50} values for 5-HT (low potency portion of the curve) and 2-methyl-5-HT were $4.1 \pm 1.0 \times 10^{-6}$ M ($n = 8$) and $1.23 \pm 0.24 \times 10^{-5}$ M ($n = 8$) respectively. α-Methyl-5-HT (1×10^{-8}–1×10^{-4} M) also induced concentration-related contractions and a biphasic concentration-response curve yielding an EC_{50} (for the whole curve) of $1.7 \pm 0.23 \times 10^{-5}$ M ($n = 7$); the maximum response was $104.5 \pm 2.6\%$ ($n = 8$) that of 5-HT (Figure 1a). 5-CT (1×10^{-6}–1×10^{-4} M) induced very small contractions (not shown), the maximum being less than 30% that of 5-HT. 8-OH DPAT (1×10^{-6}–3×10^{-5} M) had no significant effect.

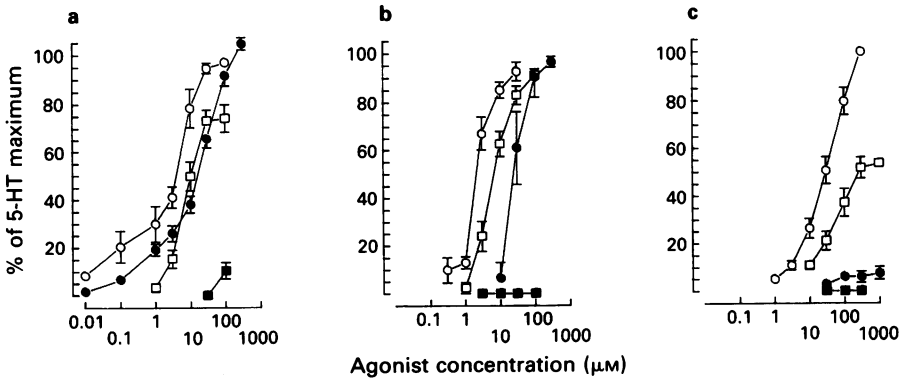

Figure 1 The effects of 5-hydroxytryptamine (5-HT, ○), 2-methyl-5-HT (□), α-methyl-5-HT (●) and phenylbiguanide (■) on the guinea-pig isolated ileum (a), isolated colon (b) and isolated vagus nerve (c). Results are expressed as the % maximum contraction to 5-HT (ileum and colon) or depolarization (% of depolarization at 3×10^{-4} M 5-HT; vagus nerve). Results are the mean of at least 4 separate observations; s.e.mean shown by vertical bars

Phenylbiguanide had no effect at concentrations up to 3×10^{-5} M but caused small contractions at 1×10^{-4} M (Figure 1a).

Because of the biphasic nature of the 5-HT concentration-response curve, 5-HT_3 receptor antagonist potencies were determined against 2-methyl-5-HT. Control concentration-response curves constructed 1 h apart on the same tissue preparation were not different from each other (not shown). Because of the low solubility and potency of 2-methyl-5-HT, the maximum shift in concentration-response curves that could be measured was small: the maximum usable concentration of 2-methyl-5-HT was 3×10^{-4} M. Selective 5-HT_3 receptor antagonists caused parallel, rightward displacements of the 2-methyl-5-HT concentration-response curve with no depression of maximum response (e.g. Figures 2a and 3a). pK_B values calculated from these shifts are shown in Table 2. Ondansetron (1×10^{-6} M) also caused a rightward shift of the low-potency portion of the α-methyl-5-HT concentration-response curve, yielding a pK_B of 7.0 which was similar to the pK_B value obtained against 2-methyl-5-HT (not shown).

Ketanserin (1×10^{-6} M), methysergide (1×10^{-5} M) (Figure 4a) and phenylbiguanide (1×10^{-4} M) (not shown) had no effect on contractions induced by 2-methyl-5-HT.

Guinea-pig isolated descending colon

5-HT (3×10^{-7}–3×10^{-5} M) and 2-methyl-5-HT (1×10^{-6}–1×10^{-4} M) induced concentration-related contractions of the guinea-pig isolated colon as exemplified by 5-HT in Figure 5. The concentration-contraction curves for both agonists appeared monophasic (Figure 1b). The EC_{50} values for 5-HT and 2-methyl-5-HT (when examined in the same tissues) were $2.4 \pm 0.4 \times 10^{-6}$ M ($n = 7$) and $7.2 \pm 1.0 \times 10^{-6}$ M ($n = 7$) respectively. The maximum response to 2-methyl-5-HT was $91.0 \pm 2.6\%$ of that to 5-HT. α-Methyl-5-HT (1×10^{-6}–1×10^{-4} M) also induced concentration-related contractions of the guinea-pig colon (Figure 1b); the EC_{50} was $2.97 \pm 0.97 \times 10^{-5}$ M ($n = 3$). The maximum response to α-methyl-5-HT was $99.2 \pm 3.8\%$ ($n = 3$) that of 5-HT. 5-CT induced small contractions ($24.3 \pm 4.4\%$ of the 5-HT maximum at 1×10^{-4} M; $n = 3$; not shown). Neither 8-OH DPAT nor phenylbiguanide caused contractions at concentrations up to 1×10^{-4} M (Figure 1b). Concentration-response curves for 5-HT constructed 30 min apart on the same tissue preparation were not significantly different from each other (not shown). 5-HT_3 receptor antagonists caused parallel, rightward shifts of the 5-HT concentration-contraction curve. The pK_B value for ondansetron was 7.1 ± 0.1 ($n = 12$). Schild analysis yielded a slope (95% confidence interval) of 1.26 (0.97–1.56) (Figure 2b). MDL 72222 (Figure 3b), also caused parallel, rightward shifts of the 5-HT concentration-contraction curve. MDL 72222 (1×10^{-6} M) had no significant effect on contractions induced by the nicotinic agonist, DMPP (1×10^{-6}–1×10^{-4} M; not shown). pK_B values calculated for 5-HT_3 receptor antagonists against 5-HT responses are given in Table 2. Ketanserin (1×10^{-6} M), methysergide (1×10^{-5} M) or phenylbiguanide (1×10^{-4} M) had no effect on contractions induced by 5-HT (Figure 4b). Atropine (1×10^{-6} M) blocked the response to 5-HT in an insurmountable manner: approximately 15% of the 5-HT maximum response remained (not shown).

Guinea-pig isolated vagus nerve

5-HT (1×10^{-6}–3×10^{-4} M) induced concentration-dependent depolarizations of the guinea-pig isolated vagus nerve. Reproducible curves to 5-HT could be obtained with up to 2 h between curves and single responses were repro-

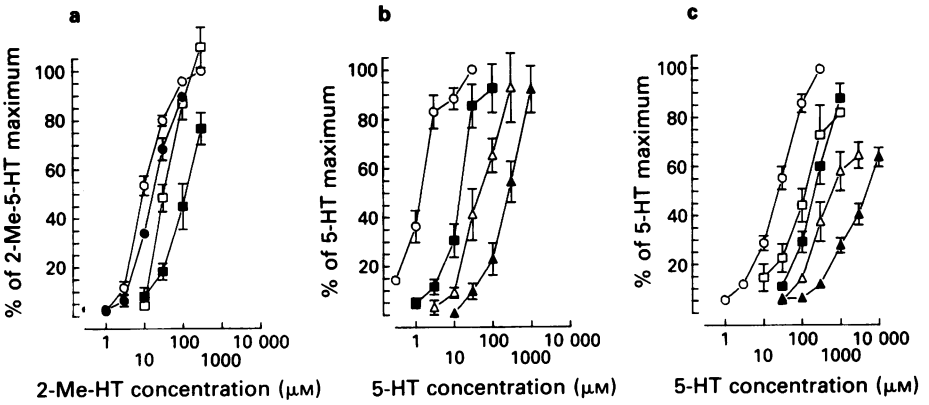

Figure 2 Antagonism by ondansetron of 2-methyl-5-HT-induced responses in guinea-pig isolated ileum (a) and 5-HT responses in guinea-pig isolated colon (b) and guinea-pig vagus nerve (c). Symbols indicate control (○) or in the presence of ondansetron 1×10^{-7} M (●), 3×10^{-7} M (□), 1×10^{-6} M (■), 3×10^{-6} M (△), and 1×10^{-5} M (▲). pK_B values derived from these experiments are presented in Table 2. Results are the mean of at least 4 separate observations; s.e. shown by vertical bars.

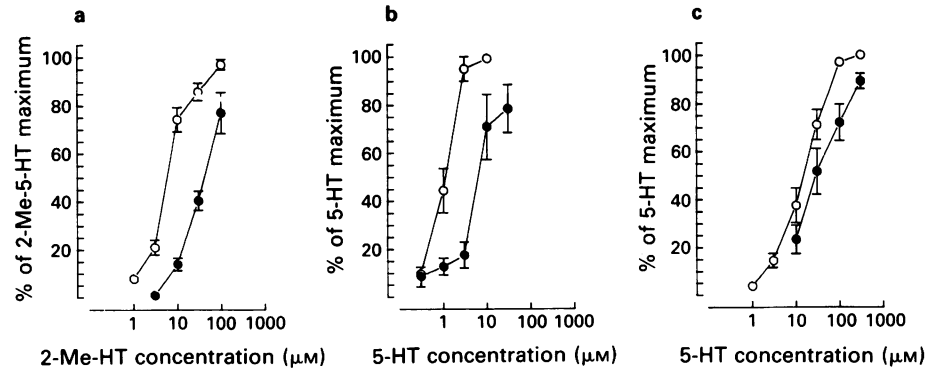

Figure 3 Antagonism by MDL 72222 of 2-methyl-5-HT responses in guinea-pig isolated ileum (a) and 5-HT responses in guinea-pig isolated colon (b) and guinea-pig isolated vagus nerve (c). Symbols indicate control (○) or in the presence of MDL 72222 at 1×10^{-6} M (●). Results are the mean of at least 3 separate observations; s.e. shown by vertical bars.

Table 2 Comparison of the affinities of 5-HT$_3$ receptor antagonists in guinea-pig isolated ileum, colon and vagus nerve and rat isolated vagus nerve

Antagonist	*Guinea-pig vs-2-Me-5-HT* pK_B	*Guinea-pig colon* pK_B	*Guinea-pig vagus nerve* pK_B	*Rat vagus nerve* pK_B
Metoclopramide	5.5 ± 0.1	5.7 ± 0.1	5.4 ± 0.1*	6.6†
MDL 72222	6.7 ± 0.1	6.7 ± 0.3	6.4 ± 0.1*	7.9†
Ondansetron	7.3 ± 0.1	7.1 ± 0.1	7.0 ± 0.1*	8.6 ± 0.1
R-GR38032	7.2 ± 0.1	NT	7.0 ± 0.1*	9.0 ± 0.1
S-GR38032	6.3 ± 0.1	NT	6.3 ± 0.1*	8.6 ± 0.1
GR80284	7.8 ± 0.1	7.6 ± 0.1	7.2 ± 0.1*	7.9 ± 0.1
GR65630	7.5 ± 0.1	7.5 ± 0.3	7.2 ± 0.1*	9.9 ± 0.1
ICS 205-930	8.0 ± 0.2↓	8.0 ± 0.1	7.8 ± 0.1*	11.0†
Granisetron	8.1 ± 0.1	8.1 ± 0.2	7.9 ± 0.1*	9.8 ± 0.1↓
Zacopride	8.1 ± 0.1	8.3 ± 0.1	8.0 ± 0.1*	9.9 ± 0.2↓
GR67330	9.0 ± 0.1	8.7 ± 0.1	8.0 ± 0.2*	10.2 ± 0.1↓

Results are the mean ± s.e. of at least four separate observations
↓ decrease in maximum observed response
NT Not tested
† From Ireland & Tyers (1987)
$P < 0.05$ (Two sample t test compared with rat vagus nerve values).
Refer to Table 1 for concentration(s) used.

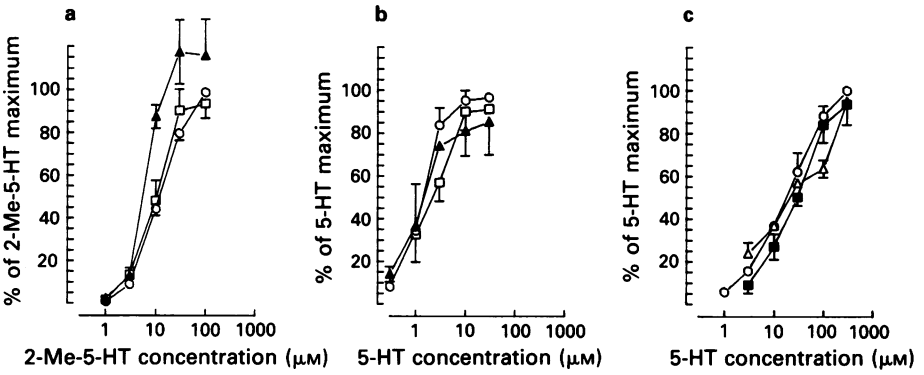

Figure 4 The effect of ketanserin and methysergide on responses to 2-methyl-5-HT in the guinea-pig isolated ileum (a) and 5-hydroxytryptamine (5-HT) in the guinea-pig isolated colon (b) and vagus nerve (c). Symbols indicate control (○) or in the presence of ketanserin at 1×10^{-6} M (□), or 1×10^{-5} M (■) and methysergide at 1×10^{-6} M (△) or 1×10^{-5} M (▲). Results are the mean of at least 3 separate observations; s.e. shown by vertical bars.

ducible with an interval between doses of 15–40 min. At higher concentrations the depolarization was followed by an after-hyperpolarization (Figure 6). Maximum depolarizations obtained were between 0.8 and 1.2 mV. True maximum responses to 5-HT were not routinely determined because the membrane potential did not recover from depolarizations evoked by concentrations of 5-HT above 3×10^{-4} M. Because of this, results are presented as a percentage of the depolarization obtained with 5-HT at 3×10^{-4} M. 2-Methyl-5-HT (1×10^{-5}–1×10^{-3} M) also induced concentration-dependent depolarizations and after-hyperpolarizations with an apparent EC_{50} for the depolarizations of $7.1 \pm 1.6 \times 10^{-5}$ M ($n = 9$). 2-Methyl-5-HT appeared to be a partial agonist, but because the true maximum response to 5-HT was not determined, a maximal response to 2-methyl-5-HT as a percentage of that to 5-HT could not be reliably calculated. Phenylbiguanide (up to 3×10^{-4} M), 5-CT and 8-OHDPAT (up to 10^{-4} M) did not induce depolarizations or hyperpolarizations of guinea-pig isolated vagus nerve. Furthermore, phenylbiguanide (1×10^{-4} M) did not antagonize 5-HT-induced responses.

The 5-HT reuptake inhibitor, paroxetine (3×10^{-7}–3×10^{-6} M) had no significant effect on the concentration-response curves for 5-HT (data not shown).

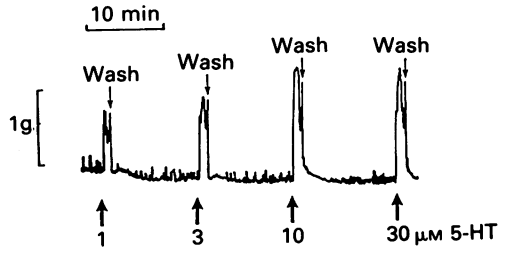

Figure 5 Typical contraction responses of the guinea-pig isolated colon to 5-hydroxytryptamine (5-HT). The traces are a continuous recording from a single preparation. Arrows indicate the application of 5-HT.

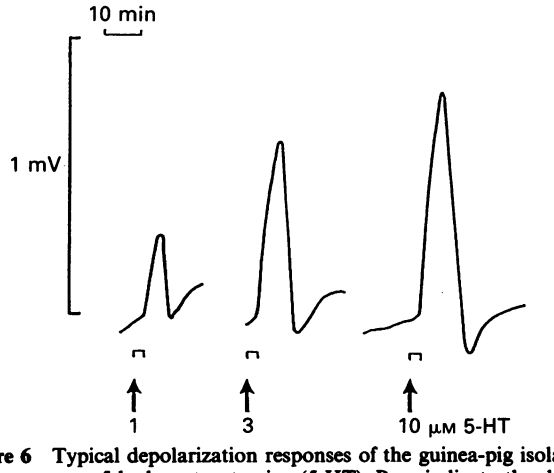

Figure 6 Typical depolarization responses of the guinea-pig isolated vagus nerve to 5-hydroxytryptamine (5-HT). Bars indicate the duration of application of 5-HT. The traces are a discontinuous recording from a single preparation.

5-HT_3 receptor antagonists caused parallel, rightward shifts of the 5-HT concentration-depolarization curves. MDL 72222 (1×10^{-6} M) caused a small shift in the concentration-response curve to 5-HT (Figure 3c). Full Schild analysis was performed on ondansetron (Figure 2c), metoclopramide, granisetron and ICS 205-930 (see Table 2 for pK_B values). For these antagonists the slopes obtained from the Schild plots did not differ significantly from unity (ondansetron, 0.89, 0.68–1.10; metoclopramide, 1.13, 0.94–1.31; granisetron 1.05, 0.85–1.25; ICS 205-930, 0.97, 0.78–1.16; slope, 95% confidence interval). For other compounds, the pK_B values were calculated from single antagonist concentrations and are shown in Table 2. The after-hyperpolarizations were also reduced by the 5-HT_3 antagonists, although this effect was not quantified. Methysergide (1×10^{-6} M) and ketanserin (1×10^{-5} M) (Figure 4c) had no effect on 5-HT-induced depolarizations in guinea-pig isolated vagus nerve.

Rat vagus nerve

As shown previously (Ireland & Tyers, 1987), 5-HT and 2-methyl-5-HT caused concentration-related depolarizations of the rat isolated vagus nerve. 5-HT_3 receptor antagonists caused rightward shifts of the concentration-depolarization response curves to 5-HT (see Table 2, Ireland & Tyers, 1987 and Butler *et al.*, 1988). For some compounds (MDL 72222, ICS 205-930, GR65630, granisetron, zacopride and GR67330) the rightward shifts were accompanied by a fall in maximum response. In these cases (except MDL 72222 and ICS 205-930, where published values are cited) a pK_B was calculated by the method of Kennedy & Roberts (1985).

[^{3}H]-GR67330 binding

Sixteen regions of guinea-pig brain were dissected (for list see Kilpatrick *et al.*, 1989). Specific binding, (defined by the inclusion of metoclopramide, 30 μM), could not be detected in any of these brain areas. Similarly, no specific binding of [^{3}H]-GR67330 could be detected with homogenates of guinea-pig ileum (longitudinal muscle-myenteric plexus).

Discussion

This study describes the pharmacological characterization of responses to 5-HT in three different tissues from the guinea-pig.

The response of the guinea-pig isolated ileum to 5-HT is well documented (Gaddum & Picarelli, 1957; Buchheit *et al.*, 1985; Richardson *et al.*, 1985; Butler *et al.*, 1988) and it is widely accepted that these responses are largely mediated via neuronally located 5-HT_3 receptors. The concentration-response curve to 5-HT is biphasic; the upper (low-potency) part of the response curve is mediated by 5-HT_3 receptors because the responses are mimicked by the selective 5-HT_3 receptor agonist, 2-methyl-5-HT and antagonized by highly selective 5-HT_3 antagonists such as ICS 205-930 and ondansetron. The lower (high potency) part of the concentration-response curve is resistant to blockade by 5-HT_3 antagonists. Craig & Clarke (1990) and Hill *et al.* (1990) have recently suggested that this response is mediated by a novel 5-HT receptor type which is also coupled positively to adenylate cyclase (Dumuis *et al.*, 1988).

The depolarizing action of 5-HT on guinea-pig isolated vagus nerve has been described previously (Burridge *et al.*, 1989; Lattimer *et al.*, 1989). Contractile effects of 5-HT in guinea-pig isolated descending colon have been described to the British Pharmacological Society (Grossman *et al.*, 1989). In contrast to responses of the guinea-pig isolated ileum, 5-HT effects in these tissues appeared to be mediated by a single 5-HT receptor type since the responses were mimicked by the selective 5-HT_3 agonist, 2-methyl-5-HT, whilst the monophasic concentration-response curves to 5-HT were shifted in a parallel fashion to the right by highly selective 5-HT_3 receptor antagonists. Furthermore, antagonists of other 5-HT receptors, such as ketanserin (5-HT_2) and methysergide (5-HT_1-like and 5-HT_2) were inactive. The depolarizing response of the guinea-pig isolated vagus nerve differs from the colon and ileum in being a directly mediated response. In the guinea-pig isolated ileum longitudinal muscle preparation and isolated descending colon preparation responses are mediated at least partially by acetylcholine because both responses are blocked insurmountably by the inclusion of atropine. Hence, estimations of agonist and antagonist potencies against the direct depolarizing response in the vagus nerve are not influenced by errors that may arise, for example, through depletion of the neurotransmitter.

In the guinea-pig isolated vagus nerve the depolarization induced by 5-HT at higher concentrations was followed by an after-hyperpolarization. This has been observed previously in other neuronal tissues in which depolarization is mediated by 5-HT_3 receptors, for example, the rabbit isolated superior cervical ganglion (Wallis & Nash, 1987) and rat isolated vagus nerve (Ireland, 1987). In the guinea-pig vagus nerve the after-hyperpolarizations, like 5-HT induced depolarizations were blocked by 5-HT_3 antagonists. This suggests that as in the rat vagus nerve, after-hyperpolarization is a consequence of depolarization and is not mediated by a different 5-HT receptor type (see Ireland, 1987).

The availability of different 5-HT_3 receptor responses in tissues from one species allows comparisons to be made between agonist potencies and antagonist affinity values. In each of the three tissues 5-HT and 2-methyl-5-HT were agonists. The EC_{50} values for 5-HT were similar in the ileum and colon ($\sim 2 \times 10^{-6}$ M) but slightly lower in the vagus nerve ($\sim 1.3 \times 10^{-5}$ M). The lower value in the vagus nerve may be due to a lower receptor reserve in this tissue which was also reflected in the lower maximum response to 2-methyl-5-HT and α-methyl-5-HT. 2-Methyl-5-HT was some three times less potent than 5-HT in each tissue which is typical of 5-HT_3 receptor-mediated responses in other species.

Phenylbiguanide has been identified as a potent 5-HT_3 receptor agonist in the rat isolated vagus nerve (Ireland & Tyers, 1987) and evokes the von Bezold-Jarisch reflex in rats (Fastier *et al.*, 1959) which has also been characterized as a 5-HT_3 receptor-mediated response. However, in each of the guinea-pig tissues described here phenylbiguanide was inactive either as an agonist or antagonist.

Conversely, α-methyl-5-HT has little effect in rat vagus nerve (Richardson *et al.*, 1985) and guinea-pig vagus nerve but we have shown it to act as a full agonist in guinea-pig ileum and guinea-pig colon. This may be explained if α-methyl-5-HT is a partial agonist and the receptor reserve in the vagus nerve is much lower than in ileum and colon.

Selective 5-HT_3 receptor antagonists inhibited the effects of 5-HT and 2-methyl-5-HT in each of the tissues described. MDL 72222 has been reported to be a weak antagonist of neuronal 5-HT contractions in the guinea-pig ileum (Fozard, 1984) and in the present study MDL 72222 was a weak antagonist in each of the three tissues. The affinity values for the 5-HT_3 antagonists were very similar in each of the guinea-pig tissues, implying that the receptors are the same. However, differences in pK_B values between the three tissues were noted for GR67330; further studies would be required before receptor heterogeneity could be suggested.

Comparison of antagonist affinities obtained in the guinea-pig tissues with those obtained against 5-HT-induced depolarization of the rat vagus nerve revealed marked differences. It is possible that the apparently low affinity of antagonists in guinea-pig tissues results from uptake and, perhaps, subsequent metabolism of agonist: the rat vagus nerve does not accumulate 5-HT actively (Ireland *et al.*, 1987). On the guinea-pig vagus nerve, the action of 5-HT was not potentiated by the 5-HT uptake inhibitor paroxetine. In addition, in no guinea-pig tissue did Arunlakshana & Schild plots of antagonism data appear non-linear or have slopes significantly less than unity. This would be expected were the apparent affinity of antagonists reduced by saturation of agonist uptake (see

Furchgott, 1972; Ireland *et al.*, 1987). In the guinea-pig, antagonist affinities were generally 10–100 fold lower. However, some compounds, such as GR80284 had similar affinities for the receptors in the guinea-pig and rat tissues whilst GR65630 had some five hundred times lower affinity for the guinea-pig receptor. These marked differences in antagonist affinities coupled with the lack of effect of phenylbiguanide in guinea-pig tissues provide convincing evidence that the 5-HT$_3$ receptors mediating responses in the guinea-pig tissues differ from those in the rat. Since all of the compounds having antagonist affinity for the guinea-pig 5-HT receptor also have affinity for the 5-HT$_3$ receptor of the rat, and are highly selective, it is reasonable to suggest that the responses in the guinea-pig tissues are also mediated by 5-HT$_3$ receptors; but these differ from the 5-HT$_3$ receptors in the rat.

This observation raises the question as to whether 5-HT$_3$ receptors also co-exist as subtypes within a species. Antagonist affinity values derived from the rat isolated vagus nerve correlate well with other affinities for 5-HT$_3$ receptor-mediated responses in other tissues in the rat such as the 5-HT$_3$-receptor binding sites in the brain (Kilpatrick *et al.*, 1987; Barnes *et al.*, 1988; Milburn & Peroutka, 1989) and vagus nerve (Kilpatrick *et al.*, 1989). There is no evidence for the 'guinea-pig-like' 5-HT$_3$ receptor in the rat. Equally, no 'rat-like' 5-HT$_3$ receptor response has been reported for guinea-pig tissues. Furthermore, using the 5-HT$_3$ receptor ligand [^{3}H]-GR67330, which labels the 5-HT$_3$ receptor in the rat (Kilpatrick *et al.*, 1990a), we could find no specific binding sites in any guinea-pig tissue tested. This could result from a lower affinity for 5-HT$_3$ receptors in the guinea-pig or from a low density of receptors.

In conclusion, the antagonist affinities of 5-HT$_3$ receptor antagonists are similar for three functional tissue responses mediated by 5-HT or 2-methyl-5-HT in the guinea-pig. The differences from affinities obtained in the rat isolated vagus nerve and the lack of effect of phenylbiguanide cannot be explained in terms of interference from other receptor types or neurotransmitter depletion. Therefore, the 5-HT$_3$ receptor in the guinea-pig tissues is almost certainly different from that in the rat. The guinea-pig and rat 5-HT$_3$ receptors can probably be termed 5-HT$_3$ receptor subtypes in the same way that 5-HT$_{1B}$ and 5-HT$_{1D}$ receptor subtypes appear to be species variants and have similar functions in rodent (excluding the guinea-pig) and non-rodent species respectively. There are limited data on the affinities of 5-HT$_3$ receptor antagonists in other species: however, there is some indication that there may be other species variants of the 5-HT$_3$ receptor (for example in the rabbit vagus nerve, Richardson *et al.*, 1985). These observations need to be fully investigated before reaching firm conclusions, but the large differences in affinities of 5-HT$_3$ receptor antagonists between species offer a new dimension for discussion on the criteria for the classification of receptor subtypes.

We would like to thank Lorraine Fallaize for expert technical assistance.

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(Received April 10, 1990
Revised June 28, 1990
Accepted July 4, 1990)

Br. J. Pharmacol. (1990), 101, 599–604

Acetylcholinesterase activity in regions of mouse brain following acute and chronic treatment with a benzodiazepine inverse agonist

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1 Chronic administration of the benzodiazepine inverse agonist FG 7142 has previously been shown to induce seizure activity in mice. In the present study we have investigated the effects of acute and chronic treatment with FG 7142 in mice on the levels of acetylcholinesterase activity in cortex, hippocampus, midbrain and striatum. We have also investigated the effects of acute and chronic stress in the form of handling (vehicle-injection) on acetylcholinesterase levels.

2 A single dose of FG 7142 produced a marked elevation of total acetylcholinesterase activities in the hippocampus and midbrain when compared with vehicle-injected control levels, but the levels were not different from those in unhandled animals.

3 Acute stress, in the form of vehicle-injection produced decreases in cortical and hippocampal soluble acetylcholinesterase activity but FG 7142 had no effect upon these stress-induced changes.

4 Total cortical and hippocampal acetylcholinesterase activities were increased by 56% and 16% respectively in the chronic FG 7142-treated mice that exhibited seizure activity (compared with vehicle-injected controls).

5 Soluble acetylcholinesterase activity in the midbrain was decreased to 82% of control levels only in animals that had undergone FG 7142-induced kindling. Smaller or no changes in acetylcholinesterase activity in the midbrain were observed in chronically FG 7142-treated animals that exhibited no seizure activity.

6 Mice that did not demonstrate seizure activity in response to chronic FG 7142 treatment showed alterations in the soluble acetylcholinesterase activities of the hippocampus and midbrain.

7 It is concluded that chronic treatment with the benzodiazepine inverse agonist FG 7142 produces alterations in the acetylcholinesterase activities of various brain regions, in a manner related to the kindling that can be produced by this treatment.

8 Chronic mild stress, in the form of repeated handling (vehicle injection), induced changes in brain activity with decreases in total activity occurring in the cortex and hippocampus, and an increase in soluble acetylcholinesterase activity occurring in the midbrain.

9 All these stress-induced changes appeared to be prevented by administration of FG 7142 at the time of the stress. It would appear therefore that FG 7142 can prevent the effects of chronic stress on brain acetylcholinesterase activity.

Introduction

Chronic administration of inverse agonists at the benzodiazepine receptor has been shown to produce chemical kindling (Little *et al.*, 1984; Morin, 1984) i.e. repeated administration of a dose of FG 7142 which is initially proconvulsant (but which does not itself produce convulsions) reliably produces generalised seizures in 60% of mice so treated. This effect persists for at least six months after cessation of treatment. The development of this chemical kindling phenomenon is prevented by concurrent administration of benzodiazepine antagonist Ro 15-1788 (Little *et al.*, 1984) indicating that FG 7142 kindling is mediated via the benzodiazepine receptor. Ro 15-1788 also prevents electrically kindled seizures (Robertson & Riives, 1983).

Chemical kindling can also be induced by repeated cholinergic stimulation of the limbic regions. The development and manifestation of this cholinergic kindling is prevented by atropine treatment (Wasterlain *et al.*, 1978; Olney *et al.*, 1983; Turski *et al.*, 1983). Similarly atropine also retards electrically-induced kindling (Arnold *et al.*, 1973) indicating that cholinergic hypersensitivity may be responsible for the development of seizures. Supersensitivity to endogenous acetylcholine (ACh) may also be a factor in cortical epileptogenesis: the chronically isolated cortex shows both spontaneous and prolonged epileptiform after discharges, and increased sensitivity to topically applied ACh after several weeks (Echlin & Battista, 1963). This increase in the sensitivity of the isolated cortex parallels a decrease in the acetylcholinesterase (EC 3.1.1.7; AChE) activity of the tissue (Hebb *et al.*, 1963; Rosenberg & Echlin, 1965; Chu *et al.*, 1971) prompting Girgis (1981) to propose that AChE may have a protective function in limbic structures prone to epileptiform activity.

Convulsions induced with a single electroconvulsive shock have previously been found to induce transient increases in the AChE activity of cortex and striatum, and sustained decreases in the AChE activity of the hippocampus and midbrain of rats (Appleyard *et al.*, 1986). We have therefore examined the AChE activities of various brain regions from mice that were kindled with chronic FG 7142 treatment. The effects produced by FG 7142 kindling were compared with those produced by a single dose of FG 7142. A preliminary report of some of these findings has been published as an abstract (Appleyard *et al.*, 1985).

Acute and repeated stress have also been reported to have marked effects upon the AChE activity of numerous brain nuclei. Increased levels of AChE activity have been observed in the cortex, thalamus and hypothalamus of rats that had been stressed by application of electric foot shocks (Singh *et al.*, 1979). Acute and chronic immobilization stress induced

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increases in the AChE activity of certain hypothalamic and brain stem nuclei (Romero, 1981). Repeated handling can be regarded as a form of mild chronic stress (Graham-Jones *et al.*, 1983; Stanford *et al.*, 1984) and has been shown to induce long-lasting changes in several cortical chemical parameters, such as adrenoceptor number. Therefore the effects of acute and chronic handling upon the AChE activity of various brain regions were also investigated by comparing vehicle-injected mice with handled animals.

Methods

Animals and seizure administration

Male CD1 mice (Charles River) weighing between 30–35 g were used for all experiments. They were housed in groups of 8–12 under conditions of controlled lighting and temperature, and with free access to food and water. Acute and chronic experiments were performed; in each case there were three experimental groups of mice consisting of FG 7142-treated, vehicle-injected (handled) and unhandled animals.

FG 7142 (A/S Ferrosan, Denmark) was suspended in distilled water with one drop of Tween 80 per 10 ml, and was administered in a volume of 10 ml kg^{-1}, i.p.

In the acute experiments mice received a single injection of 40 mg kg^{-1} FG 7142 i.p. and were killed seven days later by cervical dislocation. Vehicle-injected (handled) animals received a single injection of the Tween 80 vehicle and were also killed seven days later. Unhandled mice that arrived in the department with, and were housed under the same conditions as the experimental groups, but which received no treatments and remained unhandled during their stay in the department, were killed at the same time as the other groups.

In the chronic treatment experiments, a single dose of 40 mg kg^{-1} FG 7142 (i.p.) was administered daily at 10 h 00 min for 12 days. On days 10, 11 and 12 the mice were observed for 1 h after each injection in open top cages (30 cm × 60 cm). Observations were made of their behaviour during this time and the incidence of myoclonic jerks and full generalized seizures were recorded. A full generalized seizure was defined as clonic or tonic contractions of all limbs plus loss of posture (i.e. the mice fell onto their side or back). All injections and observations were made by the same observer and all injections were performed in the same room. Vehicle-injected (handled) animals received daily injections of the Tween 80 vehicle. On day 19, seven days after the last injection, all the mice were killed by cervical dislocation. Unhandled mice that had arrived in the department on the same day, and were housed under the same conditions, as the treatment groups were also killed by cervical dislocation at the same time as the other groups.

Measurement of acetylcholinesterase activity in brain regions

After the mice were killed by cervical dislocation their brains were removed and rapidly dissected at 0°C to obtain total cortex, hippocampus, midbrain and striatum. After weighing the dissected areas were stored at −20°C until analysis which was within four weeks.

Brain regions were homogenized in 20 volumes per wet weight of ice-cold 0.03 M sucrose, and homogenates were frozen and thawed once to liberate, as far as possible, the soluble AChE. Aliquots of this total homogenate were then centrifuged at 50,000 *g* for 120 min to sediment the membranes and their bound AChE.

Acetylcholinesterase activities of the total homogenate and supernatant were measured by a stopped assay version (Chubb & Smith, 1976) of the Ellman assay (Ellman *et al.*, 1961) using 1.0 mM acetylthiocholine as substrate, and the specific AChE inhibitor BW 284c51 to distinguish between AChE and non-specific cholinesterase activities. Protein was measured in the soluble and total fractions by the method of Lowry *et al.* (1951) after precipitation by 6% trichloroacetic acid.

Statistics

Results are expressed as mean ± s.e.mean and were analyzed by use of Student's two-tailed *t* test and Welch's d approximation where appropriate.

In order to determine the effects of FG 7142 treatment, levels in FG 7142-treated animals were compared with those found in vehicle-injected animals. The possible effects of stress (in the form of handling) were determined by comparing levels in the vehicle-injected mice with those found in unhandled mice.

Drug

FG 7142 (N-methyl-β-carboline-3-carboxamide) was obtained from A/S Ferrosan, Denmark.

Results

Acetylcholinesterase activities following acute administration of FG 7142

The total and soluble AChE activities per mg protein were determined in homogenates of cortex, hippocampus, midbrain and striatum seven days after administration of a single dose of FG 7142 or the Tween vehicle.

In the cortex there were no significant differences in the total AChE activity between these groups of mice (Figure 1).

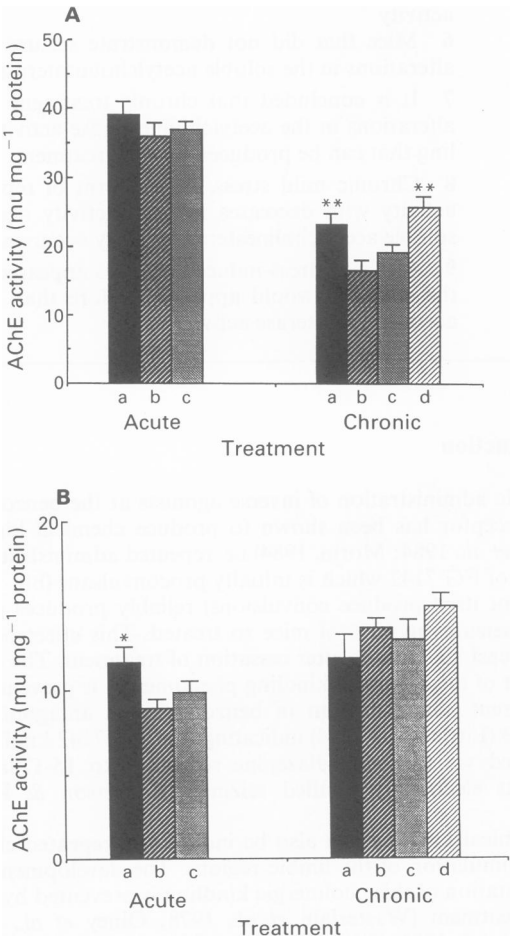

Figure 1 Acute and chronic effects of FG 7142 administration upon acetylcholinesterase activities in (A) the total homogenate (B) the soluble fraction of the cortex: (a) unhandled animals; (b) vehicle-injected controls; (c) FG 7142-treated animals not demonstrating seizure activity; (d) FG 7142-kindled animals. Acetylcholinesterase activity is expressed in nmol acetylthiocholine hydrolysed $min^{-1} mg^{-1}$ total or soluble protein, as appropriate. Data are presented as mean ± s.e.mean. Significantly different from controls with * $P < 0.05$ and ** $P < 0.001$.

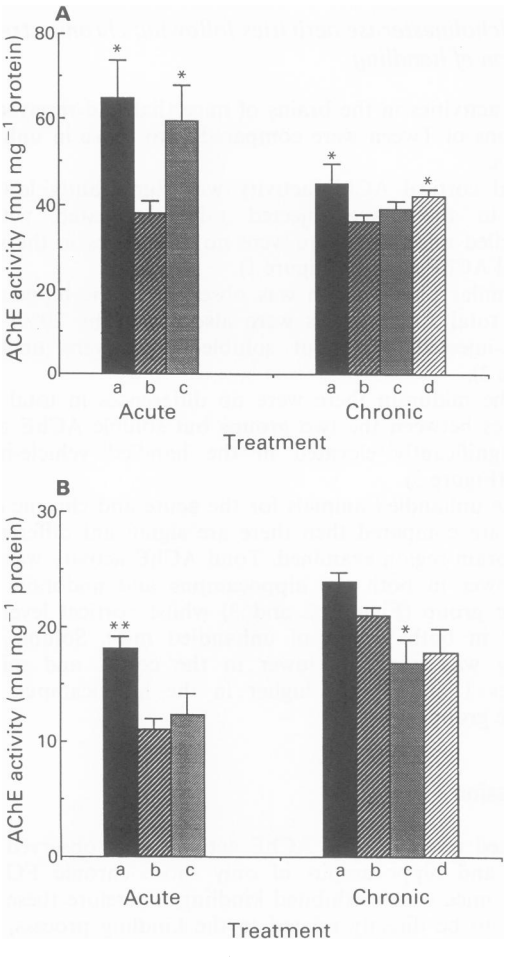

Figure 2 Acetylcholinesterase activities (expressed in nmol acetylthiocholine hydrolysed $min^{-1} mg^{-1}$ protein) in (A) the total homogenate and (B) the soluble fraction of the hippocampus following acute and chronic administration of FG 7142: (a) unhandled animals; (b) vehicle-injected controls; (c) FG 7142-treated animals not showing seizure activity; (d) FG 7142-kindled animals. Significantly different from vehicle injected controls with * $P < 0.05$ and ** $P < 0.005$.

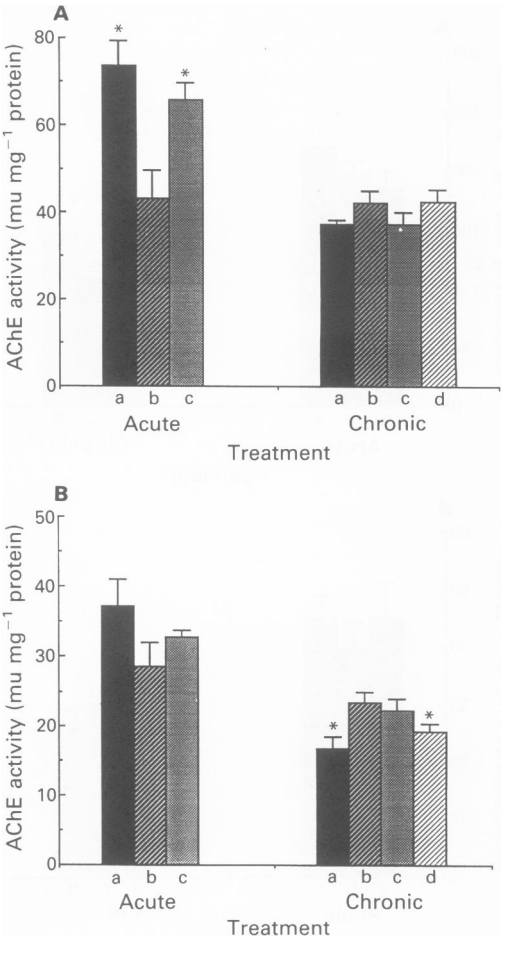

Figure 3 Acetylcholinesterase activities (expressed in nmol acetylthiocholine hydrolysed $min^{-1} mg^{-1}$ protein) in (A) the total homogenate and (B) the soluble fraction of the midbrain following acute and chronic administration of FG 7142; (a) unhandled animals; (b) vehicle-injected controls; (c) FG 7142-treated animals not showing seizure activity; (d) FG 7142-kindled animals. Significantly different from vehicle-injected controls with * $P < 0.05$ and ** $P < 0.005$.

The soluble AChE activity in the cortex of FG 7142-treated mice was not significantly different from that observed in the vehicle-injected animals (Figure 1).

In both hippocampal and midbrain regions the total AChE activities of mice treated acutely with FG 7142 were significantly higher (by 57% and 52% respectively) than those in the vehicle-injected group (Figures 2 and 3). In contrast, no changes in soluble AChE activity were observed in the hippocampus and midbrain of FG 7142-treated mice when compared to levels in vehicle-injected animals (Figures 2 and 3).

No differences in either total or soluble striatal AChE activity were observed between the two groups (Figure 4).

Acetylcholinesterase activities following acute stress in the form of handling

AChE activities found in the brains of vehicle-injected (handled) mice were compared with those found in unhandled mice.

In the cortex there were no significant differences in the total AChE activity, but the soluble AChE activity of the handled Tween-injected mice was significantly ($P < 0.05$) lower (by 24%) than that in the unhandled mice (Figure 1).

Total AChE levels in the hippocampus and midbrain were significantly lower (by 41% in both cases) in the vehicle-injected mice compared with levels in unhandled mice. Significantly lower levels (by 39%) of soluble AChE activity were also observed in the hippocampus of vehicle-injected mice (Figures 2 and 3).

No differences in either total or soluble AChE activity were observed between the two groups (Figure 4).

Acetylcholinesterase activities following chronic administration of FG 7142

The total and soluble AChE activities per mg protein were determined in several brain regions (cortex, hippocampus, midbrain and striatum) from mice that received a single daily injection of FG 7142 for 12 days and were killed seven days later. No behavioral effects were obvious after a single dose of FG 7142 but by day 5, animals showed brief myoclonic jerks of the head and neck in response to the FG 7142 injection. This progressed to generalized seizure activity with squeaking, and the number of mice convulsing increased throughout the treatment schedule, until by day 12 seizure activity was observed in 65% of the animals. The identity of mice that exhibited seizure activity was noted. In general, seizures were observed only once after each injection with a latency which tended to be constant for a given mouse but which varied between mice. The duration of the seizure was 10–20 s.

Total cortical AChE activity was significantly ($P < 0.001$) higher (by 56%) in the chronic FG 7142-treated animals that exhibited seizure activity (i.e. that had undergone kindling) compared with vehicle-injected animals. However, this effect

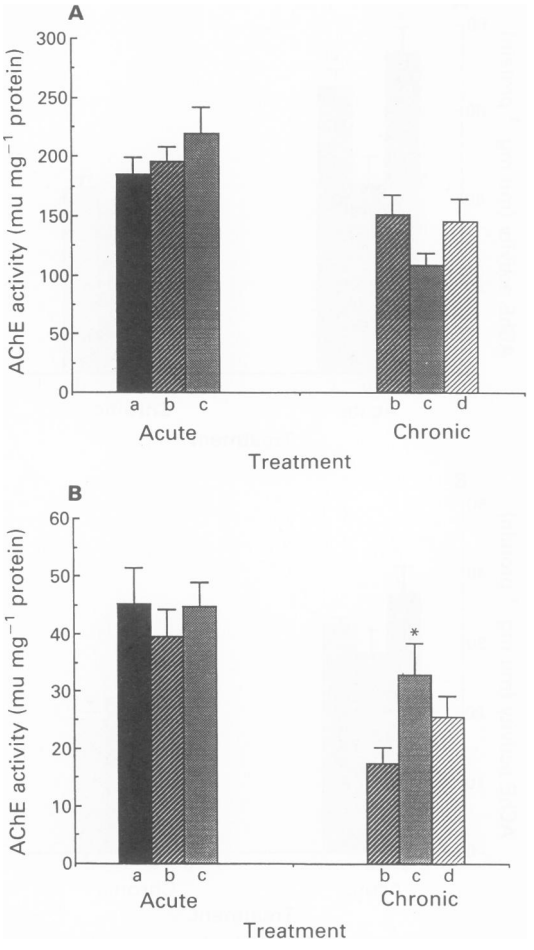

Figure 4 Acetylcholinesterase activities (expressed in nmol acetylthiocholine hydrolysed $min^{-1} mg^{-1}$ protein) in (A) the total homogenate and (B) the soluble fraction of the striatum following acute and chronic administration of FG 7142: (a) unhandled animals; (b) vehicle-injected controls; (c) FG 7142-treated animals not showing seizure activity; (d) FG 7142-kindled animals. Significantly different from vehicle-injected controls with $*P < 0.05$ and $**P < 0.005$ respectively.

was absent from the FG 7142-treated animals that did not exhibit seizure activity. In contrast, there were no differences in the soluble cortical AChE activity between any of the groups (Figure 1).

A similar phenomenon was also observed in the hippocampus, with a significant increase of 16% in the total AChE activity occurring in the kindled group compared with the vehicle-injected group but there was no difference in the mean total activity of vehicle-injected mice and FG 7142-treated mice that did not exhibit seizure activity. No changes in the soluble AChE activity of the hippocampus were observed, apart from a small (20%) but statistically significant decrease in the FG 7142-treated animals that did not exhibit seizure activity (Figure 2).

In the midbrain region there were no alterations in the total AChE activity induced by chronic FG 7142 treatment compared with the vehicle-injected group (Figure 3). However, significantly lower levels (by 18%) of soluble AChE activity were observed in the midbrain of FG 7142 kindled mice. No such difference was apparent in the FG 7142-treated animals that did not exhibit seizure activity (Figure 3).

No differences in the total AChE activity of the striatum were induced by chronic FG 7142 treatment, however, a significantly ($P < 0.02$) higher (by 90%) level of soluble striatal AChE activity was observed in the FG 7142-treated animals that did not exhibit seizure activity, but not in the FG 7142-kindled animals when compared with the vehicle-injected group (Figure 4).

Acetylcholinesterase activities following chronic stress in the form of handling

AChE activities in the brains of mice that had received daily injections of Tween were compared with those in unhandled animals.

Total cortical AChE activity was significantly lower (by 29%) in the Tween-injected mice compared with the unhandled mice, but there were no differences in the soluble cortical AChE activity (Figure 1).

A similar phenomenon was observed in the hippocampus where total AChE levels were also lower (by 20%) in the vehicle-injected mice, but soluble levels were unchanged (Figure 2).

In the midbrain there were no differences in total AChE activities between the two groups but soluble AChE activity was significantly elevated in the handled vehicle-injected group (Figure 3).

If the unhandled animals for the acute and chronic experiments are compared then there are significant differences in every brain region examined. Total AChE activity was markedly lower in both the hippocampus and midbrain of the chronic group (Figures 2 and 3) whilst cortical levels were similar in both groups of unhandled mice. Soluble AChE activity was markedly lower in the cortex and midbrain (Figures 1 and 3) but higher in the hippocampus of the chronic group.

Discussion

Increased levels of total AChE activity were observed in the cortex and hippocampus of only those chronic FG 7142-treated mice which exhibited kindling. Therefore these effects appear to be directly related to the kindling process, or the occurrence of seizures and are not a consequence simply of the chronic administration of FG 7142. However an increased level of hippocampal AChE activity was also observed following acute administration of FG 7142, a treatment which did not induce seizure activity. It is possible that such an effect reflected initiation of the kindling process.

A decrease in total hippocampal AChE activity has previously been observed in rats for up to 3 h immediately following a convulsion induced by acute electroconvulsive shock treatment (Appleyard *et al.*, 1986). It suggests that the changes in AChE activity which occur in the hippocampus are indeed related to the seizure process since susceptibility is decreased for a similar time period immediately following a single electroconvulsive shock (Nutt *et al.*, 1981) and increased (at least to FG 7142) following FG 7142 kindling. This could explain why the changes in AChE activity produced by these two treatments are in opposite directions. Cortical and midbrain changes in AChE activity may not be as important for seizure susceptibility since increased levels of total cortical activity and decreased levels of midbrain soluble AChE activity were observed following both FG 7142 kindling in mice and a single electroconvulsive shock in rats (Appleyard *et al.*, 1986) despite their opposite effects on seizure susceptibility. Species differences could contribute to this discrepancy. It should be noted however that FG 7142 kindling does not appear to be associated with a general decrease in seizure threshold since it produced less change in the seizure threshold to convulsant drugs such as pentylenetetrazol and bicuculline than the effects of benzodiazepine receptor ligands (Little *et al.*, 1986; 1987). Furthermore the changes seen in AChE activity after a single electroconvulsive shock are probably not causally related to the concurrent increase in seizure threshold since no such changes in AChE activity were observed following the last of a series of ten electroconvulsive shocks (Appleyard & Green, 1988) despite the rise in seizure threshold produced by this treatment. Hence, although the present study demonstrates changes in AChE activity in several brain regions which are specific to animals exhibiting kindling in response

to chronic FG 7142 treatment the precise contribution of these changes to the kindling process is at present unclear.

Changes in AChE activity that occurred only in animals chronically treated with FG 7142 but which did not exhibit seizure activity were also apparent, such as a decrease in soluble hippocampal activity. Similar changes have been observed in rats following a single electroconvulsive shock, and could reflect a compensatory mechanism that raises the seizure threshold, so preventing the development of kindling. No such changes in AChE activity were caused by acute administration of FG 7142.

Chronic mild stress, in the form of repeated handling, induced changes in the AChE activity of all three brain regions studied. Repeated handling makes animals tame and has been shown to decrease the emotional reactivity of rats in the open field test (Broadhurst, 1960; Coscina *et al.*, 1975). The changes in brain AChE activity observed in the handled animals could therefore be a direct result of the repeated stress, or could reflect a gradual adaptation to repeated stimulation of an initially stressful nature. Comparison of the effects of acute and chronic handling should distinguish between these two possibilities. A single injection of vehicle had no effect upon total cortical AChE activity, unlike repeated injections, so the changes in AChE activity observed in this brain region probably reflected adaptation to the repeated mild stress, as did changes in the midbrain. However, in the hippocampus, similar decreases in total AChE activity were observed following both a single injection of vehicle and repeated injections, and so this is probably a direct effect of the stress itself. It should be noted that there were marked differences in the AChE activity of all brain regions of unhandled animals for the acute and chronic experiments. These two groups of animals differed only in the amount of time they spent in the department.

Mild acute stress, in the form of a single vehicle injection induced long-lasting decreases in the AChE activity of both the hippocampus and the midbrain. Administration of a single dose of FG 7142 at the time of handling appeared to abolish this effect of stress since there was no difference between these FG 7142-treated mice and unhandled mice. Chronic FG 7142 treatment also appeared to prevent the appearance of chronic stress-induced changes in AChE activities. It is puzzling that FG 7142 appears to prevent the stress-induced changes in AChE activity, since its anxiogenic properties would be expected to intensify the effect of the stress. However, similar effects have been observed with adrenoceptors. Chronic mild stress in the form of repeated saline injections resulted in a decrease in cortical adrenoceptor binding in rats (Stanford *et al.*, 1984) whilst chronic FG 7142 treatment produced an elevation of cortical adrenoceptor binding over vehicle-injected levels in mice (Stanford *et al.*, 1986). It could be that these alterations in adrenoceptors and AChE reflect an adaptive process for coping with the stress, and that this is prevented by the anxiogenic agent.

What is the relevance of the alterations in AChE activity observed in animals kindled with FG 7142? The increases in total AChE activity that occurred in the cortex and hippocampus of kindled mice were due to increased levels of membrane-bound AChE, since levels of soluble enzyme remained unchanged. In cholinergic regions such as the cortex and hippocampus, such AChE would primarily function to terminate cholinergic transmission by hydrolysis of acetylcholine. Hence these altered levels of AChE activity could reflect, or result in, disruptions of cholinergic transmission that have been induced by, or contribute to, the kindling process. It is obviously important therefore to determine whether other cholinergic system markers are also affected by FG 1742 kindling. The influence of cholinomimetic drugs on the development of FG 7142 kindling should also be investigated.

In the midbrain, FG 7142 kindling led to decreased levels of soluble AChE. One of the main AChE-containing regions of the midbrain is the substantia nigra, a region where there is good evidence that soluble AChE is secreted and can affect the activity of pars compacta neurones (Greenfield, 1984; Greenfield *et al.*, 1988). Alterations of the level of soluble AChE could reflect, or result in, an altered secretion of the protein, and hence of nigral activity. Indeed, previous studies have shown that an increased secretion of AChE during a seizure (induced by electroconvulsive shock) (Appleyard *et al.*, 1987) resulted in decreased levels of midbrain AChE (Appleyard *et al.*, 1986). It has been suggested that the substantia nigra functions as a gate in the propagation of seizures (McNamara *et al.*, 1983; Garant & Gale, 1983). Obviously, if such a mechanism exists, any alteration of nigral activity could affect the incidence of seizure activity and so contribute to the kindling process.

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(Received April 12, 1990
Revised June 26, 1990
Accepted July 2, 1990)

Br. J. Pharmacol. (1990), **101**, 605–614

Haemodynamic profile of the potassium channel activator EMD 52692 in anaesthetized pigs

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1 The systemic and regional haemodynamic effects of the potassium channel activator EMD 52692 or its solvent were investigated after intravenous and after intracoronary administration in anaesthetized pigs.

2 Consecutive intravenous 10 min infusions of EMD 52692 (0.15, 0.30, 0.60, 1.20 $\mu g\,kg^{-1}\,min^{-1}$; $n = 7$) dose-dependently decreased mean arterial blood pressure by up to 50%. This was entirely due to peripheral vasodilatation, since cardiac output did not change. Heart rate increased by up to 50%, while left ventricular end diastolic pressure decreased dose-dependently from 6 ± 1 mmHg to 3 ± 1 mmHg ($P < 0.05$), and stroke volume decreased from 30 ± 2 ml to 21 ± 2 ml ($P < 0.05$). Left ventricular dP/dt_{max} was not affected.

3 Although cardiac output did not change, EMD 52692 caused a redistribution of blood flow from the arteriovenous anastomoses to the capillary channels. Blood flow to the adrenals, small intestine, stomach, bladder, spleen and brain increased, while renal blood flow decreased and blood flow to several muscle groups and skin were not altered. Vascular conductance was increased dose-dependently in all organs, except for the kidneys, where after the initial increase, vascular conductance returned to baseline with the highest dose. Particularly striking were the effects on the vasculature of the brain. With the highest dose of EMD 52692 blood flow more than doubled, while vascular conductance increased four fold.

4 Transmural myocardial blood flow increased slightly, which was entirely due to an increase in subepicardial blood flow. Myocardial O_2-consumption and segment length shortening were not significantly affected.

5 After consecutive 10 min intracoronary infusions (0.0095, 0.019, 0.0375 and 0.075 $\mu g\,kg^{-1}\,min^{-1}$; $n = 7$) into the left anterior descending coronary artery (LADCA), mean arterial blood pressure was maintained with the lowest two doses, but decreased by up to 15% with the higher doses, whereas heart rate increased by up to 24%. Blood flow to the LADCA-perfused myocardium doubled with the highest dose, the subepicardium benefitting the most. Coronary venous O_2-saturation increased dose-dependently from $23 \pm 2\%$ to $60 \pm 4\%$, while myocardial O_2-consumption of the LADCA-perfused myocardium was not affected by the drug.

6 It is concluded that EMD 52692 is a potent vasodilator, with particularly pronounced effects on vasculature of the brain. Its selectivity for vascular smooth muscle cells exceeds that for the myocytes, since with doses that are much higher than those of potential clinical interest no negative inotropic effects were observed. The compound primarily dilates arteries but some venodilatation may also occur.

Introduction

Diverse pharmacological treatments can have equal hypotensive effects in essential hypertension, despite apparently distinct modes of action (e.g. Ca^{2+} antagonism, angiotensin converting enzyme-inhibition). Recently, a new class of vasodilators, the potassium channel activators, has been introduced. These drugs cause hyperpolarization of the membranes of vascular smooth muscle cells, which reduces the availability of Ca^{2+} and thereby attenuates contraction (Hamilton *et al.*, 1986). Furthermore, the cells become less sensitive to depolarizing stimuli (Clapham & Wilson, 1987; Cook, 1988; 1989). The vasodilator and cardiac effects of drugs like cromakalim (Hamilton *et al.*, 1986; Weir & Weston, 1988; Cook & Hof, 1988) and pinacidil (Bray *et al.*, 1987) and also the cardiac effects of nicorandil (Weir & Weston, 1988) are mediated by opening of adenosine 5'-triphosphate (ATP)-dependent potassium channels. These potassium channels have been shown to be important in mediating the hypoxic dilatation of coronary arteries (Daut *et al.*, 1990).

Cromakalim, at doses causing similar decreases in arterial blood pressure as nifedipine, exhibits a vasodilator profile which is clearly different from that of the calcium antagonists (Buckingham *et al.*, 1986). The aim of the present study was to investigate the systemic haemodynamics and the distribution of cardiac output (radioactive microsphere technique) in response to intravenous infusions of EMD 52692 in the anaesthetized pig. EMD 52692 (Figure 1), a novel benzopyran derivative, structurally identical to SR 44866 (Findlay *et al.*, 1989), has been shown to hyperpolarize smooth muscle cells, which has been attributed to activation of ATP-dependent potassium channels (Findlay *et al.*, 1989; De Peyer *et al.*, 1989; Gericke *et al.*, 1989). In order to distinguish direct from indirect (secondary to systemic haemodynamic changes)

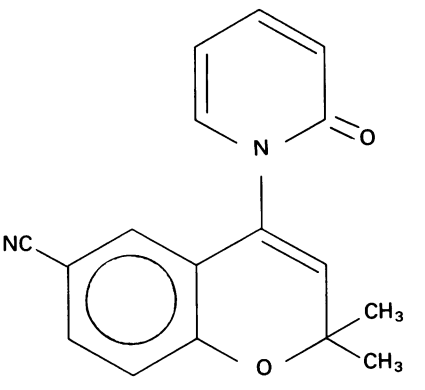

Figure 1 Chemical structure of EMD 52692.

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effects, we also studied the effects of EMD 52692 on myocardial performance after intracoronary infusion of the compound.

Methods

General

After an overnight fast, cross-bred Landrace × Yorkshire pigs of either sex (22–28 kg, $n = 29$) were sedated with 120 mg azaperone (Stresnil) i.m., anaesthetized with 150 mg metomidate (Hypnodil) i.v., intubated and connected to a ventilator for intermittent positive pressure ventilation with a mixture of O_2 and N_2O (1:2, v/v). Respiratory rate and tidal volume were set to keep arterial blood gases within the normal range: $7.41 < pH < 7.44$; $42\,mmHg < PCO_2 < 45\,mmHg$ and $130\,mmHg < Po_2 < 170\,mmHg$. 7F catheters were placed in the superior caval vein for administration of $160\,mg\,kg^{-1}$ α-chloralose followed by an infusion of a low dose of sodium pentobarbitone ($5\,mg\,kg^{-1}\,h^{-1}$); for administration of the muscle relaxant pancuronium bromide (4 mg) prior to thoracotomy; and for administration of haemaccel (Behringwerke A.G., Marburg, F.R.G.) to replace blood loss. Catheters were also positioned in the descending aorta for withdrawal of blood samples and measurement of central aortic blood pressure. A micromanometer-tipped catheter (7F Millar), inserted via the left carotid artery, was used to measure left ventricular pressure and its first derivative (LV dP/dt). After thoracotomy, an electromagnetic flow probe (Skalar, Delft, The Netherlands) was placed around the ascending aorta, while the great cardiac vein was cannulated for collection of blood in which the haemoglobin concentration and O_2-saturation were determined (OSM2, Radiometer, Copenhagen, Denmark). In 15 of the animals the proximal left anterior descending coronary artery was dissected free for placement of an electromagnetic flow probe around the vessel, while a cannula (outer diameter 0.8 mm) was inserted for intracoronary infusions of EMD 52692.

Regional blood flows

In order to determine regional blood flows, the left atrial appendage was cannulated for injection of a batch of $1–2 \times 10^6$ carbonized plastic microspheres [$15 \pm 1\,\mu m$ (s.d.) in diameter] labelled with either ^{46}Sc, ^{95}Nb, ^{103}Ru, ^{113}Sn or ^{141}Ce. Fifteen s before the injection of microspheres, blood was withdrawn from a femoral artery at a rate of $10\,ml\,min^{-1}$ until 60–65 s after completion of the injection of the microspheres. At the end of each experiment in which the animals received intracoronary infusions of either EMD 52692 or its solvent (see below), the area perfused by the left anterior descending coronary artery was identified by intracoronary injection of patent blue violet (Sigma, St. Louis, MO, U.S.A.). All the animals were killed with an overdose of sodium pentobarbitone and from the i.v. treated animals (see below) various organs (adrenals, bladder, stomach, small intestine, brains and kidneys) and tissues (abdominal skin, different muscle groups) were excised, weighed and put into vials. In the animals treated with intracoronary infusions only the heart was excised. The hearts were fixed in formaldehyde (10% v/v) and 48 h later divided into atria and right and left ventricle. The myocardium of the left ventricle was divided into three layers of equal thickness: subepicardium, mesocardium and subendocardium. The kidneys were divided into medulla and three cortical layers of equal thickness. The cerebral hemispheres, cerebellum, diencephalon and brain stem were separated in order to obtain regional blood flow data from the different parts of the brain.

The radioactivity was counted and the amount of blood flow to the various tissues (Q_{tis}) calculated as:

$$Q_{tis}(ml\,min^{-1}) = (I_{tis}/I_{art}) \times Q_{art},$$

where I_{tis} and I_{art} are, respectively, the radioactivity (c.p.m.) in a particular tissue and that of the arterial blood sample, while Q_{art} is the rate of withdrawal of the blood sample. Although the lungs receive microspheres via both peripheral arteriovenous anastomoses and bronchial arteries, the contribution via the latter route appears to be only about 1% of cardiac output (Baile et al., 1982). Thus, the values for the lung blood flow can be used as an index of the arteriovenous anastomotic flow (i.e. non-nutrient part of the cardiac output). The nutrient part of the cardiac output was calculated by subtracting 'lung flow' from the cardiac output. All regional vascular conductances were calculated as the ratio between Q_{tis} and the mean arterial blood pressure. Full details of the procedures and the calculation of flow data using this technique have been described earlier (Saxena et al., 1980).

Myocardial oxygen consumption and contractile function

Myocardial O_2-consumption ($M\dot{V}O_2$) was calculated as the product of coronary blood flow and the difference in the O_2 contents of the arterial and coronary venous blood.

Regional myocardial segment length shortening was measured with sonomicrometry (Triton Technology Inc., San Diego, CA, U.S.A.) by a pair of ultrasonic crystals implanted approximately 10–15 mm apart in the subepicardial layers (during intracoronary infusions) or the midmyocardial layers (during the intravenous infusions), oriented parallel to the short axis (Freeman et al., 1985). In order to differentiate between the effect of EMD 52692 on the different layers of the myocardium, another pair of crystals was also implanted in the subendocardial layers of the animals which received the intracoronary infusions of EMD 52692. At the end of each experiment the position of the subendocardial crystals was confirmed. From the tracings, segment length shortening (SLS) was calculated as: SLS (%) = 100 × (EDL − ESL)/EDL, in which EDL and ESL are the segment length at end-diastole and end-systole, respectively.

Experimental protocols

Four consecutive 10 min intravenous infusions of EMD 52692 (0.15, 0.3, 0.6 and $1.2\,\mu g\,kg^{-1}\,min^{-1}$; total cumulative doses of 1.5, 4.5, 10.5 and $22.5\,\mu g\,kg^{-1}$; $n = 7$) or equal volumes of the solvent ($n = 7$) or four consecutive 10 min intracoronary infusions of EMD 52692 (0.0095, 0.019, 0.0375 and $0.075\,\mu g\,kg^{-1}\,min^{-1}$; total cumulative doses of 0.10, 0.29, 0.66 and $1.41\,\mu g\,kg^{-1}$; $n = 7$) or equal volumes of the solvent ($n = 8$) were administered. Systemic haemodynamics, regional myocardial function and the distribution of coronary blood flow were determined in all four series of experiments, but the distribution of cardiac output was only determined during the intravenous infusions of EMD 52692 and its solvent.

Determination of EMD 52692 plasma concentrations

At the end of each infusion step blood samples (5 ml) were collected in heparinized tubes for the determination of plasma levels of EMD 52692. The blood samples were centrifuged at 1500 *g* at 0°C for 10 min and the plasma (2–3 ml) was stored at −80°C until further analysis. Known amounts of hexadeuterated EMD 52692 were added as internal standard to the plasma samples. EMD 52692 and internal standard were extracted with isopropylether. The extracts were evaporated to dryness under nitrogen, taken up in 20 to 60 μl of methanol, and aliquots of 1 μl analysed by gas chromatography/mass fragmentography (Hewlett Packard GC 5890 II and autoinjector HP 7673).

Drugs

For the i.v. infusions, EMD 52692 (4-(1,2-dihydro-2-oxo-1-pyridyl)-2,2-dimethyl-2*H*-1- benzopyran-6-carbonitrile; courtesy Dr P. Schelling, Merck Darmstadt, F.R.G.) was dissolved in 4% (v/v) polyethylene glycol for the two highest (0.60 and $1.20\,\mu g\,kg^{-1}\,min^{-1}$) doses and subsequently further diluted

with NaCl (0.9%) to give the two lower (0.15 and 0.30 $\mu g\,kg^{-1}\,min^{-1}$) doses. The infusion rate was 1 ml min^{-1} for the lowest and the third, and 2 ml min^{-1} for the second and the highest dose. For the intracoronary infusions EMD 52692 was dissolved in 1% (v/v) polyethylene glycol and the required doses were reached by adjusting the infusion rate (from 0.25 ml min^{-1} to 2 ml min^{-1}).

Statistical evaluation

All data are presented as the arithmetic mean ± s.e.mean. The significance of the effects of the solvent or EMD 52692 on the different variables was evaluated by Duncan's new-multiple range-test once an analysis of variance (randomized block design) had revealed that the samples represented different populations. Statistical significance was defined as $P < 0.05$ (two-tailed).

Results

Intravenous infusions of EMD 52692

Plasma concentrations of EMD 52692 As the infusion rates (0.15, 0.30, 0.75 and 1.5 $\mu g\,kg^{-1}\,min^{-1}$) of EMD 52692 were increased, the arterial plasma concentrations reached levels of 5.9 ± 0.5, 14.6 ± 1.1, 30.8 ± 1.1 and 62.5 ± 2.3 $\mu g\,l^{-1}$, respectively.

Systemic haemodynamics No significant changes occurred during infusion of the solvent in any of the systemic haemodynamic variables (Table 1). EMD 52692 lowered the arterial blood pressure dose-dependently (by 51% with the highest dose). This fall in arterial blood pressure was caused by systemic vasodilatation (fall in systemic vascular resistance of 52%) as cardiac output was not affected. Cardiac output was maintained, but stroke volume decreased (by up to 30%, $P < 0.05$), most likely secondary to the fall in left ventricular filling pressure (from 6 ± 1 mmHg to 3 ± 1 mmHg, $P < 0.05$). Heart rate increased dose-dependently from 103 ± 7 beats min^{-1} to 151 ± 13 beats min^{-1} ($P < 0.05$) with the highest dose. LV$dP/dt_{max}$ was not affected.

Left ventricular blood flow and performance The solvent had no effect on the transmural blood flow and its distribution over the subendocardial and the subepicardial layers or on the coronary vascular conductance (Figure 2). Infusions of EMD 52692 had no significant effect on transmural left ventricular blood flow, but with the highest two doses there was a redistribution in favour of the subepicardium, as the flow to the subepicardial layers increased dose-dependently from 154 ± 14 ml min^{-1} 100 g^{-1} to 257 ± 18 ml min^{-1} 100 g^{-1} ($P < 0.05$), and flow to the subendocardium did not change. Vasodilatation occurred in all layers of the myocardium as indicated by an increase in vascular conductance. The increase in the subepicardium (by up to 268 ± 41%) was more pronounced than the increase (by up to 65 ± 16%) in the subendocardium (Figure 2).

In the solvent-treated animals there was a minor narrowing

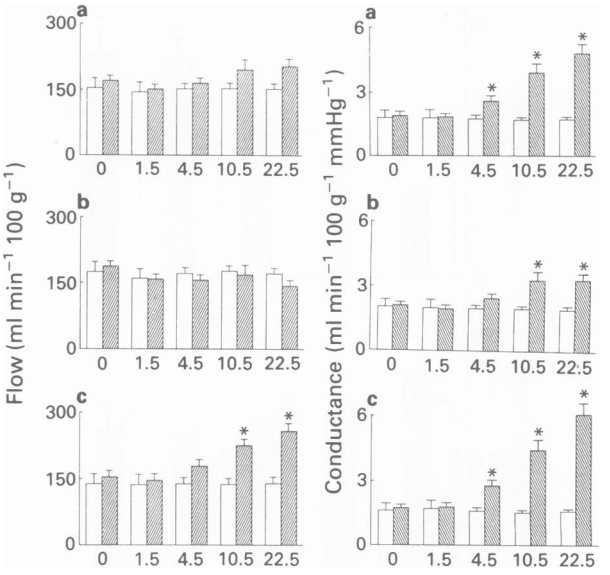

Figure 2 Effect of cumulative 10 min intravenous infusions (total dose 0, 1.5, 4.5, 10.5 and 22.5 $\mu g\,kg^{-1}$) of EMD 52692 (hatched columns, $n = 7$) or equal volumes of its solvent (open columns, $n = 7$) on transmural (a), subendocardial (b) and subepicardial (c) left ventricular blood flows and conductances in anaesthetized pigs. Data are presented as mean and the bars show s.e.mean. * Indicates that the drug-induced changes from baseline are significantly different ($P < 0.05$) from the solvent-induced changes from baseline.

Table 1 Systemic haemodynamics after cumulative intravenous 10 min infusions of EMD 52692 or its solvent in anaesthetized pigs

		EMD 52692 ($\mu g\,kg^{-1}$) or equal volumes of its solvent				
		0	*1.5*	*4.5*	*10.5*	*22.5*
MAP	Solvent	89 ± 5	88 ± 4	88 ± 4	90 ± 4	88 ± 4
	EMD 52692	90 ± 3	82 ± 2*	65 ± 2*	51 ± 4*	44 ± 4*
APP	Solvent	43 ± 2	46 ± 2	45 ± 2	46 ± 2	45 ± 2
	EMD 52692	42 ± 3	39 ± 3	39 ± 3	42 ± 3	44 ± 4
CO	Solvent	2.8 ± 0.2	2.8 ± 0.2	2.8 ± 0.2	2.7 ± 0.2	2.7 ± 0.2
	EMD 52692	3.0 ± 0.2	3.1 ± 0.2	3.0 ± 0.2	3.0 ± 0.3	3.1 ± 0.2
SVR	Solvent	34 ± 4	33 ± 4	33 ± 3	35 ± 3	34 ± 3
	EMD 52692	31 ± 3	28 ± 2	22 ± 2*	18 ± 2*	15 ± 2*
HR	Solvent	99 ± 6	96 ± 6	99 ± 8	98 ± 6	99 ± 6
	EMD 52692	103 ± 7	111 ± 8*	129 ± 7*	141 ± 12*	151 ± 13*
LV$dP/dt_{max}$	Solvent	2670 ± 170	2610 ± 130	2650 ± 130	2540 ± 150	2530 ± 140
	EMD 52692	2710 ± 120	2630 ± 120	2880 ± 130	2730 ± 280	2800 ± 300
LVEDP	Solvent	6 ± 1	6 ± 1	6 ± 1	6 ± 1	7 ± 1
	EMD 52692	6 ± 1	5 ± 1*	4 ± 1*	3 ± 1*	3 ± 1*
SV	Solvent	28 ± 1	29 ± 1	29 ± 2	27 ± 1	27 ± 1
	EMD 52692	30 ± 2	28 ± 2*	23 ± 2*	22 ± 2*	21 ± 2*

Data are mean ± s.e.mean; $n = 7$ for both groups. MAP = mean arterial blood pressure (mmHg); APP = arterial pulse pressure (mmHg); CO = cardiac output (1 min^{-1}); SVR = systemic vascular resistance (mmHg 1 min^{-1}); HR = heart rate (beats min^{-1}); LV$dP/dt_{max}$ = maximal rate of rise of left ventricular pressure (mmHg s^{-1}); LVEPD = left ventricular end diastolic pressure (mmHg); SV = stroke volume (ml).

* Changes versus baseline in the EMD 52692-treated animals are significantly ($P < 0.05$) different from those in the solvent-treated animals.

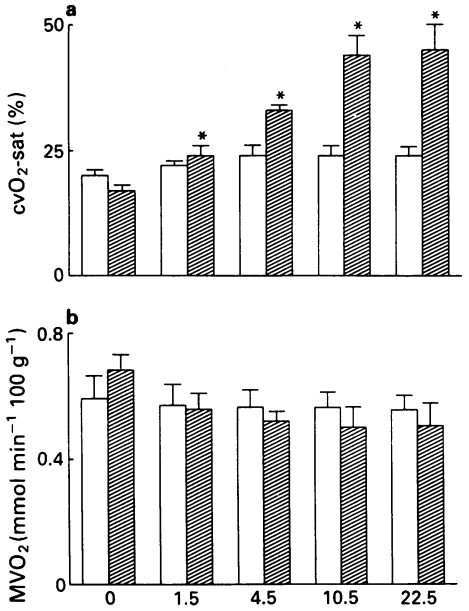

Figure 3 Effect of cumulative 10 min intravenous infusions of EMD 52692 (hatched columns, total dose 0, 1.5, 4.5, 10.5 and 22.5 $\mu g\,kg^{-1}$, $n = 7$) or equal volumes of its solvent (open columns, $n = 7$) on (a) coronary venous O_2-saturation (cvO_2-sat) and (b) left ventricular O_2-consumption ($M\dot{V}O_2$). Data are presented as mean and the bars show s.e.mean. * Indicates that the drug-induced changes from baseline are significantly different ($P < 0.05$) from the solvent-induced changes from baseline.

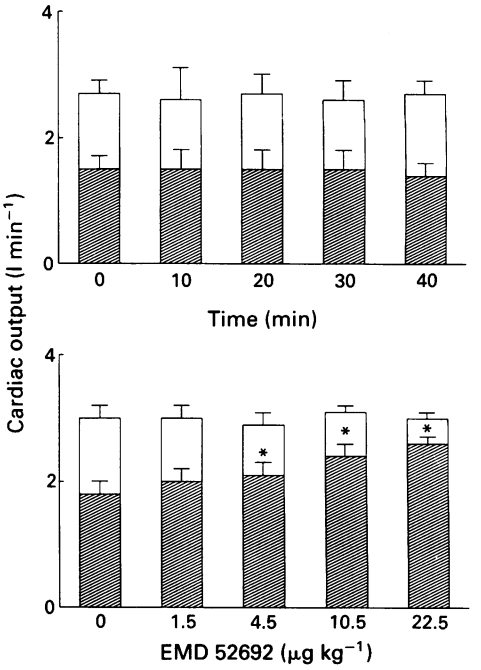

Figure 4 Fractionation of cardiac output in arteriovenous anastomotic (open columns) and capillary (hatched columns) blood flow during cumulative 10 min intravenous infusions of EMD 52692 (total dose 0, 1.5, 4.5, 10.5 and 22.5 $\mu g\,kg^{-1}$, $n = 7$) or equal volumes of its solvent ($n = 7$) in anaesthetized pigs. Data are presented as mean and the bars show s.e.mean. * Indicates that the drug-induced changes from baseline are significantly different ($P < 0.05$) from the solvent-induced changes from baseline.

of the difference in the O_2 contents of the arterial and coronary venous blood (5%, $P < 0.05$), reflected by the small increase (from 20 ± 1% to 24 ± 2%) in the O_2-saturation in the great cardiac vein (Figure 3). With EMD 52692, the decrease (35% after the highest infusion rate) in O_2-extraction was considerably large as the coronary venous O_2-saturation almost tripled (from 17 ± 1% to 45 ± 5% with the highest dose, $P < 0.05$). Myocardial O_2-consumption was not affected by the solvent or by the drug. Data are presented in Fig. 3.

Myocardial segment length shortening, determined from the crystals placed in the midmyocardium, did not change either

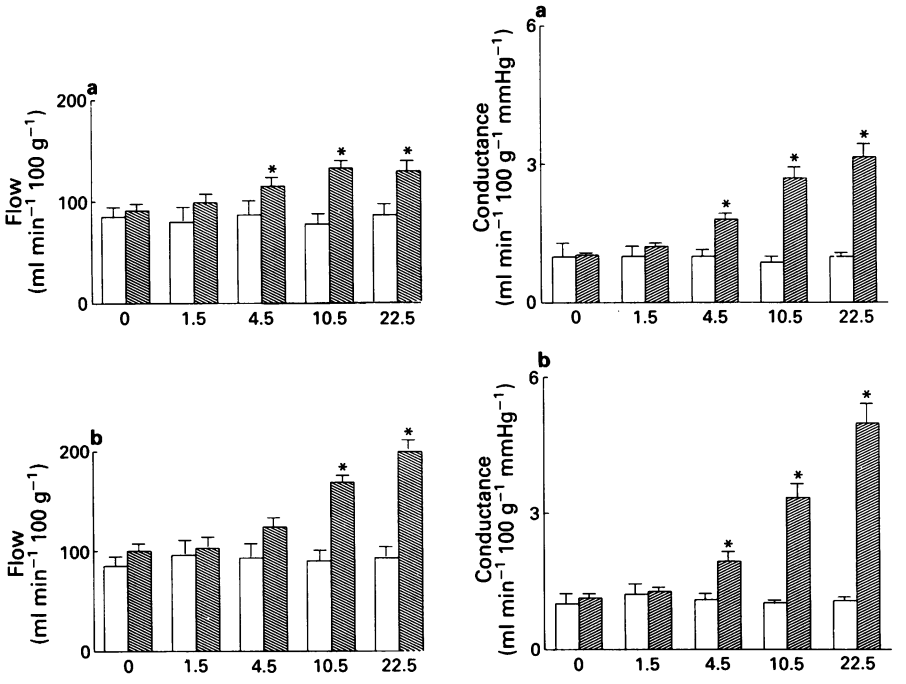

Figure 5 Effect of cumulative 10 min intravenous infusions (total dose 0, 1.5, 4.5, 10.5 and 22.5 $\mu g\,kg^{-1}$) of EMD 52692 (hatched columns, $n = 7$) and equal volumes of its solvent (open columns, $n = 7$) on atrial (a) and right ventricular blood flow (b) and conductance in anaesthetized pigs. Data are presented as mean and the bars show s.e.mean. * Indicates that the drug-induced changes from baseline are significantly different ($P < 0.05$) from the solvent-induced changes from baseline.

during infusion of the solvent (19 ± 1%, 18 ± 1%, 19 ± 1%, 19 ± 1% and 20 ± 2% at baseline and after 10, 20, 30 and 40 min, respectively) or during infusion of EMD 52692 (20 ± 1%, 19 ± 1%, 18 ± 2% and 18 ± 1% at baseline, and after cumulative infusions of 0.15, 0.30, 0.60 and 1.2 $\mu g\,kg^{-1}\,min^{-1}$ of EMD 52692, respectively).

Distribution of cardiac output

Fractionation of cardiac output into arteriovenous anastomotic and capillary flows In anaesthetized animals a large fraction of the cardiac output is shunted through arteriovenous anastomoses (Kaihara *et al.*, 1968; Van Woerkens *et al.*, 1990) and without affecting total cardiac output, antihypertensive drugs may affect this non-nutritional flow differently (Hof & Hof, 1989). Figure 4 illustrates that, under baseline conditions, in both groups approximately 40% of the cardiac output was shunted through arteriovenous anastomoses. This fraction did not change during infusion of the solvent. Although EMD 52692 did not affect cardiac output, there was a redistribution in favour of the capillary flow, which increased to 85% of cardiac output after the highest dose.

Regional blood flows Blood flow to the atria and the right ventricle increased dose-dependently during the EMD 52692 infusions (Figure 5). Taking into account the decrease in perfusion pressure it is clear that the increments in vascular conductance were even more pronounced (Figure 5). EMD 52692 caused pronounced increases in total cerebral blood flow (32 ± 3, 34 ± 2, 43 ± 3, 66 ± 7 and 69 ± 7 $ml\,min^{-1}\,100\,g^{-1}$ at baseline and after 1.5, 4.5, 10.5 and 22.5 $\mu g\,kg^{-1}$, respectively). Although flow to all parts of the brain increased (Figure 6), the increase was more pronounced in the brain stem (216 ± 24%) and diencephalon (178 ± 40%) than in the cerebellum (113 ± 14%) and the hemispheres (100 ± 10%). The increases in regional conductances were even more impressive than the increases in flow (Figure 6).

Total renal blood flow remained constant with the two lowest doses, but decreased by 43 ± 7% after infusion of the highest dose (Table 2). Further analysis revealed that this was predominantly due to a dose-dependent decrease in glomerular blood flow of the outer cortex, as the glomerular blood flow of the inner cortex decreased only slightly and flow to the medulla even increased (Table 2). The decreases in flow were secondary to the fall in arterial blood pressure, as total renal conductance increased significantly by as much as 110 ± 50% after the third dose. With the highest dose renal vascular conductance started to return to baseline values. The increases in vascular conductance, observed in the inner

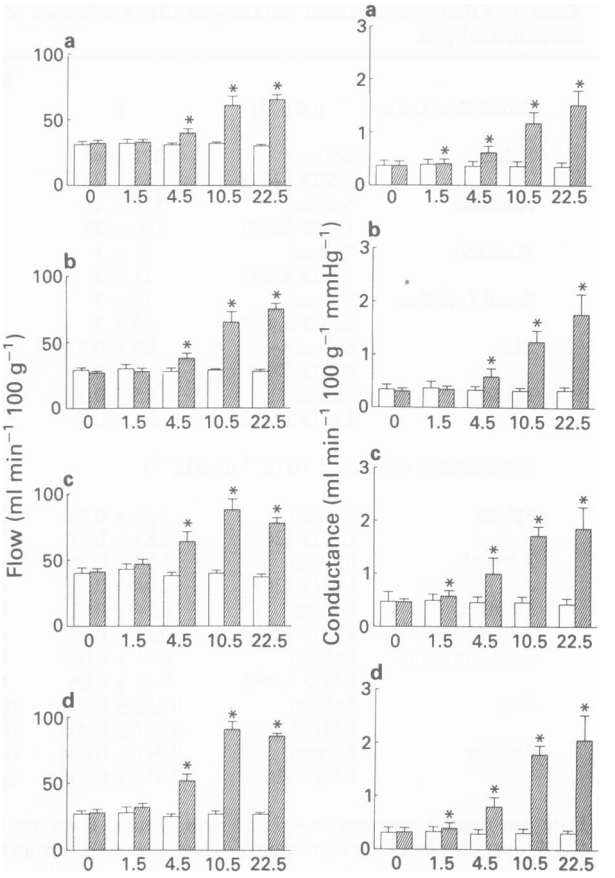

Figure 6 Effect of cumulative 10 min infusions of EMD 52692 (hatched columns, total dose 0, 1.5, 4.5, 10.5 and 22.5 $\mu g\,kg^{-1}$, $n = 7$) or equal volumes of its solvent (open columns; $n = 7$) on blood flow and conductance of the cerebral hemispheres (a), diencephalon (b), cerebellum (c) and brain stem (d) in anaesthetized pigs. Data are presented as mean and the bars show s.e.mean. * Indicates that the drug-induced changes from baseline are significantly different ($P < 0.05$) from the solvent-induced changes from baseline.

Table 2 Effect of continuous intravenous 10 min infusions of EMD 52692 or its solvent on renal blood flow and conductance in anaesthetized pigs

		EMD 52692 ($\mu g\,kg^{-1}$) or equal volumes of its solvent				
Blood flow ($ml\,min^{-1}\,100\,g^{-1}$)		*0*	*1.5*	*4.5*	*10.5*	*22.5*
Kidneys	Solvent	252 ± 22	267 ± 35	253 ± 24	249 ± 24	236 ± 15
	EMD 52692	247 ± 19	225 ± 14	228 ± 16	191 ± 11*	137 ± 17*
Glomeruli of the inner cortex	Solvent	210 ± 18	210 ± 19	202 ± 16	190 ± 13	183 ± 11
	EMD 52692	163 ± 10	161 ± 10	178 ± 9	155 ± 9	115 ± 13
Glomeruli of the outer cortex	Solvent	393 ± 25	407 ± 32	405 ± 32	412 ± 33	387 ± 26
	EMD 52692	386 ± 31	333 ± 19	316 ± 25*	258 ± 18*	179 ± 23*
Medulla	Solvent	58 ± 7	59 ± 6	53 ± 5	46 ± 5	46 ± 5
	EMD 52692	42 ± 5	44 ± 5	55 ± 7*	58 ± 5*	55 ± 6*
Conductance ($ml\,min^{-1}\,100\,g^{-1}\,mmHg^{-1}$)						
Kidneys	Solvent	2.89 ± 0.31	3.14 ± 0.25	2.95 ± 0.34	2.79 ± 0.31	2.67 ± 0.17
	EMD 52692	2.78 ± 0.23	2.74 ± 0.17	3.35 ± 0.33*	3.88 ± 0.33*	3.19 ± 0.34
Glomeruli of the inner cortex	Solvent	2.40 ± 0.25	2.54 ± 0.22	2.33 ± 0.22	2.12 ± 0.18	2.08 ± 0.14
	EMD 52692	1.84 ± 0.13	1.96 ± 0.12	2.79 ± 0.18*	3.18 ± 0.35*	2.73 ± 0.36*
Glomeruli of the outer cortex	Solvent	4.43 ± 0.34	4.89 ± 0.32	4.62 ± 0.41	4.62 ± 0.42	4.37 ± 0.20
	EMD 52692	4.32 ± 0.35	4.05 ± 0.19	4.95 ± 0.48	5.22 ± 0.43	4.14 ± 0.45
Medulla	Solvent	0.67 ± 0.08	0.68 ± 0.07	0.62 ± 0.07	0.51 ± 0.06	0.53 ± 0.06
	EMD 52692	0.48 ± 0.05	0.53 ± 0.06	0.84 ± 0.09*	1.16 ± 0.11*	1.27 ± 0.11*

Data are mean ± s.e.mean; $n = 7$ for both groups. * Changes versus baseline in the EMD 52692-treated animals are significantly ($P < 0.05$) different from those in the solvent-treated animals.

Table 3 Effect of continuous intravenous 10 min infusions of EMD 52692 or its solvent on regional blood flow and conductance in anaesthetized pigs

		EMD 52692 (μg kg⁻¹) or equal volumes of its solvent				
Blood flow (ml min⁻¹ 100 g⁻¹)		*0*	*1.5*	*4.5*	*10.5*	*22.5*
Spleen	Solvent	271 ± 46	317 ± 52	273 ± 43	254 ± 55	267 ± 56
	EMD 52692	255 ± 23	311 ± 31	453 ± 36	375 ± 42	272 ± 31
Adrenals	Solvent	131 ± 16	139 ± 14	142 ± 7	144 ± 12	149 ± 22
	EMD 52692	180 ± 27	220 ± 23	332 ± 20*	291 ± 19*	231 ± 14*
Stomach	Solvent	21 ± 1	20 ± 1	23 ± 2	21 ± 2	20 ± 2
	EMD 52692	21 ± 2	20 ± 1	19 ± 2	21 ± 3	29 ± 5
Small Intestine	Solvent	38 ± 3	37 ± 2	36 ± 2	39 ± 2	36 ± 2
	EMD 52692	44 ± 5	46 ± 8	51 ± 9	62 ± 9	77 ± 7*
Skin	Solvent	1.9 ± 0.5	1.9 ± 0.9	1.5 ± 0.4	2.0 ± 0.7	2.3 ± 1.0
	EMD 52692	2.4 ± 0.4	2.7 ± 0.5	2.2 ± 0.5	2.6 ± 0.4	2.5 ± 0.3
Bladder	Solvent	3.5 ± 0.3	4.3 ± 0.4	4.0 ± 0.4	3.9 ± 0.3	3.9 ± 0.2
	EMD 52692	5.0 ± 0.5	4.9 ± 0.5	4.5 ± 0.6	4.5 ± 0.5	7.6 ± 0.9
Conductance (ml min⁻¹ 100 g⁻¹ mmHg⁻¹)						
Spleen	Solvent	3.13 ± 0.60	3.70 ± 0.60	3.16 ± 0.51	2.84 ± 0.65	3.05 ± 0.64
	EMD 52692	2.83 ± 0.23	3.79 ± 0.35	7.00 ± 0.38*	7.30 ± 0.54*	6.18 ± 0.39*
Adrenals	Solvent	1.53 ± 0.22	1.72 ± 0.14	1.64 ± 0.11	1.60 ± 0.14	1.67 ± 0.19
	EMD 52692	2.04 ± 0.32	2.70 ± 0.30	5.16 ± 0.36*	5.83 ± 0.42*	5.45 ± 0.44*
Stomach	Solvent	0.24 ± 0.02	0.25 ± 0.02	0.26 ± 0.02	0.23 ± 0.02	0.23 ± 0.03
	EMD 52692	0.24 ± 0.03	0.25 ± 0.02	0.31 ± 0.03	0.42 ± 0.06*	0.67 ± 0.11*
Small Intestine	Solvent	0.44 ± 0.05	0.44 ± 0.03	0.42 ± 0.030	0.44 ± 0.03	0.42 ± 0.03
	EMD 52692	0.49 ± 0.06	0.56 ± 0.09	0.81 ± 0.017	1.23 ± 0.16*	1.79 ± 0.18*
Skin	Solvent	0.022 ± 0.007	0.022 ± 0.012	0.018 ± 0.005	0.023 ± 0.008	0.028 ± 0.013
	EMD 52692	0.027 ± 0.005	0.033 ± 0.007	0.036 ± 0.009	0.051 ± 0.007*	0.058 ± 0.006*
Bladder	Solvent	0.041 ± 0.004	0.066 ± 0.006	0.046 ± 0.002	0.044 ± 0.004	0.044 ± 0.003
	EMD 52692	0.056 ± 0.006	0.059 ± 0.006	0.072 ± 0.013	0.090 ± 0.011*	0.178 ± 0.022*

Data are mean ± s.e.mean; $n = 7$ for both groups. Data are in ml min⁻¹ 100 g⁻¹ mmHg⁻¹; * changes versus baseline in the EMD 52692-treated animals are significantly different ($P < 0.05$) from those in the solvent-treated animals.

cortex and the medulla were not observed in the outer cortex (Table 2).

Flow to a number of other organs was well maintained (Table 3). Increases were observed in flow to the small intestine (by 81 ± 19% with the highest dose, $P < 0.05$) and the adrenals (by up to 38 ± 15%, $P < 0.05$). Since the fall in arterial perfusion pressure outweighed the decrease in flow, vascular conductance was increased in all these organs (Table 3).

The blood flow to eight different muscle groups was measured, of which only flow to the M. sternocleidomastoideus increased from 5.6 ± 0.7 ml min⁻¹ 100 g⁻¹ at baseline to 12.4 ± 2.7 ml min⁻¹ ($P < 0.05$), with the highest dose. Flow to all the other muscles (M. iliopsoas, pars costalis diaphragmatis, M. pectoralis, M. erector spinae, M. gluteus, M. masseter and the M. rectus abdominis) remained constant (baseline values between 2 and 6 ml min⁻¹ 100⁻¹). Vasodilatation occurred in all muscle groups as vascular conductance had increased 2 to 5 fold after infusion of the highest dose.

Intracoronary infusions of EMD 52692

Systemic haemodynamics No arrhythmias were observed during infusions of either the solvent or EMD 52692. Infusion of the solvent had no effect on systemic haemodynamics (Table 4). During infusion of the lowest two doses of EMD 52692 the only change was a 5% increase in heart rate. With the higher infusion rates there was a further increase in heart rate (by up to 22 ± 4%) and a 16 ± 3% decrease in mean arterial blood pressure, due to peripheral vasodilatation (decrease in systemic vascular resistance of 16 ± 4%). Cardiac output and left ventricular filling pressure did not change, while LV dP/dt_{max} showed an increase (24 ± 8%, $P < 0.05$) after infusion of the highest dose.

Table 4 Systemic haemodynamics after continuous intracoronary 10 min infusions of EMD 52692 or its solvent in anaesthetized pigs

		EMD 52692 (μg kg⁻¹) or equal volumes of its solvent				
		0	*0.10*	*0.29*	*0.66*	*1.41*
HR	Solvent	111 ± 6	111 ± 6	111 ± 5	113 ± 5	114 ± 6
	EMD 52692	116 ± 5	117 ± 5	123 ± 6*	130 ± 8*	144 ± 10*
MAP	Solvent	87 ± 4	85 ± 3	84 ± 3	85 ± 4	84 ± 5
	EMD 52692	93 ± 4	94 ± 4	89 ± 5	86 ± 4*	79 ± 5*
CO	Solvent	2.6 ± 0.3	2.6 ± 0.2	2.6 ± 0.3	2.6 ± 0.3	2.5 ± 0.3
	EMD 52692	2.3 ± 0.1	2.4 ± 0.2	2.3 ± 0.2	2.3 ± 0.1	2.3 ± 0.1
SVR	Solvent	35 ± 3	35 ± 3	34 ± 3	35 ± 3	35 ± 3
	EMD 52692	42 ± 3	41 ± 3	39 ± 3	38 ± 2*	35 ± 1*
LV$dP/dt_{max}$	Solvent	2490 ± 290	2460 ± 280	2460 ± 290	2610 ± 370	2580 ± 370
	EMD 52692	2270 ± 160	2330 ± 190	2500 ± 250	2680 ± 300	2830 ± 320*
LVEDP	Solvent	7 ± 1	6 ± 1	6 ± 1	6 ± 1	6 ± 1
	EMD 52692	7 ± 1	7 ± 1	7 ± 1	7 ± 1	6 ± 1

Data are mean ± s.e.mean; $n = 8$ for the solvent-treated and $n = 7$ for the EMD 52692-treated animals. HR = heart rate (beats min⁻¹); MAP = mean arterial blood pressure (mmHg); CO = cardiac output (l min⁻¹); SVR = systemic vascular resistance (mmHg min l⁻¹); LV$dP/dt_{max}$ = maximal rate of rise of left ventricular pressure (mmHg s⁻¹); LVEDP = left ventricular end-diastolic pressure (mmHg). * Changes versus baseline in the EMD 52692-treated are significantly different ($P < 0.05$) from those in the solvent-treated animals.

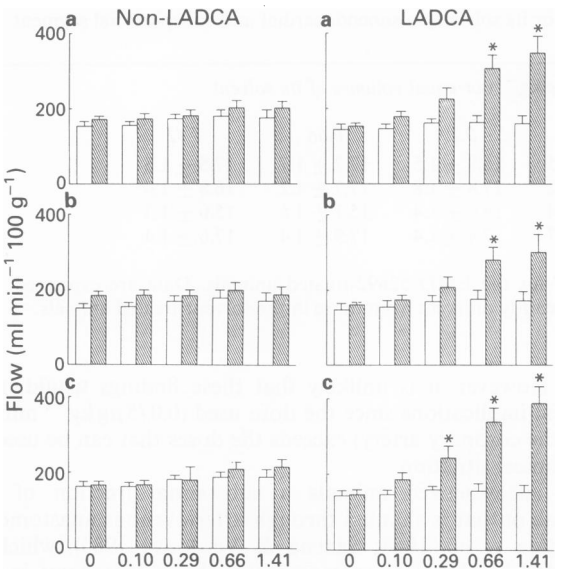

Figure 7 Transmural (a), subendocardial (b) and subepicardial (c) blood flow after 10 min cumulative intracoronary infusions of EMD 52692 (hatched columns, total doses 0, 0.10, 0.29, 0.66 and 1.41 $\mu g\,kg^{-1}$, $n = 7$) or its solvent (open columns, $n = 8$) directly into the left anterior descending coronary artery (LADCA). Non-LADCA = area not perfused by the LADCA (posterior wall of the left ventricle). Data are presented as mean and the bars show s.e.mean. * Indicates that the EMD 52692-induced changes from baseline are significantly different ($P < 0.05$) from solvent-induced changes from baseline.

Myocardial performance Intracoronary infusions of the solvent had no effect on myocardial perfusion (Figure 7). A representative example of the effects of EMD 52692 is presented in Figure 8, which clearly shows the EMD 52692-induced increases in coronary blood flow. EMD 52692 dose-dependently increased transmural blood flow of the myocardium perfused by the left anterior descending coronary artery by up to 128% (Figure 7). Although all layers benefitted from the increase in transmural flow, the increments in the subepicardium ($242 \pm 55\,ml\,min^{-1}\ 100\,g^{-1}$) were considerably larger than in the subendocardium ($140 \pm 46\,ml\,min^{-1}\ 100\,g^{-1}$). There were no significant changes in myocardial blood flow (Figure 7) or vascular conductance (not shown) of the posterior wall of the left ventricle (the myocardium not perfused by the left anterior descending coronary artery).

The solvent had no effect on myocardial O_2-consumption (499 ± 39, 486 ± 41, 593 ± 29, 529 ± 52, $523 \pm 59\,\mu mol\,min^{-1}\ 100\,g^{-1}$ at baseline and after 10, 20, 30 and 40 min of solvent infusion, respectively) or on regional subendo- and subepicardial segment length shortening (Table 5). Subendocardial segment length shortening decreased from 18.2 $\pm$ 1.6% to 16.8 $\pm$ 1.0% with the highest dose of EMD 52692 ($P < 0.05$). Subepicardial segment length shortening tended to increase, but the increments did not reach levels of significance (Table 5).

Myocardial O_2-consumption of the LADCA-perfused myocardium was not affected by the drug (542 ± 43, 522 ± 26, 560 ± 33, 618 ± 17 and $521 \pm 46\,\mu mol\,min^{-1}\ 100\,g^{-1}$ at baseline and after 0.095, 0.29, 0.66 and 1.41 $\mu g\,kg^{-1}$, respectively).

Discussion

In the present study the plasma levels of EMD 52692 ranged from 5.1 to 62 $\mu g\,l^{-1}$. This range compares well with the effective *in vitro* concentrations described by De Peyer and colleagues (1989). In this last study the EC_{50} of EMD 52692 to hyperpolarize the membrane of the vascular smooth muscle cells of porcine coronary arteries was $0.6–1 \times 10^{-7}$ M, which corresponds with a concentration of 16–26 $\mu g\,l^{-1}$.

EMD 52692 is a potent systemic vasodilator. Its potency exceeds that of nicorandil, tested in the same model by a factor of 100 (Verdouw *et al.*, 1987). Furthermore EMD 52692 was shown to be 5 and 50 times more potent in anaesthetized

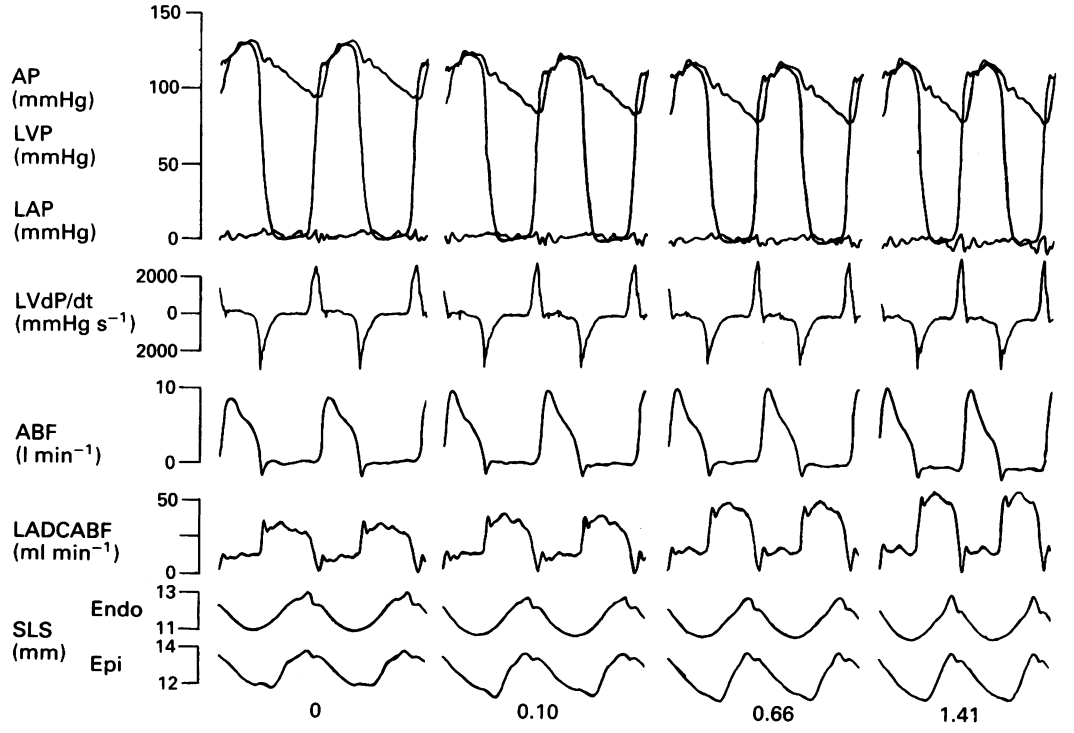

Figure 8 Representative tracing of the effects of intracoronary (left anterior descending coronary artery) infusions of EMD 52692 on systemic haemodynamics and regional myocardial blood flow and function in an anaesthetized pig. AP, LVP and LAP are central aortic, left ventricular and left atrial blood pressure, respectively; LV*dP/dt* = first derivative of LVP; ABF = ascending aortic blood flow; LADCABF = left anterior descending coronary artery blood flow; SLS(Endo) = subendocardial segment length; SLS(Epi) = subepicardial segment length. The infusion rates of EMD 52692 (total dose, $\mu g\,kg^{-1}$) are given at the bottom of the figure.

Table 5 Effect of continuous intracoronary 10 min infusions of EMD 52692 or its solvent on subendocardial and subepicardial segment length shortening in anaesthetized pigs

		EMD 52692 ($\mu g\,kg^{-1}$) or equal volumes of its solvent				
		0	*0.10*	*0.29*	*0.66*	*1.41*
Subendocardium	Solvent	17.3 ± 1.4	17.7 ± 1.5	16.8 ± 1.5	17.2 ± 1.7	17.5 ± 1.5
	EMD 52692	18.2 ± 1.6	18.3 ± 1.2	17.8 ± 1.8	17.7 ± 1.2	16.8 ± 1.0*
Subepicardium	Solvent	15.1 ± 1.1	14.9 ± 1.4	14.6 ± 1.4	15.1 ± 1.6	15.0 ± 1.3
	EMD 52692	16.7 ± 1.7	17.1 ± 1.7	17.4 ± 1.4	17.9 ± 1.4	17.6 ± 1.4

Data are mean ± s.e.mean; $n = 8$ for the solvent-treated animals and $n = 7$ for the EMD 52692-treated animals. Data are expressed as %. * Changes versus baseline in the EMD 52692-treated animals are significantly different from those in the solvent-treated animals.

dogs than cromakalim and pinacidil, respectively (Schliep *et al.*, 1989), two other compounds of which the pharmacological actions have been ascribed to potassium channel opening (Hamilton *et al.*, 1986; Bray *et al.*, 1987; Weir & Weston, 1988). The potency of EMD 52692 to dilate the systemic vascular bed, also compares favourably with that of the most potent representatives of the dihydropyridine calcium antagonists such as nisoldipine (Duncker *et al.*, 1986) and elgodipine (Sassen *et al.*, 1990).

Contraction of vascular smooth muscle cells in capacitance vessels depends on a rise in intracellular free calcium, mediated by activation of receptor-operated rather than voltage-operated events (Bolton, 1979). Webb and colleagues (1989) found that cromakalim produced dose-dependent dilatation of forearm resistance vessels, but did not dilate dorsal hand veins preconstricted with noradrenaline and concluded that this potassium channel activator was arterioselective. However, we observed at similar increments in heart rate (e.g. compare the second infusion rate of the intravenous and the highest infusion rate of the intracoronary experiments), that left ventricular end-diastolic pressure decreased after the intravenous, but not after the intracoronary infusions. The dose-dependent decrease in left ventricular filling pressure during the intravenous infusions can therefore not solely be attributed to the tachycardia and EMD 52692 most likely caused some venodilatation during the intravenous infusions.

The increase in heart rate, which has also been demonstrated for cromakalim (Buckingham *et al.*, 1986), was most likely a reflex-mediated response, and is a common feature of acute administration of vasodilators. Despite the tachycardia, cardiac output did not increase. We have shown that in the same model vasodilators such as nisoldipine (Duncker *et al.*, 1986) and nicorandil (Verdouw *et al.*, 1987) elicited similar responses. In conscious pigs these compounds caused marked increases in heart rate, left ventricular dP/dt_{max} and cardiac output (Verdouw *et al.*, 1987; Duncker *et al.*, 1988), indicating that the anaesthesia may have attenuated the reflex-mediated responses.

Potassium channel activators have potentially negative inotropic properties (Yanagisawa *et al.*, 1988), although the selectivity for vascular smooth muscle outweighs that for the myocardium (Cohen & Colbert, 1986; Longman *et al.*, 1988; Gotanda *et al.*, 1988). SR 44866, a compound which is structurally identical to EMD 52692 has been shown to have inhibitory effects on the electrical and mechanical activity of cardiac muscle (Findlay *et al.*, 1989), but the required dose is much higher for cardiac muscle than for smooth muscle. We did not see any changes in $LVdP/dt_{max}$, a frequently used measure of myocardial contractility. This index is, however, also influenced by changes in heart rate and pre- and afterload, all of which were affected considerably by EMD 52692. However, the two lowest intracoronary infusion rates (where heart rate, pre- and afterload were only slightly affected, with no changes in segment length shortening) demonstrated that at comparable intravenous infusions EMD 52692 exhibits no negative inotropic action. With the highest intracoronary dose subendocardial layers segment length shortening decreased significantly, while subepicardial segment length shortening was not affected. At present these results are difficult to interpret. However, it is unlikely that these findings would have clinical implications since the dose used ($0.075\,\mu g\,kg^{-1}\,min^{-1}$, into the coronary artery) exceeds the doses that can be used in the clinical situation.

In anaesthetized animals a substantial fraction of the cardiac output is shunted through arteriovenous anastomoses (Kaihara *et al.*, 1968; Saxena & Verdouw, 1985), which is reflected by the large entrapment of the microspheres in the lungs after induction of anaesthesia (Van Woerkens *et al.*, 1990). Figure 4 shows that although cardiac output did not change, the nutritional fraction increased with increasing doses of EMD 52692. This increase was most prominent in the brain, adrenals and small intestine, but absent in skeletal muscle. This study therefore confirms earlier investigations that potassium channel activators at equihypotensive dosages have a different vasodilator profile than for instance the calcium antagonists (Duncker *et al.*, 1986; Buckingham *et al.*, 1986; Sassen *et al.*, 1990). When comparing the vasodilator profile of EMD 52692 with two compounds that were tested in the same model, nicorandil (Verdouw *et al.*, 1987) and elgodipine (Sassen *et al.*, 1990), a calcium antagonist very similar to nisoldipine (Duncker *et al.*, 1986), it appears that EMD 52692 closely resembles nicorandil; similar increases in adrenal, cerebral, left ventricular and intestinal blood flow occurred while no effect on skeletal muscle blood flow at comparable increases in systemic vascular conductance was observed. Furthermore, it can be concluded that EMD 52692 dilates cerebral and adrenal vascular beds much more potently than elgodipine, but that the increase in vascular conductance of skeletal muscle by the potassium channel activator is less than by the calcium antagonist. Of interest is that the initial vasodilatation in the kidneys, as observed with elgodipine and EMD 52692 was followed by a decrease with the highest dose although systemic vascular conductance still increased. These vascular responses in the kidneys differed markedly in the inner and outer cortex and the medulla, as a result of which blood flow was redistributed away from the outer to the inner layers. A similar redistribution of renal flow during hypotension has been obtained by other investigators (see Carrière, 1975) and may thus be a perfusion pressure-dependent response rather than a direct drug action. The reduction in cortical glomerular blood flow might serve to enhance fluid retention to counterbalance the severe hypotension (Wright & Briggs, 1979).

Only a few studies have described the vasodilator profiles of other potassium channel activators (Arrigoni-Martelli *et al.*, 1980; Buckingham *et al.*, 1986; Cook & Hof, 1988). Cook & Hof (1988) studied the effects of cromakalim on regional blood flows in anaesthetized rabbits, and showed at doses inducing a blood pressure drop of up to 20% that renal blood flow decreased, skeletal muscle flow was maintained and blood flow to the stomach and small intestine were enhanced. These responses correspond well with our experiments. At variance with our findings, cerebral blood flow did not change significantly in their study. Arrigoni-Martelli and colleagues (1980) showed that pinacidil also did not affect blood flow through the femoral artery (representing muscle blood flow).

Myocardial O_2-consumption did not change after both the intravenous and the intracoronary infusions. In both experi-

ments the increase in O_2-demand caused by the increase in heart rate was fully compensated for by the decrease in arterial blood pressure. The increase in flow has therefore no metabolic origin and indicates that the coronary blood flow response is the result of a direct action of the drug on the coronary vascular bed. The finding that with the intravenous infusions the increase in flow was almost exclusively confined to the subepicardial layers is not surprising in view of the tachycardia and hypotension (Domenech & Goich, 1976); this has also been demonstrated for nicorandil (Verdouw *et al.*, 1987). Chronic treatment often leads to a reduction of the reflex tachycardia (Kiowski *et al.*, 1983). If the coronary vasodilator action were to be sustained with chronic treatment, the lower heart rate would most likely not only lower myocardial O_2-demand but also shift coronary blood flow in favour of the subendocardial layers.

In conclusion, EMD 52692 is a potent arterial vasodilator with particularly pronounced effects on the cerebral vasculature. At doses exceeding the clinically relevant range, negative inotropic actions were not observed. The cardiovascular profile suggests that the drug may be useful in the treatment of hypertension and, in view of the lack of negative inotropic actions, of heart failure. The pronounced coronary, cerebral and mesenteric vasodilatation warrants investigations with this compound in syndromes of myocardial, cerebral and intestinal ischaemia.

The authors gratefully acknowledge Miss Marjo van Ee for her assistance in the preparation of this manuscript.

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(Received April 24, 1990
Revised June 21, 1990
Accepted June 25, 1990)

Br. J. Pharmacol. (1990), **101**, 615–620

Pharmacological and biochemical comparison of thyrotropin releasing hormone (TRH) and di-methyl proline-TRH on pituitary GH_3 cells

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1 The binding of [^{3}H]-thyrotropin releasing hormone ([^{3}H]-TRH) and [^{3}H]-RX77368 (di-methyl proline TRH) and the ability of these peptides to stimulate phosphoinositide hydrolysis were investigated in the GH_3 pituitary cell line.

2 For both peptides binding was found to be saturable with a single component (Hill slopes were, for TRH, 0.98 and for RX77368, 1.13). TRH bound with greater affinity than RX77368 K_d values were 16 nM and 144 nM respectively. B_{max} values were 227 fmol mg^{-1} protein for TRH and 123 fmol mg^{-1} protein for RX77368.

3 The rank order of potency of a series of TRH analogues to inhibit binding was the same versus each peptide. However, unlike with saturation analysis, Hill slopes of all displacing ligands were less than 1.0 against both TRH and RX77368 suggeting either multiple binding sites, alteration of affinity state, negative co-operativity or some allosteric interaction.

4 Both peptides stimulated phosphoinositide hydrolysis in a dose-dependent fashion. TRH was more potent than RX77368, EC_{50} values were 7.9 ± 1 nM and 96.3 ± 3 nM respectively.

5 These *in vitro* data suggest that the greater *in vivo* potency of RX77368 is not the result of enhanced receptor affinity but is more probably due to its greater metabolic stability.

Introduction

The tripeptide thyrotropin releasing hormone, TRH, (Figure 1) is widely distributed throughout the central nervous system. It exhibits a broad spectrum of stimulatory effects (Griffiths, 1987) which are probably mediated via receptors very similar to those moderating the endocrine function of the peptide in the pituitary gland (Burt & Taylor, 1980; Dettmar *et al.*, 1983a,b; Sharif *et al.*, 1983). TRH has been found capable of preventing neuronal damage (Freedman *et al.*, 1986; 1989), promoting recovery following spinal trauma (Faden *et al.*, 1981) and relieving weakness and spasticity in motor neurone disease (MND) (Engel *et al.*, 1983). However, its clinical potential in these areas has not been realised, possibly a consequence of the peptide's short biological half life due to its rapid metabolism by pyroglutamyl aminopeptidases and deamidase enzymes in body tissues and fluids (Bassiri & Utiger, 1981; Coggins *et al.*, 1987). RX77368 is one of several analogues synthesized in an attempt to overcome this lability. Here the substitution of two methyl groups at position 3 on the proline ring (Figure 1) renders the molecule much more resistant to enzymatic degradation (Brewster *et al.*, 1981; Griffiths *et al.*, 1982; Metcalf, 1983). RX77368, which has also been found beneficial for the treatment of MND (Guiloff *et al.*, 1987a,b), is endocrinologically equipotent to TRH but shows greater biological activity in several neuropharmacological tests (Dettmar *et al.*, 1980; 1981; 1983a; Metcalf *et al.*, 1981; Sharp *et al.*, 1984a,b). The latter has been attributed to the greater metabolic stability hence increased bioavailability of the analogue; however, other factors, for example active metabolites or enhanced potency at the receptor, may play a role. While there are no known active metabolites of RX77368, indeed much of a dose is excreted unchanged (Brewster *et al.*, 1981), there has to date been no data published concerning the direct binding characteristics of the drug, although Hawkins *et al.* (1986) and more recently Sharif *et al.* (1989) have shown indirectly that RX77368 has a lower affinity than TRH for mammalian spinal cord and brain receptors. Here we have had the unique opportunity of investigating the binding characteristics of tritiated RX77368 and comparing them to those of the parent compound in whole GH_3 pituitary cells, a clonal cell line having receptors for TRH that are coupled to phosphoinositide (PI) hydrolysis and prolactin release (Tashjian *et al.*, 1971; Drummond & Macphee, 1981; Sutton & Martin, 1982). We have also studied the ability of the peptides to stimulate PI hydrolysis in this cell line. A preliminary account of this work has already been published (McDermott *et al.*, 1989).

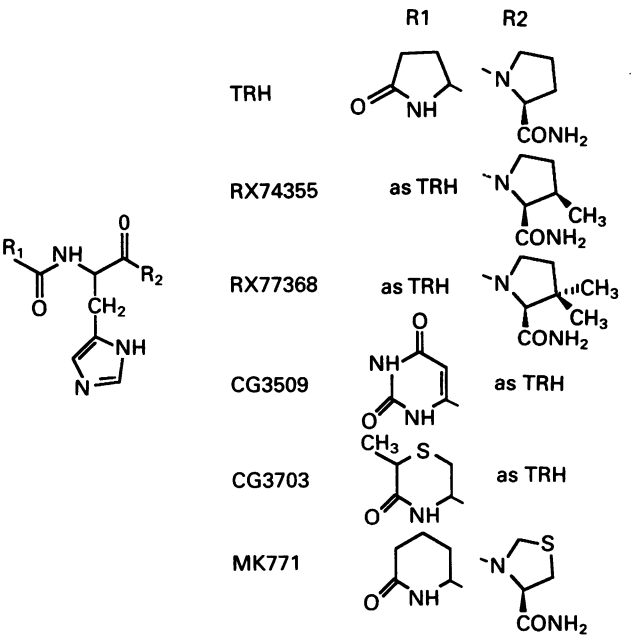

Figure 1 The structure of thyrotropin releasing hormone (TRH) and some of its analogues.

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Methods

Cell culture

GH_3 cells were routinely grown in monolayer culture in 10 cm dishes at 37°C, under 95% air/5% CO_2 in Ham's F10 culture medium supplemented with 15% donor horse serum, 2.5% foetal calf serum and 40 $\mu g\,ml^{-1}$ gentamicin. Cells were cultured to about 80% confluency and passaged using 0.05% trypsin in Tris/EDTA. For binding and PI hydrolysis studies, cells were seeded at a concentration of $10^5\,ml^{-1}$ on to 13 mm diameter glass coverslips coated with 0.5 $\mu g\,ml^{-1}$ poly-L-lysine in multiwell plates. The culture medium was changed every 3 days and the cells allowed to grow for 9–11 days before use.

Receptor binding studies

The growth medium was aspirated and the cells washed twice with 0.5 ml of Ham's F10 medium, supplemented with 25 mM HEPES, 5 mM magnesium sulphate, 14 mM sodium bicarbonate and 400 $\mu g\,ml^{-1}$ bovine serum albumin, pH 7.4. The cells were then incubated for up to 1 h at 37°C in a rotary shaker, in 0.5 ml of medium also containing either [^{3}H]-TRH or [^{3}H]-RX77368. Non-specific binding was determined by the addition of 100 μM TRH to the medium. The incubation was terminated by aspirating the medium and the cells were then washed 3 times with 1 ml of ice cold medium. The coverslips were then transferred to vials for scintillation counting.

Tritiated peptide stability studies

On the manufacturer's recommendation, paper chromatography followed by liquid scintillation counting was used to assess the stability of the tritiated ligands. Aliquots of medium containing radioligand that had been incubating with cells (with or without the presence of 40 $\mu g\,ml^{-1}$ bacitracin) for 1 h at either 37°C, ambient temperature or 4°C were compared to radioligand incubated without cells.

Phosphoinositide hydrolysis studies

Cells were pre-labelled for 2–3 days with 1 $\mu Ci\,ml^{-1}$ [^{3}H]-inositol. Under these conditions labelling of the inositol lipids has reached isotopic equilibrium (Drummond *et al.*, 1984; confirmed in our laboratory, data not shown). The growth medium was aspirated and the cells washed twice with 0.5 ml of medium, as described for binding studies but with the addition of 10 mM lithium chloride and a mixture of peptidase inhibitors routinely used in our laboratory (leupeptin 2 $\mu g\,ml^{-1}$, chymostatin 1 $\mu g\,ml^{-1}$ and bacitracin 40 $\mu g\,ml^{-1}$), then pre-incubated in 0.5 ml of this medium for 30 min at 37°C on a rotary shaker. Peptides were added in a volume of 10 μl to give the required final concentration, and the incubation continued for a further 15 min. The reaction was terminated by transferrence of the coverslips to vials containing 1.5 ml chloroform/methanol 2:1 (v/v) then adding 0.5 ml water. The mixture was vortexed then left to separate; 0.5 ml of the upper aqueous phase was removed for the determination of total inositol phosphates as described by Cholewinski *et al.* (1988) following the method of Berridge *et al.* (1982). An aliquot (200 μl) of the lower phase was removed, dried and counted for the estimation of [^{3}H]-inositol incorporation into phospholipids.

Protein estimation

Cellular protein content was determined by the method of Lowry *et al.* (1951).

Materials

Culture media and sera were from Imperial Laboratories. Antibiotics and tissue culture plasticware were from Flow Laboratories. All other tissue culture reagents and TRH were from Sigma. [1,2-^{3}H(N)]myo-inositol, [^{3}H]-inositol, (45–80 Ci $mmol^{-1}$) and [L-proline-2,3,4,5,^{3}H(N)]-pGlu-His-Pro-NH_2, [^{3}H]-TRH, (119.2 Ci $mmol^{-1}$) were purchased from New England Nuclear. GH_3 cells were a gift from Dr A. Drummond. pGlu-[2,5-$^3H_2$] His-3,3'dimethyl Pro-NH_2, [^{3}H]-RX77368, (32.7 Ci $mmol^{-1}$, purified and characterized by P.M. Taylor), RX77368 and RX74355 were provided by Reckitt and Colman. MK771 was provided by Merck Sharp and Dohme. CG3509 and CG3703 were from Chemie Grunenthal. All other reagents and solvents were of analytical grade.

Results

Receptor binding studies

Incubation of [^{3}H]-TRH or [^{3}H]-RX77368 with whole GH_3 cells resulted in a time-dependent increase in specific binding at 37°C (Figure 2). [^{3}H]-TRH bound more rapidly than [^{3}H]-RX77368. Maximal binding of both peptides was achieved by 60 min. The data obtained from experiments in which cells were incubated with various concentrations of [^{3}H]-TRH or [^{3}H]-RX77368 for 1 h, revealed that under these conditions binding of both peptides was saturable (Figure 3a,b). Non-specific binding was less than 35% for [^{3}H]-TRH and 50% for [^{3}H]-RX77368. Hanes analysis of the data indicated a single binding component for each peptide (Figure 4a,b). K_d and B_{max} values were, for TRH 16 nM and 227 fmol mg^{-1} protein, and for RX77368 144 nM and 123 fmol mg^{-1} protein. Hill coefficients were 0.98 and 1.13 for TRH and RX77368 respectively.

The ability of a number of TRH analogues (Figure 1) to inhibit binding was determined by incubating GH_3 cells with either [^{3}H]-TRH or [^{3}H]-RX77368 in the presence of various concentrations of analogue (Figure 5). Table 1 shows the concentrations of ligand required to displace 50% of the specific binding. The orders of potency of the analogues to inhibit binding were as follows: versus [^{3}H]-TRH: RX74355 > TRH >= MK771 > = CG3703 > RX77368 > CG3509; versus [^{3}H]-RX77368: RX74355 > = TRH > MK771 > =CG3703 > RX77368 > CG3509. Slopes calculated from Hill plots of the competition curves yielded values of less than 1 for all competing ligands and for both peptides (Table 1). These were confirmed by non-linear regression analysis (GraphPAD INPLOT).

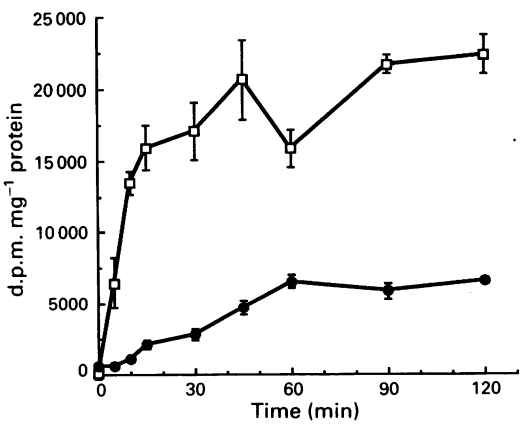

Figure 2 Time course of specific binding of [^{3}H]-thyrotropin releasing hormone ([^{3}H]-TRH, □) and [^{3}H]-dimethyl proline TRH ([^{3}H]-RX77368, ●) to GH_3 cells. The cells were incubated at 37°C with either [^{3}H]-TRH (5 nM) or [^{3}H]-RX77368 (50 nM) for various times. Maximal binding of both peptides was achieved by 60 min. Each value represents the mean of quadruplicate samples from a single experiment; vertical bars show s.e.mean. The discrepancy between actual d.p.m. values reflects the different specific activities of the two radioligands.

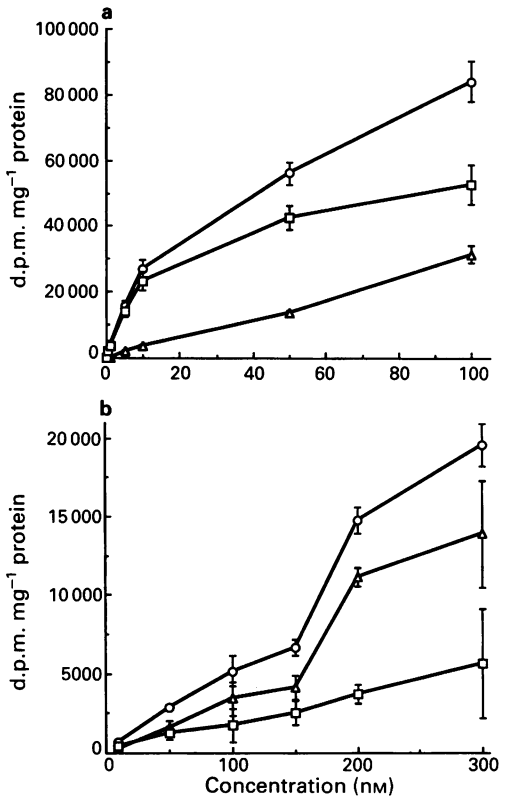

Figure 3 Saturation isotherms for (a) [^{3}H]-thyrotropin releasing hormone ([^{3}H]-TRH) and (b) [^{3}H]-dimethyl proline TRH ([^{3}H]-RX77368). Cells were incubated at 37°C for 1 h with various concentrations of radioligand alone (total binding ○) or in the presence of 100 μM cold TRH (non-specific binding △). Specific binding (□) was determined by subtracting non-specific from total binding. Each value represents the mean of quadruplicate samples from a single experiment; vertical bars show s.e.mean.

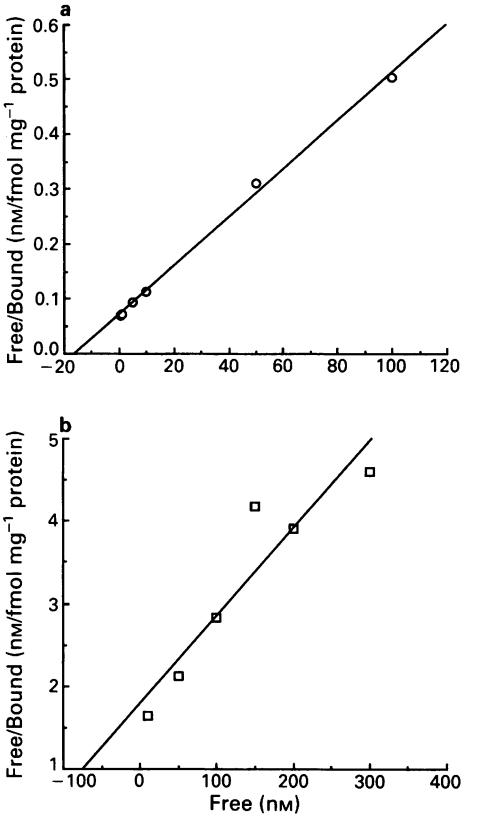

Figure 4 Hanes analysis of (a) [^{3}H]-thyrotropin releasing hormone ([^{3}H]-TRH) and (b) ([^{3}H]-dimethyl proline TRH ([^{3}H]-RX77368) binding data. Binding was saturable with a single component (correlation coefficients for TRH and RX77368 were 0.99 and 0.88 respectively). Each value represents the mean of quadruplicate samples from a single experiment. K_d and B_{max} values for TRH were 16 nM and 227 fmol mg^{-1} protein (one experiment) and for RX77368 144 nM and 123 fmol mg^{-1} protein (mean of two experiments).

Peptide stability studies

No significant differences in the proportion of counts occurring in a position equivalent to that of the cold ligand, were observed between radioligand that had been incubated with or without cells indicating that the peptides remained stable throughout the incubation conditions. Changes of temperature or presence of the peptidase inhibitor bacitracin had no effect.

Phosphoinositide hydrolysis studies

Both peptides stimulated inositol phosphate metabolism in GH_3 cells. The accumulation of [^{3}H]-phosphates rose in a linear fashion for at least 15 min (Figure 6) by which point a maximum of 16% of the [^{3}H]-inositol-containing phospholipids had been depleted. The response to stimulation by TRH and RX77368 was dose-dependent (Figure 7). TRH was more potent than RX77368, EC_{50} values were 7.9 ± 1 nM (mean ± s.e.mean) and 96.3 ± 3 nM respectively.

Table 1 Inhibition of [^{3}H]-thyrotropin releasing hormone ([^{3}H]-TRH) and [^{3}H]-dimethyl proline TRH ([^{3}H]-RX77368) binding to GH_3 cells by various TRH analogues

	[^{3}H]-TRH		[^{3}H]-RX77368	
	IC_{50}	Hill slope	IC_{50}	Hill slope
RX74355	2.0 ± 0.3	0.62	58 ± 21	0.55
TRH	23 ± 4	0.55	80 ± 16	0.40
MK771	30 ± 4	0.71	241 ± 41	0.42
CG3703	44 ± 5	0.60	316 ± 0	0.47
RX77368	295 ± 42	0.49	2009 ± 230	0.37
CG3509	1061 ± 218	0.42	2990 ± 172	0.31

Mean ± s.e.mean from 1 to 3 experiments in quadruplicates. Concentrations are expressed as nM.

Discussion

Saturation analysis revealed that TRH and its dimethyl analogue RX77368 each bind to a single site on GH_3 pituitary cells. The dissociation constant obtained for TRH was very similar to that previously reported by Hinkle & Tashjian (1973), although our B_{max} value was lower. The B_{max} value obtained for RX77368, although of a similar magnitude to that of TRH, was somewhat lower indicating the possibilities either that RX77368 binds only to a proportion of the total TRH-binding sites or that TRH binds to some site which is inaccessible to RX77368. The likelihood of two sites is not supported by the similarities of the Hill coefficients for the two ligands. The affinity of RX77368 was nine times lower than that of the parent compound, the latter agreeing well with results obtained by Hawkins *et al.* (1986) and Sharif *et al.* (1989) for the inhibition of [^{3}H]-methyl-TRH binding to spinal cord and brain membrane preparations. There was an excellent correlation between the rank order of potency of various analogues to inhibit [^{3}H]-TRH or [^{3}H]-RX77368 binding, strongly suggesting that RX77368 binds to the GH_3 cell TRH receptor. Remarkably however, despite saturation analysis indicating a single binding site, under these incubation conditions, the Hill slopes of all the competing ligands against TRH and against RX77368 were lower than 1. This is often taken as an indication that (a) multiple binding sites

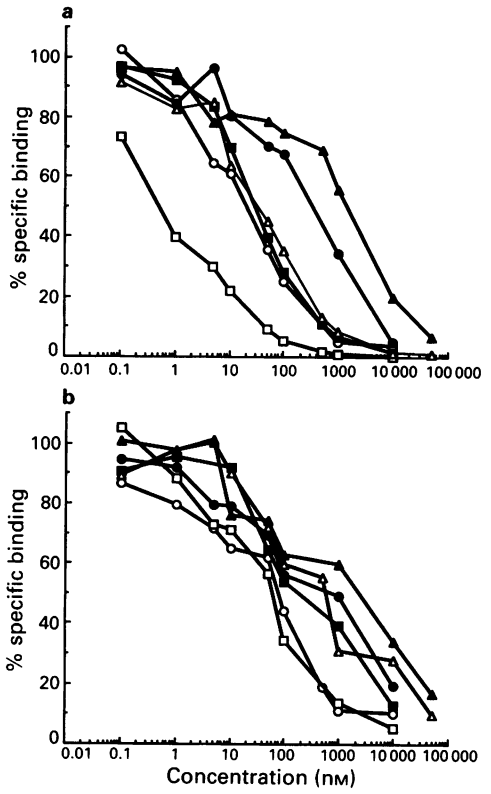

Figure 5 Inhibition of binding of (a) [^{3}H]-thyrotropin releasing hormone ([^{3}H]-TRH) and (b) [^{3}H]-dimethyl proline TRH ([^{3}H]-RX77368) by various TRH analogues. GH_3 cells were incubated at 37°C for 1 h with either [^{3}H]-TRH (5 nM) or [^{3}H]-RX77368 (50 nM) in the presence of various concentrations of TRH analogue. TRH (○), RX77368 (●), RX74355 (□), MK771 (■), CG3703 (△), CG3509 (▲). Values plotted for the inhibition of binding are the mean results of 3 determinations for [^{3}H]-TRH and of 2 determinations for [^{3}H]-RX77368.

exist, (b) there is negative co-operativity between binding sites or (c) some allosteric interaction has occurred. The latter two options are the least likely. Indeed, Hinkle *et al.* (1980) have previously shown that TRH binding is not co-operative. However, while it has been proposed that GH_3 cells possess both high affinity membrane bound receptors and possible low affinity uptake or intracellular sites (Gourdji *et al.*, 1973) the contribution of these sites in the present experiments was

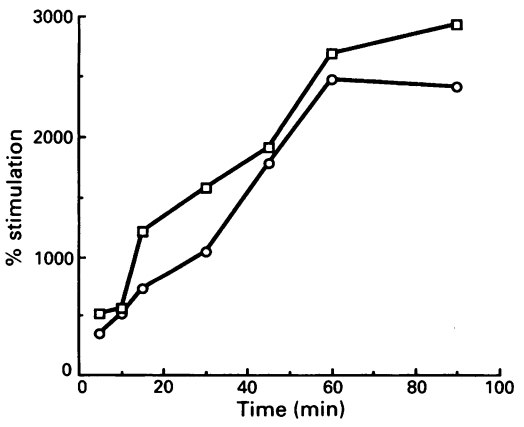

Figure 6 Time course of the accumulation of [^{3}H]-inositol phosphates in GH_3 cells stimulated by thyrotropin releasing hormone (TRH, ○) and dimethyl proline TRH (RX77368, □). Cells pre-labelled with [^{3}H]-inositol were incubated at 37°C with either 1 μM TRH or RX77368 for the times indicated. Total inositol phosphates were extracted and measured as described under Methods. Values plotted are the mean results from quadruplicate samples of a representative experiment. Two further experiments gave comparable results.

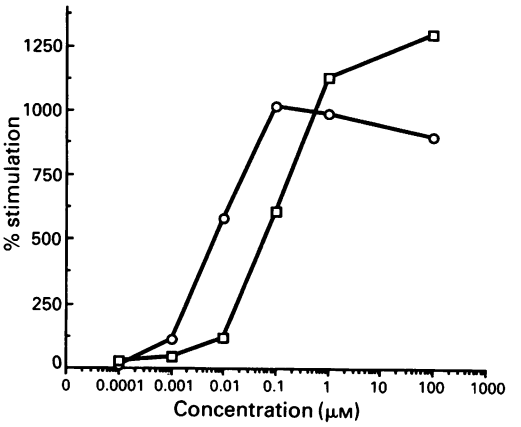

Figure 7 Dose-dependent stimulation of phosphoinositide hydrolysis in GH_3 cells by thyrotropin releasing hormone (TRH, ○) and dimethyl proline TRH (RX77368, □). Cells pre-labelled with [^{3}H]-inositol were incubated with various concentrations of TRH or RX77368 for 15 min at 37°C. Total inositol phosphates were extracted and measured as described under Methods. Values plotted are the mean results from quadruplicate samples from a representative experiment. Two further experiments gave comparable results.

thought to be minimised by the chosen incubation conditions, as indicated by their apparent lack of influence on the saturation curves. Nevertheless, the shape of the inhibition curves, as shown in Figure 5, in particular those using [^{3}H]-RX77368 as the radioligand, appear to be biphasic. Indeed, subsequent non-linear regression analysis indicated that the inhibition data could be better described by a two site rather than one site model. Similar anomalies between association (monophasic) and dissociation kinetics (biphasic) have been reported for rat amygdala membranes (Sharif & Burt, 1983) and GH_3 cells (Hinkle *et al.*, 1980). Also Hinkle & Kinsella (1982) have reported a temperature-dependent transformation of the TRH receptor complex in GH_4C_1 cells, another member of the GH family. In their studies, TRH was found to bind initially to a form of the receptor from which it dissociated rapidly, then over the course of 5 min the complex was found to be converted to a form with slow dissociation kinetics. Whilst it may be tempting to speculate about the existence of multiple TRH-receptor subtypes (as has been suggested in brain tissue, Funatsu *et al.*, 1985), given that the available ligands for this receptor are all agonists, the biphasic nature of the competition curves could equally well result from agonist-induced affinity changes and receptor kinetics. Indeed, other workers (Hawkins *et al.*, 1987) have questioned the existence of more than one TRH-receptor subtype.

It is interesting to note, from a structure-activity point of view, that the order of potency of the displacing ligands seems not to be related to simple structural modifications of the parent compound. This is demonstrated by the relative K_i values for RX77368 and RX74355, the monomethyl proline derivative, which are of very different magnitudes. Similarly, those of CG3703 and CG3509, which are modifications at the opposite N-terminal end of the tripeptide are very different. The data suggest that both ends of the tripeptide are functionally important and yet small structural changes at either end can have large effects on potency.

Both peptides were found to stimulate PI hydrolysis in a dose-dependent fashion, with TRH being twelve times more potent than RX77368. The EC_{50} value for TRH is similar to that obtained by Hinkle & Tashjian (1973) for prolactin release, the functional correlate for TRH receptor stimulation in this cell line. It is worth noting that the EC_{50}s for both TRH and RX77368 are very similar to their dissociation constants. This may imply little if any TRH receptor reserve in these cells. Also, it is of interest that, in at least 6 separate experiments in which TRH-stimulated PI turnover had plateaued, RX77368 was capable of achieving a still greater response. Although these intra-experimental findings are con-

sistent within cell-matched experiments carried out at the same time, a statistical difference in efficacy is difficult to show due to inter-experimental variation. Possible differences in efficacy may indicate that TRH is only a partial agonist at its own receptor in GH_3 cells, that TRH may regulate, or an active metabolite inhibit, the PI system or, less likely, that TRH is inactivated by a selective uptake process. Differences in metabolism can be ruled out as the experiments were carried out in the presence of peptidase inhibitors.

The reduced potency of RX77368 compared with TRH on GH_3 cells, may be a direct result of the presence of two methyl groups on the proline ring, since RX74355, the monomethyl derivative, has greater affinity than the parent compound, as indicated by the inhibition studies, and was found to be four times more potent than TRH at stimulating PI turnover (unpublished observation).

In conclusion, the greater *in vivo* potency of RX77368 as established in earlier studies (Dettmar *et al.*, 1980; 1981; Metcalf *et al.*, 1981) cannot be explained, *in vitro*, by greater affinity or potency at the TRH receptor but is more likely to be due to its enhanced bioavailability, as a result of its reported increased resistance to enzymatic degradation (Brewster *et al.*, 1981; Griffiths *et al.*, 1982; Metcalf, 1983).

A.M.McD. is an SERC-CASE student in association with Reckitt & Colman. We thank Dr G. Schofield for the use of his tissue culture facilities, Dr G.W. Bennett for the supply of some materials and Dr D.J. Nutt for helpful discussions.

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(Received May 2, 1990
Revised June 28, 1990
Accepted July 11, 1990)

Br. J. Pharmacol. (1990), **101**, 621–624

Characterization of histamine H_3-receptors in guinea-pig ileum with H_3-selective ligands

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1 The effect of the selective histamine H_3-receptor agonist **R**-(α)-methylhistamine has been investigated on the contractile responses of the longitudinal smooth muscle of guinea-pig ileum elicited by electrical field stimulation of acetylcholine release from myenteric nerve endings.

2 **R**-(α)-methylhistamine produced a concentration-dependent ($EC_{50} = 1.4 \pm 0.2 \times 10^{-8}$ M) inhibition of the response to electrical field stimulation which was insensitive to inhibition by mepyramine (1 μM) and tiotidine (2.4 μM).

3 This response to **R**-(α)-methylhistamine could be inhibited in a competitive fashion by a range of H_3-receptor antagonists including thioperamide ($K_B = 1.1$ nM), impromidine ($K_B = 65$ nM), norburimamide ($K_B = 380$ nM) and SKF 91486 ($K_B = 34$ nM). Burimamide was also a potent inhibitor of this response but the Schild slope obtained (1.3) was significantly greater than unity.

4 The estimated K_B values were all within a factor of three of those values reported for the histamine H_3-receptor mediating inhibition of histamine release in rat cerebral cortex.

5 These data suggest that the histamine receptor mediating inhibition of cholinergic neurotransmission by **R**-(α)-methylhistamine in guinea-pig ileum is the same as the H_3-receptor present in rat cerebral cortex.

Introduction

Histamine receptors have been divided into three major subtypes (H_1, H_2 and H_3) on the basis of quantitative *in vitro* studies in isolated tissues (Hill, 1987; 1990). In guinea-pig ileum, histamine produces a well characterized contractile response via H_1-receptor stimulation (Arunlakshana & Schild, 1959; Ash & Schild, 1966; Hill & Young, 1981; Hill, 1990). However, there is evidence to suggest that H_2- (Barker & Hough, 1983) and H_3- (Trzeciakowski, 1987) receptors may additionally be present in this tissue and can modulate neurotransmitter-induced smooth muscle contraction. Thus, H_2-receptor stimulation has been shown to induce the release of contractile substances from the myenteric plexus of guinea-pig ileum (Barker & Hough, 1983), whilst a novel receptor resembling the H_3-subtype has been implicated in the inhibitory effect of histamine on the contractile response to electrical field stimulation in this tissue (Ambache *et al.*, 1973; Fjalland, 1979; Trzeciakowski, 1987).

The characterization and definition of histamine H_3-receptors were originally performed in slices of rat cerebral cortex where these receptors perform an autoreceptor function in inhibiting neurotransmitter release from histamine-containing nerve endings (Arrang *et al.*, 1983; 1985). Several existing H_1- and H_2-receptor ligands are particularly effective as antagonists of this response including the potent H_2-agonist impromidine (Arrang *et al.*, 1983), which was the compound used to define the novel H_3-like receptor regulating cholinergic neurotransmission in myenteric plexus-containing preparations of guinea-pig ileum (Trzeciakowski, 1987).

Recently, the identification of H_3-receptor responses has been facilitated by the development of H_3-selective agonists and antagonists (Arrang *et al.*, 1987). These compounds have been particularly useful in detecting the presence of H_3-receptors on cholinergic postganglionic vagal nerve endings in human bronchi and guinea-pig trachea (Ichinose *et al.*, 1989; Barnes & Ichinose, 1989; Ichinose & Barnes, 1989). The aim of the present investigation was to characterize the receptor responsible for the inhibitory effects of histamine on cholinergic neurotransmission in myenteric plexus-containing guinea-pig ileal preparations by use of the H_3-selective agonist **R**-(α)-methylhistamine and the H_3-antagonist thioperamide.

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Methods

Transmural electrical stimulation of guinea-pig ileal segments

Guinea-pigs (200–400 g, either sex) were killed by cervical dislocation. The ileum was removed and placed in Krebs Henseleit solution (composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgCl$ 1.2, $NaHPO_4$ 1.2, $NaHCO_3$ 25 and glucose 5.5, pH 7.4) gassed with O_2/CO_2 (95:5). A portion of the whole ileum (5 cm segments) was then mounted on a coaxial electrode assembly in a water jacketed organ bath such that one electrode protruded into the lumen and the other was located outside the lumen of the ileum. The electrodes were 12 mm apart. The tissue was then equilibrated in 50 ml Krebs Heseleit medium, continously gassed with O_2/CO_2 (95:5) for 1 h at 37°C before experimentation. The preparation was electrically stimulated with a submaximal voltage (chosen to produce 80–90% of the maximal stimulus) at 0.1 Hz and a pulse width of 1 ms; contractile responses of the longitudinal smooth muscle were recorded isotonically. The stimulus voltage varied between 2 and 5 V with a mean value of 2.3 ± 0.1 V ($n = 32$). With these stimulus parameters, the discrete twitch contractile responses elicited by electrical stimulation were mediated via the release of acetylcholine and could be completely inhibited by the addition of 1 μM atropine.

Cumulative concentration-response curves for inhibition of electrically stimulated contractions were determined for the H_3-selective antagonist **R**-(α)-methylhistamine in the presence and absence of increasing concentrations of H_3-receptor antagonists. Antagonists were allowed to equilibrate with the tissue for 30 min before the redetermination of agonist concentration-response curves. In most experiments mepyramine (1 μM) was added at the beginning of the experimental period in order to prevent activation of histamine H_1-receptors (and hence contractile responses to H_3-receptor agonists at the high concentrations used in the presence of H_3-antagonists).

Longitudinal smooth muscle-myenteric plexus preparation

Longitudinal smooth-muscle strips containing adherent myenteric plexus were prepared essentially as described by Rang (1964). Muscle strips were suspended between two parallel platinum electrodes (5 mm apart) in Krebs Henseleit medium

and equilibrated for 60 min prior to agonist administration. Contractile responses were elicited at a frequency of 0.1 Hz by electrical field stimulation at submaximal voltages (80–90% of maximal stimulus; 8–15 V) with a pulse width of 1 ms. The effect of R-(α)-methylhistamine on this response was determined as described above in the presence and absence of 10 nM thioperamide. As with the ileal segment preparation, the contractile response to electrical field stimulation of the longitudinal muscle strips was abolished by 1 μM atropine.

In some experiments the effect of R-(α)-methylhistamine was determined on the contractile response elicited by a submaximal concentration of carbachol (1 μM). R-(α)-methylhistamine (0.1 μM) was added to the preparation 5 min before reapplication of carbachol. In each preparation, at least three pairs of measurements were made in the presence and absence of R-(α)-methylhistamine. Carbachol was in contact with the tissue for 15–25 s and applied at 5 min intervals.

Data analysis

Concentration-response curves for R-(α)-methylhistamine were fitted to a Hill equation using the programme Graphpad (ISI). The equation fitted was:

$$\% \text{ of maximal response} = \frac{E_{max} \times D^n}{D^n + (EC_{50})^n}$$

where D is the agonist concentration, n is the Hill coefficient, EC_{50} is the concentration of agonist giving half-maximal response and E_{max} is the maximal response. Dissociation constants for antagonists were obtained from the parallel shifts of the log concentration response curve for R-(α)-methylhistamine using the relationship:

$$\text{dose-ratio} = A/K_B + 1$$

where A is the concentration of antagonist, K_B is the antagonist dissociation constant and the dose-ratio is the ratio of the concentration of agonist necessary to give a specified response in the presence of antagonist to the concentration of agonist required for the same response in the absence of antagonist. Where the data were adequate (i.e. in experiments in which three concentrations of antagonist were used) the dose-ratios obtained were used to determine Schild slopes (m) and K_B values by unweighted linear regression of the Schild equation (Arunlakshana & Schild, 1959):

$$\log (\text{dose-ratio} - 1) = m \times \log A - \log KB$$

Data are expressed as mean ± s.e.mean and *n* in the text and figure legends represents the number of experiments performed on different ileal preparations (i.e. separate animals).

Drugs

Mepyramine maleate and atropine were purchased from Sigma. Carbachol chloride was obtained from BDH and pentolinium tartrate was from May & Baker. Gifts of R-(α)-methylhistamine, thioperamide, burimamide, impromidine, norburimamide (SKF 91582; N-methyl-N'-[3-(imidazol- 4-yl)-propyl]thiourea), SKF 91486 (3-(imidazole-4-yl)- propylguanidine sulphate) (all from Smith Kline Beecham) and tiotidine (ICI) are gratefully acknowledged.

Results

Inhibition by R-(α)-methylhistamine

R-(α)-methylhistamine produced a marked inhibition of the twitch contractions elicited by 0.1 Hz electrical transmural stimulation of segments of guinea-pig ileum (EC_{50} 6.7 ± 1.8 nM, maximal inhibition = 88.9 ± 3.7%, *n* = 4). R-(α)-methylhistamine appeared to be mediating its effect by inhibiting the release of acetylcholine from presynaptic myenteric nerve terminals since it had no effect on the contractile response to submaximal concentrations of exogenously applied carbachol (*n* = 3; data not shown).

The H_1-receptor antagonist mepyramine (1 μM) did not alter the concentration-response curve to R-(α)-methylhistamine (Figure 1) and was routinely added into the Krebs-Henseleit medium for all subsequent determinations in order to prevent the appearance of H_1-receptor-mediated contractile responses to the H_3-agonist at very high concentrations. In the presence of mepyramine the average reduction in the size of the transmural response was 87.7 ± 1.6% (*n* = 39). A combination of mepyramine and the H_2-antagonist tiotidine (2.4 μM) was similarly without effect on the response to R-(α)-methylhistamine (Table 1).

Inclusion of the ganglion blocker pentolinium (4 μM) did not alter the inhibitory potency of R-(α)-methylhistamine (Table 2) but it did reduce the size of the transmurally-stimulated contractions by 42.5 ± 1.9% (*n* = 6). These data suggest that approximately 40% of the response to electrical stimulation in this preparation is due to activation of preganglionic fibres. A similar finding has been reported previously by Blair-West *et al.* (1967).

H_3-receptor antagonists

The H_3-antagonist thioperamide produced a parallel shift in the concentration-response curve to R-(α)-methylhistamine (Figure 2). Schild analysis of the data obtained in each of six individual experiments yielded a value for the Schild slope (1.1 ± 0.1) which was compatible with thioperamide acting as a competitive antagonist. The mean dissociation constant obtained for thioperamide (1.1 ± 0.3 nM; *n* = 6) was similar to that obtained by Arrang *et al.* (1987) for antagonism of histamine H_3-receptors in rat cerebral cortical slices (Table 2). A similar value was obtained for thioperamide in guinea-pig

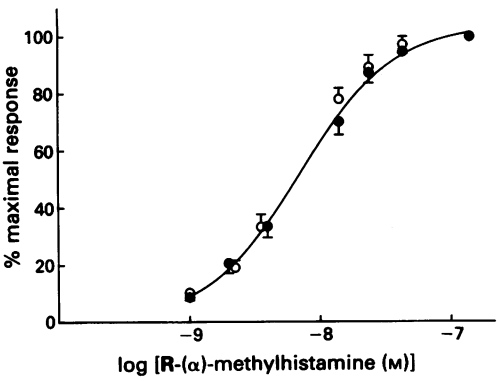

Figure 1 The effect of mepyramine on the inhibition of electrically-induced contractions of guinea-pig ileal segments elicited by R-(α)-methylhistamine. Control (●); mepyramine (1 μM; ○). Values represent mean of four determinations, with s.e.mean shown by vertical bars. Where not shown, s.e.means lie within the symbol. Data are expressed as a percentage of the maximal inhibitory response to R-(α)-methylhistamine obtained in the absence of mepyramine in each experiment.

Table 1 EC_{50} values for R-(α)-methylhistamine for inhibition of electrically-induced contractions of guinea-pig ileum in the presence of various antagonists

Antagonist	*EC_{50}* (nM)	(n)
None	6.7 ± 1.8	(4)
Mepyramine (1 μM)	14.0 ± 2.0	(39)
+ Tiotidine (2.4 μM)	19.0 ± 7.0	(5)
+ Pentolinium (4 μM)	21.0 ± 5.0	(8)

Values represent mean ± s.e.mean of *n* separate experiments.

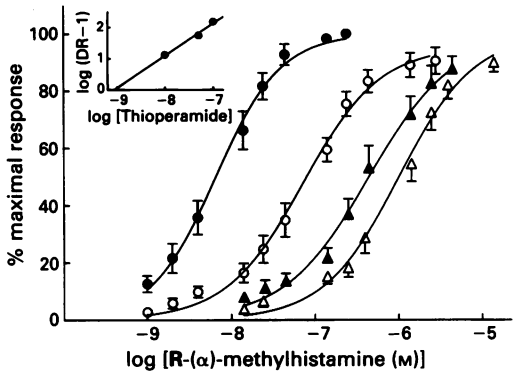

Figure 2 Antagonism by thioperamide of the inhibitory effect of R-(α)-methylhistamine on electrically-induced contractions of guinea-pig ileal segments. Concentration-response curves were determined in the absence (●) and presence of 10 nM (○), 50 nM (▲) and 100 nM (△) thioperamide. Mepyramine (1 μM) was present for every measurement. Values represent mean of six separate determinations; with s.e.mean shown by vertical bars. Data are expressed as a percentage of the maximal response to R-(α)-methylhistamine in the absence of thioperamide. Concentration-response curves were fitted by non-linear regression using the programme Graphpad. The inset shows a Schild plot of the composite data where DR represents the dose-ratio. The line through the data points has a slope of 1.03 (Schild slope) and intercepts the x axis at −9.1 (log K_B).

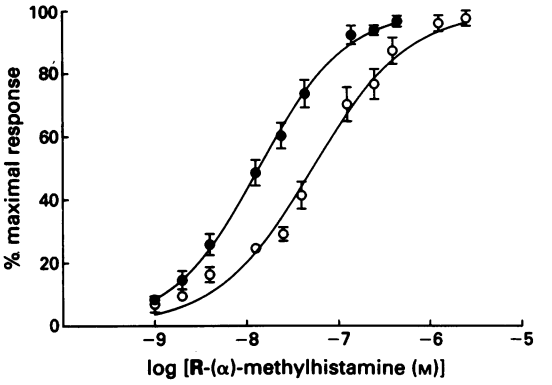

Figure 3 Antagonism by thioperamide of the inhibitory effect of R-(α)-methylhistamine on contractions elicited by electrical field stimulation of longitudinal smooth muscle strips containing adherent myenteric plexus. (●) Control; (○) 10 nM thioperamide. Values represent the mean of seven separate determinations with s.e.mean shown by vertical bars. Data are expressed as a percentage of the maximal inhibitory response to R-(α)-methylhistamine in the absence of thioperamide in each experiment.

ileum (K_B = 3.0 ± 0.9 nM; Schild slope = 1.2 ± 0.1; n = 5) in the presence of pentolinium (4 μM).

A close similarity between the dissociation constants obtained in guinea-pig ileum (Table 2) and rat cerebral cortex was also obtained with impromidine, burimamide, norburimamide and SKF 91486. For the experiments with impromidine, the H_2 antagonist tiotidine (2.4 μM) was routinely added to the incubation medium in order to antagonize any H_2-receptor-mediated contractile activity (Barker & Hough, 1983) elicited by impromidine. The Schild slope obtained for burimamide (1.3; Table 2) was significantly greater than unity ($P < 0.05$) and caution should therefore be exercised in the interpretation of the apparent K_B value determined for this particular antagonist in the present study. The values obtained for the other antagonists were, however, consistent with competitive antagonism.

Thioperamide (1×10^{-8} M) was also able to antagonize the inhibitory effect of R-(α)-methylhistamine (EC_{50} = 1.9 ± 0.3 $\times 10^{-8}$ M) on the response of longitudinal smooth muscle-myenteric plexus preparations to electrical field stimulation (Figure 3). The K_B value obtained for thioperamide in this preparation (K_B = 3.2 ± 0.7 nM; n = 7) agreed well with that obtained in ileal segments (Table 2).

Table 2 Dissociation constants (K_B) obtained for antagonists of R-(α)-methylhistamine-induced inhibition of contractile responses to electrical stimulation in guinea-pig ileum

	Guinea-pig ileum			*Rat brain*†
Antagonist	K_B (nM)	*Schild slope*		K_B (nM)
Thioperamide	1.1 ± 0.3	1.1 ± 0.1	(6)	1.1
Impromidine	65 ± 8	1.2 ± 0.1	(6)	32
Burimamide	29 ± 5	1.3 ± 0.1*	(6)	32
SKF 91486	34 ± 13	1.0 ± 0.1	(4)	88
Norburimamide	380 ± 90	1.3 ± 0.1	(3)	
Mepyramine[1]	>1000		(4)	>58
Tiotidine[1]	>2400		(5)	>12,000

Values represent mean ± s.e.mean. The number of separate determinations is given in parentheses. All measurements were made in the presence of 1 μM mepyramine (except for the measurements with mepyramine alone). Tiotidine (2.4 μM) was present in the incubation medium during the studies with impromidine.

† Data taken from Arrang *et al.* (1983, 1987).

[1] No inhibition observed with 1 μM mepyramine or 2.4 μM tiotidine.

* Schild slope significantly different from unity ($P < 0.05$; t test).

Discussion

The present study, using H_3-receptor-selective ligands, has confirmed previous suggestions regarding the existence of a non-H_1-, non-H_2-receptor-mediated inhibitory response to histamine analogues in myenteric plexus-containing preparations of guinea-pig ileum (Ambache *et al.*, 1973; Fjalland, 1979; Trzeciakowski, 1987). The selective H_3-agonist R-(α)-methylhistamine was able to inhibit contractions elicited by transmural electrical stimulation of guinea-pig ileum with an EC_{50} (14 nM) similar to that (4 nM) determined from inhibition of [^{3}H]-histamine release from rat cerebral cortical slices (Arrang *et al.*, 1987). The response to R-(α)-methylhistamine was not antagonized by a combination of the potent H_1- and H_2-antagonists mepyramine (1 μM) and tiotidine (2.4 μM), but was inhibited by the selective H_3-receptor antagonist thioperamide, in a concentration-dependent and competitive manner.

The response to R-(α)-methylhistamine was also antagonized by a range of other compounds including impromidine (an H_2-agonist and H_3-antagonist; Ganellin, 1982; Arrang *et al.*, 1983) and burimamide, norburimamide and SKF 91486 (which are all weak H_2-antagonists and rather more potent H_3-antagonists; Ganellin, 1982; Arrang *et al.*, 1983). Burimamide (H_2-receptor, K_B = 7,000), SKF 91486 (H_2-receptor, K_B = 22,000 nM) and norburimamide (H_2-receptor, K_B = 115,000 nM) are at least two orders of magnitude more potent as inhibitors of the ileal response to R-(α)-methylhistamine than they are as H_2-receptor antagonists (Table 2; Ganellin, 1982). In contrast, the K_B values obtained in guinea-pig ileum for all of the H_3-antagonists measured lie within a factor of three of the values determined against the H_3-receptor-mediated inhibition of histamine release in rat cerebral cortex (Arrang *et al.*, 1983; 1987). These data provide strong support for the contention that the receptors mediating responses to R-(α)-methylhistamine in these two tissues are identical.

Trzeciakowski (1987) has previously suggested that the H_3-receptors in guinea-pig ileum are located on presynaptic postganglionic cholinergic nerve terminals. The results obtained in the present study support this contention since inhibition of cholinergic ganglion transmission with pentolinium did not alter the pharmacological characteristics of the

inhibitory H_3-receptor response, while R-(α)-methylhistamine was without effect on contractile responses to exogenously applied carbachol. An H_3-receptor-mediated response could also be demonstrated in isolated longitudinal smooth muscle preparations which contain adherent myenteric plexus. These data confirm that H_3-receptors are located on cholinergic terminals derived from this neuronal network rather than the submucosal plexus.

It was notable, however, that pentolinium was able to reduce the contractile response to transmural electrical stimulation by some 40% indicating the presence of a significant activation of preganglionic cholinergic fibres. A similar finding has been described by Blair-West *et al.* (1967). This finding, taken together with the observation that R-(α)-methylhistamine can inhibit nearly 90% of the transmural response (in the absence of pentolinium) raises the possibility that inhibitory H_3-receptors may also be present on the preganglionic cholinergic nerve terminals. Unfortunately, this possibility cannot be resolved with the present experimental set-up since the presence of the H_3-receptors 'down stream' of the ganglia on the post-ganglionic cholinergic terminals is sufficient to inhibit the 'final' contractile response to electrical stimulation of both pre- and postganglionic fibres. Evidence for the presence of H_3-receptors on presynaptic terminals of nicotinic cholinergic synapses has been provided, however, by electrophysiological measurements of excitatory postsynaptic potentials in myenteric neurones from guinea-pig ileum (Tamura *et al.*, 1988).

In summary, this study has provided strong evidence that the novel histamine receptor mediating inhibition of cholinergic neurotransmission in guinea-pig ileum is identical to the histamine H_3-receptor in rat cerebral cortex. The guinea-pig ileal preparation should therefore prove to be an excellent system for the characterization of H_3-receptor function.

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(Received May 30, 1990
Accepted June 29, 1990)

Br. J. Pharmacol. (1990), **101**, 625–631

Regional and cardiac haemodynamic effects of N^G-nitro-L-arginine methyl ester in conscious, Long Evans rats

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1 Regional haemodynamic responses to i.v. bolus doses (0.1–10.0 mg kg^{-1}) of N^G-nitro-L-arginine methyl ester (L-NAME) were measured in conscious, Long Evans rats ($n = 8$) chronically instrumented with renal, mesenteric and hindquarters pulsed Doppler flow probes and intravascular catheters.

2 L-NAME caused dose-dependent pressor effects associated with renal, mesenteric and hindquarters vasoconstrictions. The mesenteric vascular bed showed earlier onset with more rapid, and greater, maximum vasoconstrictions than the renal or hindquarters vascular beds; however, the hindquarters vasoconstriction was more persistent. D-NAME was without significant effects ($n = 2$).

3 Primed infusion of L-arginine (100 mg kg^{-1} bolus followed by 100 mg kg^{-1} h^{-1} infusion), starting 10 min after an i.v. bolus injection of L-NAME (10 mg kg^{-1}), caused significant reversal of the pressor responses, and renal and mesenteric vasoconstrictions, but not of the hindquarters vasoconstriction. Primed infusions of L-arginine (100 mg kg^{-1}, 100 mg kg^{-1} h^{-1}) starting 5 min after L-NAME (1 mg kg^{-1}) additionally caused some reversal of the hindquarters vasoconstriction, but this effect was transient.

4 Primed infusion of L-arginine (100 mg kg^{-1}, 100 mg kg^{-1} h^{-1}) starting 30 min before i.v. bolus injection of L-NAME (10 mg kg^{-1}) caused significant attenuation of the pressor effects and the renal and mesenteric vasoconstrictions but not of the hindquarters vasoconstriction.

5 In a separate group of rats ($n = 8$) chronically instrumented with thoracic aortic electromagnetic flow probes for the measurement of cardiac haemodynamics, i.v. bolus injection of L-NAME (10 mg kg^{-1}) produced significant reductions in total peripheral conductance, cardiac output, stroke volume, peak thoracic aortic flow and the maximum rate of rise of aortic flow; these were coincident with the maximum pressor and vasoconstrictor effects.

6 These results, collectively, are consistent with L-NAME interfering with L-arginine-nitric oxide pathways that have important influences on regional vascular conductances *in vivo*. The pressor effect resulting from L-NAME-induced vasoconstrictions is offset by a substantial reduction in cardiac function that may depend on direct and/or indirect effects of L-NAME on the heart.

Introduction

Nitric oxide is the major endothelium-derived relaxing factor (see Moncada *et al.*, 1989). Inhibition of nitric oxide biosynthesis *in vivo* by intravenous (i.v.) administration of N^G-monomethyl-L-arginine (L-NMMA) causes increases in blood pressure in anaesthetized rabbits (Rees *et al.*, 1989) and guinea-pigs (Aisaka *et al.*, 1989). In conscious rats, i.v. administration of L-NMMA causes dose-dependent hypertension and widespread vasoconstrictions (renal, superior mesenteric, hindquarters and internal carotid vascular beds), although the patterns of change in vascular conductances are different in the different regions (Gardiner *et al.*, 1989b; 1990d,e,g; Bennett *et al.*, 1990). The *in vivo* vasoconstrictor effects seen following administration of L-NMMA are not observed after injection of D-NMMA, and the effects of L-NMMA are partially reversed by L-arginine but not by D-arginine (Bennett *et al.*, 1990; Gardiner *et al.*, 1990e). These results are consistent with nitric oxide generated from L-arginine being involved in the control of regional vascular conductances under basal conditions in conscious animals.

It is now known that analogues of arginine other than L-NMMA can influence endothelium-dependent vasorelaxation *in vitro*. Indeed, Moore *et al.* (1990) have found that N^G-nitro-L-arginine inhibits more potently the vasodilator effects of acetylcholine in the perfused, pre-constricted superior mesenteric vascular bed from the rat than does L-NMMA. However, there are no published data on the *in vivo* cardiovascular effects of N^G-nitro-L-arginine, but obviously such information is important for our understanding of the involvement of the L-arginine-nitric oxide system in cardiovascular regulation. Since N^G-nitro-L-arginine is a relatively insoluble compound, we investigated the effects of the highly soluble N^G-nitro-L-arginine methyl ester (L-NAME; Moore *et al.*, 1990) in conscious rats instrumented for monitoring regional or cardiac haemodynamics. Some of the results have been presented to the Physiological Society (Gardiner *et al.*, 1990f).

Methods

All studies were carried out on male, Long Evans rats (350–450 g) bred in the Animal Unit at Nottingham.

Regional haemodynamic measurements

Animals were anaesthetized (sodium methohexitone, 60 mg kg^{-1} i.p., supplemented as required) and, through a mid-line incision, miniaturized pulsed Doppler probes (Haywood *et al.*, 1981) were sutured 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). The probe wires ran from the abdominal cavity, through a small incision in the left flank, and then subcutaneously to emerge at the back of the neck where they were anchored to the skin by a suture. Following closure of the incisions all animals received an intramuscular injection of ampicillin (Penbritin, Beecham, 7 mg kg^{-1}) and were returned to their home cages for 7–14 days with free access to food and water. After this time they were briefly re-anaesthetized (sodium methohexitone, 40 mg kg^{-1} i.p.) and had intravenous (left jugular vein) and intra-arterial (distal abdominal aorta via ventral caudal artery) catheters implanted. The catheters were then run subcutaneously to emerge at the back of the neck at the same point as the probe wires. The latter were soldered

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into a 6-way micro-connector (Microtech Inc., Boothwyn, U.S.A.) that was clamped into a home-made harness worn by the rat. The catheters ran through a flexible spring connected to the harness, and a connector for the lead to the pulsed Doppler flowmeter ((Crystal Biotech, Massachussetts) modified to a pulse repetition frequency of 125 kHz (Gardiner et al., 1990c)) was taped to the spring. The latter was supported by a counterbalanced, universally-jointed lever, thereby giving the animal unrestrained movement in its home cage. Free access to water and food was allowed throughout the studies which began the following day when animals were fully conscious.

Baseline measurements were made for at least 30 min before any intervention was started. Continuous recordings (on a Gould ES 1000 recorder) of phasic arterial blood pressure, mean blood pressure (electronically derived from the phasic signal), instantaneous heart rate, phasic Doppler shift signals and mean Doppler shift signals (electronically derived from the former) were made before, during and after all interventions. At selected time points, percentage changes in mean Doppler shift signals were calculated to give an index of blood flow change relative to baseline (Haywood et al., 1981) and at those times percentage changes in regional vascular conductances were calculated from the mean Doppler shift signals and mean blood pressure (i.e. mean Doppler shift/mean blood pressure).

Cardiac output measurements

Animals were anaesthetized as above and an electromagnetic flow probe (Skalar, Delft, Netherlands) was implanted around the ascending thoracic aorta by the technique of Smith & Hutchins (1979) as modified by Smits et al. (1982). The probe lead was run subcutaneously and the plug was securely positioned at the back of the neck. Animals were given an intramuscular injection of ampicillin (Penbritin, 7 mg kg^{-1}) and returned to their home cages with free access to food and water for at least 7 days. After this time animals were briefly re-anaesthetized (as above) and had intravenous (left jugular vein) and intra-arterial (distal abdominal aorta via ventral caudal artery) catheters implanted. One intravenous catheter (i.d. 0.28 mm) was advanced until the tip lay close to the junction of the superior vena cava with the right atrium (as judged by the pressure waveform). This catheter was used for monitoring central venous pressure and, in order to ensure the quality of the recording was not impaired by the formation of blood clots around its tip, a continuous infusion (0.3 ml h^{-1}) of sterile saline (154 mM NaCl) was given through it via a fluid-filled swivel until the experiment started the next day.

Continuous recordings (on a Gould ES 1000) of phasic arterial blood pressure, mean central venous pressure, phasic and mean thoracic aortic blood flow (Skalar MDL 1401 flowmeter) and instantaneous heart rate were made. These signals were also fed into a microprocessor (designed and built in the Departments of Pharmacology and Instrument Services, University of Limburg, Netherlands) interfaced with a microcomputer (Tandon 386). This system digitized and averaged data over 2 s epochs and also derived values for mean blood pressure, stroke volume, total peripheral conductance, maximum rate of rise of thoracic aortic flow ($+dF/dt_{max}$) and peak aortic flow; the latter two variables are indices of ventricular contractility (Schoemaker, 1989). All data were stored on disc for subsequent review and analysis. The values given in the text represent means of up to 20 observations (i.e. over a 40 s measurement period). As with the regional haemodynamic studies baseline recordings were made for at least 30 min to ensure animals were in a steady state before any interventions were carried out.

Experimental protocols

Experiment 1: Regional and cardiac haemodynamic effects of L-*NAME* Rats ($n = 8$) instrumented for recording renal, mesenteric and hindquarters blood flows were given i.v. bolus (0.1 ml) injections of L-NAME (0.1, 1.0 and 10 mg kg^{-1}). The doses were given in ascending order with at least 60 min between the first and second dose and at least 90 min between the second and third. An additional two animals were given D-NAME (10 mg kg^{-1}).

A separate group of rats ($n = 8$) instrumented for measurement of cardiac haemodynamics was given L-NAME at a dose of 10 mg kg^{-1}.

Experiment 2: Effects of post-treatment with L-*arginine on the regional haemodynamic responses to* L-*NAME* One group of animals ($n = 8$) instrumented for recording renal, mesenteric and hindquarters blood flows was given a bolus injection of L-NAME (10 mg kg^{-1}) and, starting 10 min later, a primed infusion of L-arginine hydrochloride (100 mg kg^{-1} bolus followed by 100 mg kg^{-1} h^{-1} infusion).

A separate group of animals ($n = 8$) was given a bolus injection of L-NAME (1 mg kg^{-1}) followed 5 min later by a primed infusion of L-arginine hydrochloride (100 mg kg^{-1}, 100 mg kg^{-1} h^{-1}).

Experiment 3: Effects of pretreatment with L-*arginine on the regional haemodynamic responses to* L-*NAME* A further group of animals ($n = 9$) instrumented for recording renal, mesenteric and hindquarters blood flows was given L-arginine hydrochloride by primed infusion (100 mg kg^{-1} bolus, 100 mg kg h^{-1} infusion) started 30 min before administration of L-NAME (10 mg kg^{-1}).

Drugs

All substances used were dissolved in sterile saline and injected in volumes of 0.1 ml, flushed in with 0.1 ml (this volume being the dead space of the i.v. catheter); infusions were given at a rate of 0.3 ml h^{-1}. Administration of saline alone in these volumes had no consistent, sustained cardiovascular effects. L-NAME hydrochloride and L-arginine hydrochloride were obtained from Sigma Chemical Co. and were dissolved in sterile saline (154 mM NaCl). D-NAME acetate was synthesized at Wellcome Research Laboratories (Beckenham, Kent).

Data analysis

Within-group analysis was done by Friedman's test (Theordorsson-Norheim, 1987) and between-group comparisons were made with the Mann-Whitney U test applied to areas under or over curves. These areas were quantified for each individual animal on the basis of the change relative to baseline and the time interval (using a Fortran programme written in our Department). A P value of <0.05 was taken as significant.

Results

Regional haemodynamic effects of L-*NAME*

Bolus injection of L-NAME at a dose of 0.1 mg kg^{-1} had no significant effect on mean blood pressure, but there was a slight bradycardia (Figure 1). There were no significant changes in renal or hindquarters haemodynamics (Figures 1 and 2), although mesenteric flow (Figure 1) and vascular conductance (Figure 2) showed small decreases.

Over the range 1.0 to 10 mg kg^{-1}, L-NAME caused pressor effects, the magnitudes and durations of which were dose-related (Figures 1 and 3), although following L-NAME at 10 mg kg^{-1}, mean blood pressure was still significantly elevated ($+16 \pm 2$ mmHg) after 2 h. The bradycardias after L-NAME were less clearly dose-dependent than the pressor effects (Figure 1), and it is notable that 2 h after L-NAME at a dose of 10 mg kg^{-1} heart rate was not significantly different from baseline (-6 ± 11 beats min^{-1}) in spite of the elevation in mean blood pressure (see above).

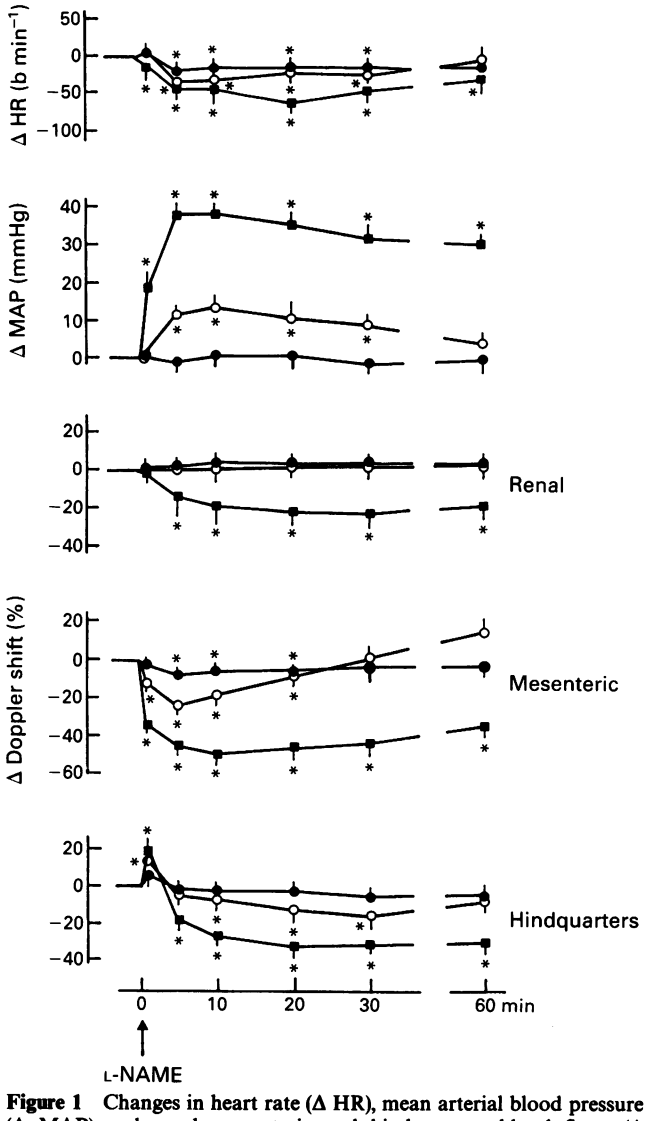

Figure 1 Changes in heart rate (Δ HR), mean arterial blood pressure (Δ MAP) and renal, mesenteric and hindquarters blood flows (Δ Doppler shift) following i.v. bolus doses of N^G-nitro-L-arginine methyl ester (L-NAME) at 0.1 mg kg^{-1} (●), 1.0 mg kg^{-1} (○) and 10.0 mg kg^{-1} (■) in conscious, Long Evans rats ($n = 8$). Values are mean with s.e.mean shown by vertical lines; * $P < 0.05$ versus baseline (Friedman's test).

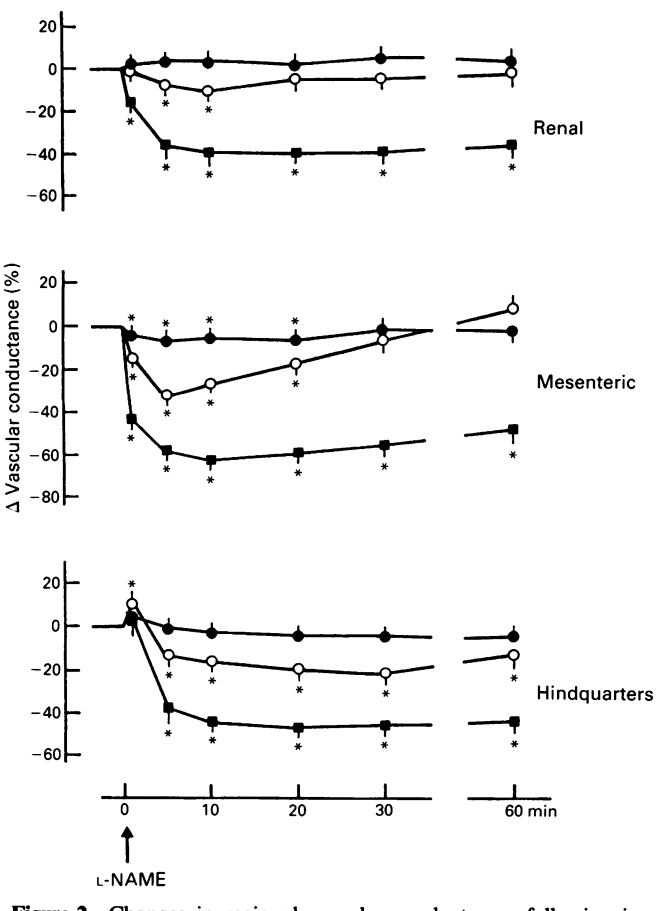

Figure 2 Changes in regional vascular conductances following i.v. bolus doses of N^G-nitro-L-arginine methyl ester (L-NAME) at 0.1 mg kg^{-1} (●), 1.0 mg kg^{-1} (○) and 10.0 mg kg^{-1} (■). Data derived from those in Figure 1. Values are mean with s.e.mean shown by vertical lines; * $P < 0.05$ versus baseline (Friedman's test).

While mesenteric blood flow (Figures 1 and 3) and vascular conductance (Figure 2) showed dose-related reductions, renal blood flow was reduced only after the 10 mg kg^{-1} dose of L-NAME (Figures 1 and 3). However, renal vasoconstriction occurred following L-NAME at 1.0 and 10 mg kg^{-1} (Figure 2). Hindquarters blood flow showed initial increases following L-NAME (1.0 and 10 mg kg^{-1}) but thereafter flow fell (Figures 1 and 3). There was an initial rise in hindquarters vascular conductance following L-NAME at 1.0 mg kg^{-1} only (Figure 2); the subsequent hindquarters vasoconstrictions were dose-dependent (Figure 2).

Following L-NAME at a dose of 10 mg kg^{-1} the mesenteric vasoconstriction occurred earlier and was more rapid in onset. Also, the nadir of mesenteric vascular conductance was lower than those seen in the renal and hindquarters vascular beds (Figures 1–3). Two h after this dose of L-NAME, renal and mesenteric blood flows were not significantly different from baseline (-5 ± 5 and -7 ± 7%, respectively), but hindquarters blood flow was reduced (-28 ± 5%). Since mean blood pressure was still elevated (see above), there were vasoconstrictions in all 3 vascular beds (renal, -16 ± 5%; mesenteric -18 ± 7%; hindquarters -36 ± 4% change in vascular conductance), although that in the hindquarters was greatest.

D-NAME at a dose of 10 mg kg^{-1} was without pressor or vasoconstrictor effects (Figure 3). There was a transient tachycardia (Figure 3) following D-NAME, but a similar effect was also seen following L-NAME (Figure 3) or saline injection, and was thus not due to the drugs administered.

Cardiac haemodynamic effects of L-*NAME*

On the basis of the changes described above, the effects of L-NAME on cardiac haemodynamics were assessed 10 min after a bolus dose of 10 mg kg^{-1}. Table 1 shows that L-NAME caused substantial bradycardia and hypertension, accompanied by reductions in cardiac output, stroke volume, $+dF/dt_{max}$, peak thoracic aortic flow and total peripheral conductance. However, central venous pressure did not change significantly. It is notable that although the increases in mean blood pressure following L-NAME (10 mg kg^{-1}) were very similar in animals in which regional haemodynamics were studied (Figure 1) and those in which cardiac haemodynamics were measured (Table 1), the bradycardia in the latter was significantly greater than that in the former. However, the resting heart rates in the 2 groups were significantly different (cardiac haemodynamics group = 378 ± 7 beats min^{-1}; regional haemodynamics group = 323 ± 10 beats min^{-1}) and hence the nadirs in heart rate were similar (272 ± 7 and 278 ± 14 beats min^{-1}, respectively).

Effects of post-treatment with L-*arginine on the regional haemodynamic effects of* L-*NAME*

The regional haemodynamic and pressor effects of L-NAME (10 mg kg^{-1}) were not significantly different in the two

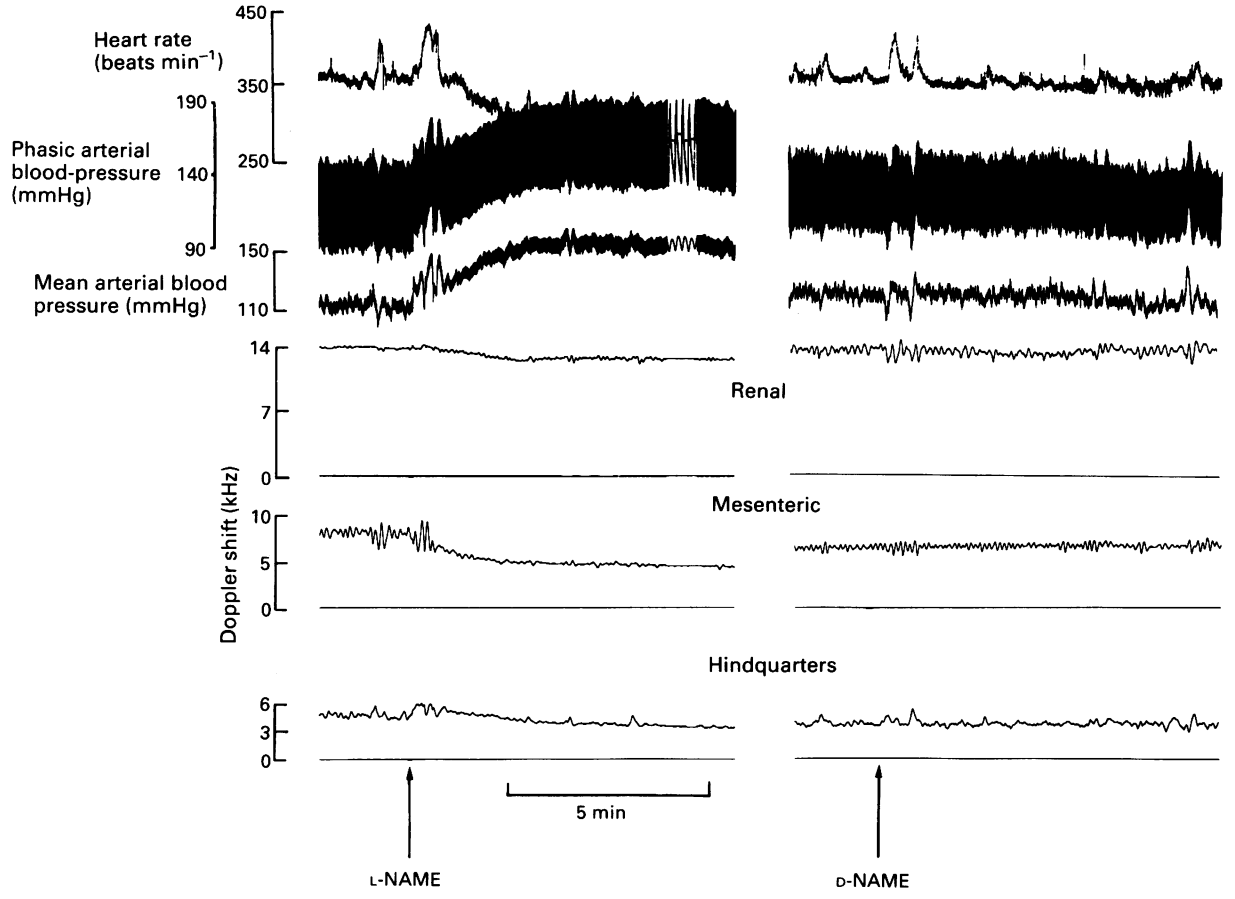

Figure 3 Original recordings of cardiovascular responses to an i.v. bolus injection (10 mg kg^{-1}) of N^{G}-nitro-L-arginine methyl ester (L-NAME) or N^{G}-nitro-D-arginine methyl ester (D-NAME) in 2 different, conscious, Long Evans rats.

separate groups of animals receiving this treatment (Figures 4 and 5). Infusion of L-arginine 10 min after bolus injection of L-NAME caused significant attenuation of the pressor effect ($P < 0.041$) and of the reductions in renal ($P = 0.014$) and mesenteric ($P < 0.019$), but not hindquarters, blood flows following L-NAME (Figure 4). Thus L-arginine caused significant reversals of the renal ($P < 0.019$) and mesenteric ($P < 0.014$), but not of the hindquarters, vasoconstrictions following L-NAME (Figure 5).

In animals receiving L-arginine (100 mg kg^{-1} bolus and 100 mg kg^{-1} h^{-1} infusion) starting 5 min after L-NAME at a dose of 1 mg kg^{-1}, renal vasoconstriction was reversed and mesenteric vasoconstriction was substantially reduced. In addition, there was a significant, albeit transient, attenuation of the hindquarters vasoconstriction (Figure 6).

Effects of pretreatment with L-*arginine on the haemodynamic effects of* L-*NAME*

Infusion of L-arginine had no consistent, sustained effect on any variable (Figures 4, 5 and 7). However, in the presence of L-arginine the pressor effect of L-NAME was significantly ($P = 0.019$) attenuated, as were the reductions in mesenteric ($P = 0.019$) and hindquarters ($P < 0.019$) blood flow (Figure 4). Although the changes in renal blood flow following L-NAME were not significantly different in the absence and presence of L-arginine, there was no significant reduction in renal blood flow following L-NAME administration in the presence of L-arginine (Figure 4). L-Arginine pretreatment caused significant attenuation of the renal ($P = 0.041$) and mesenteric ($P = 0.032$), but not of the hindquarters, vasoconstrictor responses to L-NAME (Figure 5).

Discussion

The present findings, which indicate that L-NAME has potent pressor effects associated with regional vasoconstrictions *in vivo* in conscious rats, are consistent with our previous observations on the effects of L-NMMA (Gardiner *et al.*, 1989b; 1990d,e,g) at higher doses. The findings that D-NAME was without such effects, and that some of the actions of L-NAME could either be attenuated by pretreatment with L-arginine, or completely or partially reversed by post-treatment with L-

Table 1 Cardiac haemodynamic variables before and 10 min after an i.v. bolus injection of N^{G}-nitro-L-arginine methyl ester (L-NAME, 10 mg kg^{-1}) in conscious, Long Evans rats

	Before L-*NAME*	*After* L-*NAME*
Heart rate (beats min^{-1})	378 ± 7	272 ± 7*
Mean blood pressure (mmHg)	99 ± 2	142 ± 3*
Cardiac output (ml min^{-1})	100 ± 3	61 ± 1*
Stroke volume (μl/beat)	264 ± 12	226 ± 6*
$+dF/dt$ max (1 min^{-1} min^{-1})	1732 ± 60	1000 ± 30*
Peak thoracic flow (ml min^{-1})	408 ± 13	281 ± 8*
Total peripheral conductance (μl min^{-1} mmHg^{-1})	1014 ± 25	431 ± 13*
Central venous pressure (cmH_2O)	4.54 ± 0.73	4.33 ± 0.68

Values are mean ± s.e.mean; $n = 8$; *$P < 0.05$ (Wilcoxon's test).

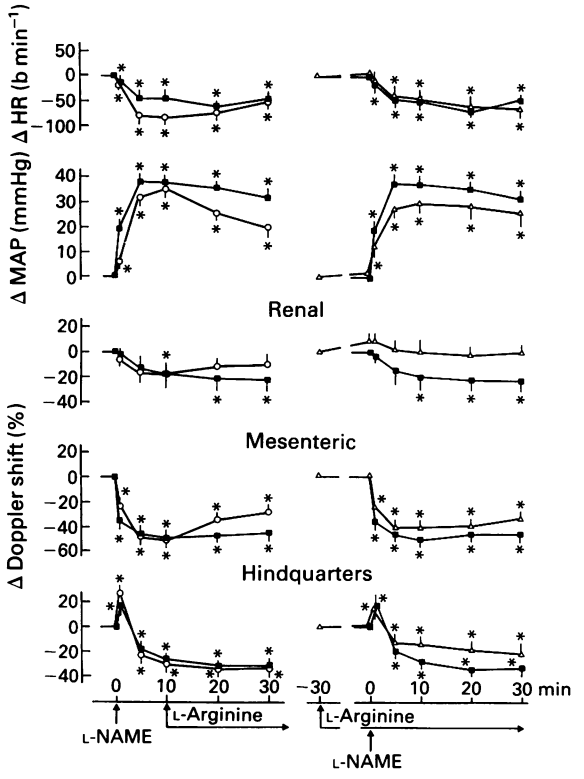

Figure 4 Left-hand panels show changes in heart rate (Δ HR), mean arterial blood pressure (Δ MAP) and renal, mesenteric and hindquarters blood flows (Δ Doppler shift) following an i.v. bolus dose of N^G-nitro-L-arginine methyl ester (L-NAME) at 10.0 mg kg^{-1} (■) in a group of conscious, Long Evans rats (n = 8). A separate group (n = 8; ○) of animals also received an i.v. bolus injection of L-NAME (10.0 mg kg^{-1}) but followed 10 min later by a primed infusion (100 mg kg^{-1} bolus, 100 mg kg^{-1} h^{-1} infusion) of L-arginine. Right-hand panels show the data in the group receiving L-NAME alone (■) compared to a separate group (n = 8; Δ) receiving a primed infusion of L-arginine starting 30 min before administration of L-NAME. Values are mean with s.e.mean shown by vertical lines; * $P < 0.05$ versus baseline (Friedman's test). Statistics for the differences between groups (as judged from areas under or over curves) are given in the results.

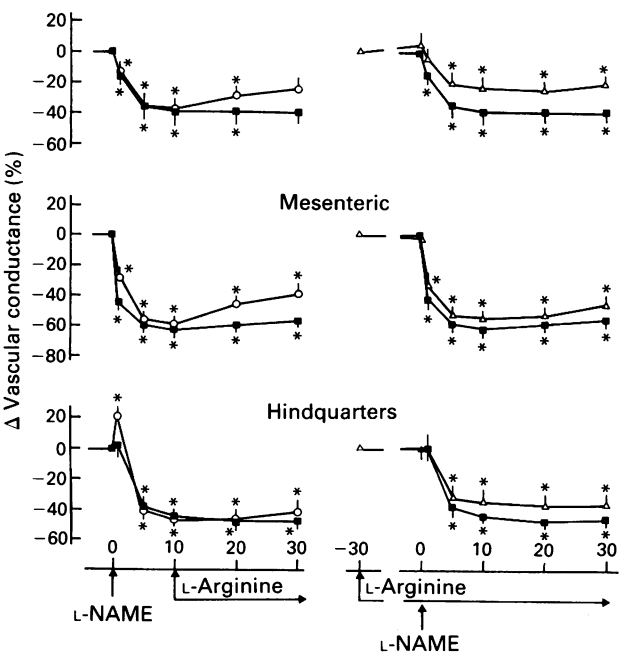

Figure 5 Changes in regional vascular conductances derived from the data in Figure 4. Animals receiving N^G-nitro-L-arginine methyl ester (L-NAME) alone (■); animals receiving L-NAME followed by L-arginine (○); animals receiving L-arginine followed by L-NAME (Δ). Values are mean with s.e.mean shown by vertical lines; * $P < 0.05$ versus baseline. Statistics for the differences between groups (as judged from areas under or over curves) are given in the results.

arginine, are further evidence for a major role of the L-arginine-nitric oxide pathway in the control of regional vascular conductances *in vivo* (Gardiner *et al.*, 1990e).

While a substantial contribution of endothelial cell nitric oxide production to this regulatory system is likely (see Moncada *et al.*, 1989), an additional influence of the L-arginine-nitric oxide pathway at the level of the efferent control of the circulation, for example, should not be dismissed. Thus, the differential profiles of effect of L-NAME in the renal, mesenteric and hindquarters vascular beds could be due to different degrees of involvement of neural mechanisms in the control of conductances in the different vascular beds. However, it is also possible, and perhaps more likely, that these differences represent different dynamics of the interactions between L-NAME and the L-arginine-nitric oxide pathway in the different vascular beds. In this regard it is noteworthy that L-NMMA also has differential effects on regional vascular conductances (Gardiner *et al.*, 1989b; 1990d,e,g) and, furthermore, the profile of haemodynamic effects of L-NMMA is similar to that seen here following administration of L-NAME. In addition, a relative resistance of the hindquarters vasoconstrictor response to reversal by L-arginine is also seen following L-NMMA (Gardiner *et al.*, 1990e; Bennett *et al.*, 1990). However, in confirmation of the *in vitro* data (Moore *et al.*, 1990), L-NAME appears to be about 10 fold more potent than L-NMMA (Figure 8), and its effect more long-lasting and more resistant to reversal by L-arginine. As yet no information is available regarding the details of the interactions between L-NAME or L-NMMA and the L-arginine-nitric oxide pathway in different vascular beds at the level of the biochemistry of the synthetic machinery.

A recent publication from Boulanger & Lüscher (1990) provides evidence that endothelial cell nitric oxide might act to inhibit endothelin release. Hence the vasoconstriction resulting from inhibition of nitric oxide synthesis could be due to loss of nitric oxide-mediated vasodilator tone together with vasoconstriction due to increased endothelin release. Elsewhere we have pointed out that the vascular beds responding most markedly to L-NMMA are those that show pronounced vasoconstriction to exogenous endothelins (Gardiner *et al.*, 1990g). In addition, it is of interest that the hindquarters vascular bed also shows an initial vasodilator response to bolus injections of endothelins (Gardiner *et al.*, 1989a; 1990a,b) sarafotoxin S6b (Gardiner *et al.*, 1990b), L-NMMA (Gardiner *et al.*, 1989b; 1990d,e,g) and L-NAME (this study).

In spite of the apparent differences in potency of L-NAME and L-NMMA, acute i.v. administration of supramaximal doses of either compound fails to increase mean blood pressure much beyond the levels reported here (S.M. Gardiner, A.M. Compton & T. Bennett unpublished results). This does not seem to be due to desensitization to repeated doses since in the present study the pressor response to L-NAME at 10 mg kg^{-1} was very similar in animals exposed to L-NAME on a single occasion and in those receiving incremental doses of the compound. Thus, the maximum pressor effect of L-NAME may represent the result of the loss of vasodilator tone (i.e. unopposed vasoconstriction) and of factors that act to buffer the resulting rise in blood pressure. As mentioned in the results, although the pressor responses to L-NAME were very consistent the associated bradycardias varied in direct relation to the resting heart rates. Furthermore, 2 h after 10 mg kg^{-1} L-NAME there was no bradycardia, although mean blood pressure was still elevated. Hence, it is unlikely that reflex slowing of the heart in itself is a major factor acting to oppose pressor changes following L-NAME. However, there were substantial reductions in cardiac output that would certainly have buffered the hypertensive effects of the

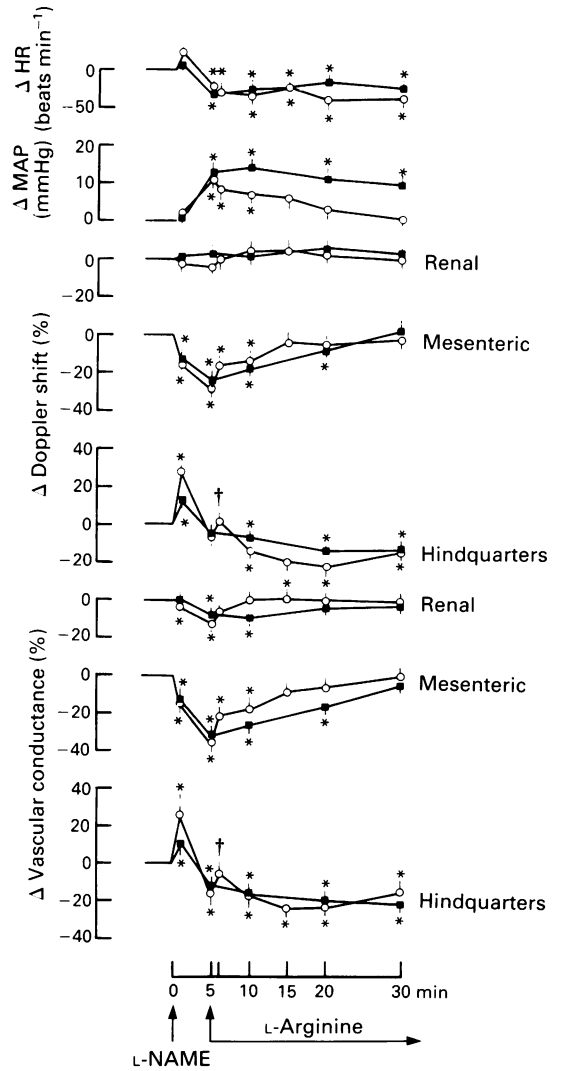

Figure 6 Changes in heart rate (ΔHR), mean arterial blood pressure (ΔMAP) and renal, mesenteric and hindquarters blood flows (Δ Doppler shift) and vascular conductances following an i.v. bolus dose of N^G-nitro-L-arginine methyl ester (L-NAME) at 1.0 mg kg^{-1} (■) in a group of conscious, Long Evans rats ($n = 8$). A separate group ($n = 8$; ○) of animals also received an i.v. bolus injection of L-NAME (1.0 mg kg^{-1}) followed by a primed infusion (100 mg kg^{-1} bolus, 100 mg kg^{-1} h^{-1} infusion) of L-arginine. Values are mean with s.e.mean shown by vertical lines; $*P < 0.05$ versus baseline (Friedman's test), $\dagger P < 0.05$ versus pre-L-arginine value (Friedman's test). Following L-arginine administration there were significant attenuations of the pressor and renal and mesenteric vasoconstrictor effects, but only a transient reduction of the hindquarters vasoconstrictor effect of L-NAME.

L-NAME-induced vasoconstriction. While it is feasible that the reduction in cardiac output was a direct consequence of the increase in afterload (i.e. decreased total peripheral conductance), the marked reductions in stroke volume, $+dF/dt_{max}$ and peak thoracic aortic flow are all consistent with negative inotropic changes following administration of L-NAME. It is possible such effects resulted from L-NAME causing coronary vasoconstriction (Amezcua *et al.*, 1988) or from a direct myocardial action or both.

Central venous pressure did not show any consistent changes following L-NAME. However, because of the substantial increase in afterload, central venous pressure may not give a reliable index of venous tone and, hence, the present results cannot be taken to indicate different degrees of involvement of the L-arginine-nitric oxide pathway in control of the arterial and venous sides of the circulation *in vivo*.

In conclusion, the present results are consistent with L-arginine-nitric oxide pathways acting tonically *in vivo* to

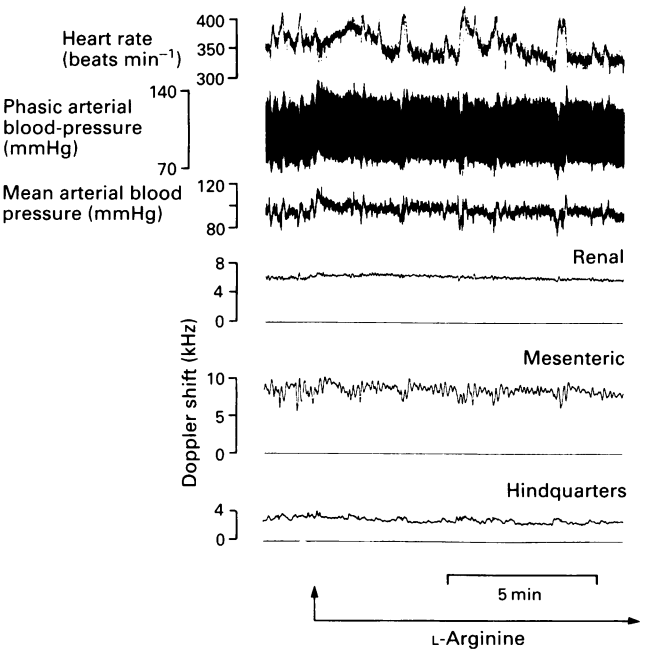

Figure 7 Original recording from a conscious, Long Evans rat. At the arrow a primed infusion (100 mg kg^{-1} bolus, 100 mg kg^{-1} h^{-1} infusion) of L-arginine was begun, but with little effect on haemodynamic status.

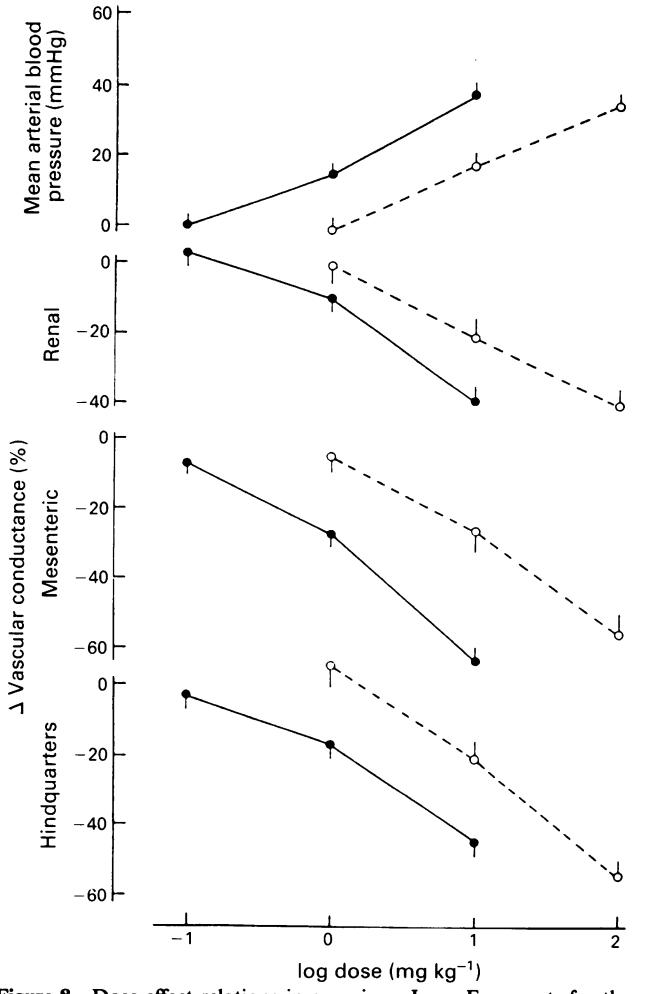

Figure 8 Dose-effect relations in conscious, Long Evans rats for the haemodynamic actions of N^G-nitro-L-arginine methyl ester (L-NAME, ●) and N^G-monomethyl-L-arginine (○). The data for the latter compound were obtained from the study of Gardiner *et al.* (1990e), whereas those for L-NAME are from the present study; measurements were made 10 min after i.v. bolus injections. Values are mean and vertical lines show s.e.mean ($n = 8$ in both groups).

oppose vasoconstrictor influences and thereby maintain regional vascular conductances appropriate for the required perfusion of the peripheral tissues. Treatment with L-NAME results in regional vasoconstrictions of variable magnitudes but which, collectively, produce a hypertension that is opposed by marked reductions in cardiac function.

We are grateful to Drs S. Moncada and R.M.J. Palmer for useful discussions during this work, and for the gift of D-NAME.

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(Received May 9, 1990
Revised June 26, 1990
Accepted July 10, 1990)

Br. J. Pharmacol. (1990), **101**, 632–639

Regional and cardiac haemodynamic responses to glyceryl trinitrate, acetylcholine, bradykinin and endothelin-1 in conscious rats: effects of N^G-nitro-L-arginine methyl ester

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1 Conscious Long Evans rats, chronically instrumented for cardiovascular measurements, were challenged with i.v. bolus doses of glyceryl trinitrate (40 nmol kg^{-1}), acetylcholine (1.2 nmol kg^{-1}), bradykinin (3.2 nmol kg^{-1}), or endothelin-1 (0.25 nmol kg^{-1}). Under control conditions these doses produced similar falls in mean arterial blood pressure (glyceryl trinitrate, -20 ± 3 mmHg; acetylcholine, -24 ± 2 mmHg; bradykinin, -21 ± 3 mmHg; endothelin-1, -25 ± 3 mmHg), associated with renal, mesenteric and hindquarters vasodilatations (except for endothelin-1 which caused mesenteric vasoconstriction).

2 In the presence of N^G-nitro-L-arginine methyl ester (L-NAME, 10 mg kg^{-1}), a potent inhibitor of nitric oxide biosynthesis and endothelium-dependent vasorelaxation *in vitro*, the hypotensive responses to glyceryl trinitrate, acetylcholine, and endothelin-1 were increased, although that to bradykinin was not. However, comparing the differences between the response to glyceryl trinitrate and that to any other agonist in the absence and presence of L-NAME showed that there were relative attenuations of the hypotensive responses to bradykinin and endothelin-1, but not to acetylcholine, in the presence of L-NAME.

3 This comparative analysis showed that the renal and hindquarters vasodilator responses to bradykinin and endothelin-1 were attenuated in the presence of L-NAME, but the renal, mesenteric and hindquarters vasodilator responses to acetylcholine were not. However, when L-NAME was administered in the presence of pentolinium, captopril and the vasopressin V_1-receptor antagonist, $d(CH_2)_5$[Tyr-(Et)]DAVP, (to abolish baroreflex and neurohumoral mechanisms), there was attenuation of the renal and mesenteric vasodilator effects of acetylcholine relative to those seen with glyceryl trinitrate. Under those conditions only the renal vasodilator effects of bradykinin and endothelin-1 were attenuated.

4 In separate experiments in conscious Long Evans rats, direct measurement of cardiac haemodynamics showed that the hypotensive responses to glyceryl trinitrate, acetylcholine, bradykinin and endothelin-1 were entirely attributable to rises in total peripheral conductance since both in the absence and presence of L-NAME there were no reductions in cardiac index in response to these substances.

5 The results indicate that measurement of systemic arterial blood pressure alone in conscious rats does not permit reliable quantitation of the influence of L-NAME on regional vasodilator responses to glyceryl trinitrate, acetylcholine, bradykinin or endothelin-1. Furthermore, these substances exert effects in different vascular beds that may be differentially influenced by baroreflex mechanisms, neurohumoral mechanisms, or both. Moreover, except in the case of the renal vasodilator response to endothelin-1 (which was abolished in the presence of L-NAME), even when L-NAME caused attenuation of the vasodilator effects of acetylcholine or bradykinin (relative to glyceryl trinitrate), substantial responses remained. It is feasible that such responses *in vivo* are nitric oxide-independent.

Introduction

Nitric oxide synthesized from L-arginine appears to be the major mediator of endothelium-dependent vasorelaxation *in vitro* (Palmer *et al.*, 1987; 1988a,b; Moncada *et al.*, 1988; 1989; Rees *et al.*, 1989a,b). The biosynthesis of nitric oxide is antagonised by N^G-monomethyl-L-arginine (L-NMMA; see Moncada *et al.*, 1989) a compound which causes widespread regional vasoconstrictions when administered to conscious rats (Gardiner *et al.*, 1989c,d; 1990c,d,f).

Whittle *et al.* (1989) reported that L-NMMA produced substantial inhibition of the falls in diastolic blood pressure induced by acetylcholine, bradykinin, substance P and endothelin-1 in pentobarbitone-anaesthetized Wistar rats, which is consistent with the hypotension being due to endothelium-derived nitric oxide causing vasodilatation. However, with endothelin-1 we had found, in conscious rats, that the initial hypotensive response was enhanced in the presence of L-NMMA and the associated hindquarters vasodilatation was not attenuated (Gardiner *et al.*, 1989d). In addition, Aisaka *et al.* (1989) reported that L-NMMA did not change the magnitude of the hypotensive response to acetylcholine in pentobarbitone-anaesthetized guinea-pigs.

It is known now that N^G-nitro-L-arginine is a more potent inhibitor than L-NMMA of endothelium-dependent vasorelaxation *in vitro* (Moore *et al.*, 1990), and this effect is associated with marked inhibition of nitric oxide production (Ishii *et al.*, 1990; Mülsch & Busse, 1990). Furthermore, oral or intravenous administration of N^G-nitro-L-arginine methyl ester (L-NAME) in conscious rats causes marked hypertension and regional vasoconstrictions (Gardiner *et al.*, 1990e,f,h), the patterns of which resemble those following L-NMMA, albeit given at a higher dose (Gardiner *et al.*, 1989c,d; 1990c,d,f). Therefore, in the present work we investigated regional haemodynamic responses to glyceryl trinitrate, acetylcholine, bradykinin and endothelin-1 in the absence and presence of L-NAME. Furthermore, in order to preclude any possible contributions from baroreflex, or neurohumoral mechanisms,

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or both, to the responses seen, the same experiments were performed in a second group of animals in the absence and presence of autonomic and neurohumoral blockade (achieved with pentolinium, captopril and an antagonist of the V_1-receptor-mediated actions of vasopressin).

In order to determine the possible contributions of changes in cardiac haemodynamics to the depressor effects of glyceryl trinitrate, acetylcholine, bradykinin and endothelin-1 in the presence and absence of L-NAME, these experiments were also performed in animals chronically instrumented for the measurement of cardiac function.

Methods

The majority of experiments were carried out on male Long Evans rats (3–4 months old; bred in Nottingham). Animals were anaesthetized (sodium methohexitone, 60 mg kg^{-1}, i.p. supplemented as required) and had pulsed Doppler probes (Haywood *et al.*, 1981) sutured 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) (Gardiner *et al.*, 1990a). Separate animals had electromagnetic flow probes (Skalar, Delft) implanted around the ascending aorta via a transthoracic approach (Smith & Hutchins, 1979; Smits *et al.*, 1982; Gardiner *et al.*, 1990g,h). Animals were given ampicillin (7 mg kg^{-1}, i.m.; Penbritin, Beechams) and left to recover for 7–14 days. Then, under brief anaesthesia (sodium methohexitone, 40 mg kg^{-1}, i.p.), intra-arterial (distal abdominal aorta via the ventral caudal artery) and intravenous (right jugular vein) catheters were implanted. In the case of animals with thoracic aortic flow probes, one of the intravenous catheters was constructed from a 3 cm length of small bore (intravascular) catheter (i.d. 0.28 mm), heat-sealed to a 150 cm length of more rigid, wider bore (i.d. 0.58 mm) nylon tubing. The tip of this catheter was positioned close to the right atrial orifice for recording central venous pressure (Gardiner *et al.*, 1990g,h).

Animals were left to recover in their home cages overnight and experiments were begun the next day, at least 24 h after anaesthesia and catheterization.

Regional haemodynamic effects of glyceryl trinitrate, acetylcholine, bradykinin and endothelin-1

In preliminary experiments based on the data of Whittle *et al.* (1989), we determined that glyceryl trinitrate (40 nmol kg^{-1}), acetylcholine (1.2 nmol kg^{-1}), bradykinin (3.2 nmol kg^{-1}) and endothelin-1 (0.25 nmol kg^{-1}) caused comparable falls in mean blood pressure. These doses were then used in the definitive experiments. It should be noted that the results obtained were similar whether measurements were made of changes in diastolic or in mean blood pressures. Animals received glyceryl trinitrate, acetylcholine, and bradykinin in random order, but received endothelin-1 last because of the prolonged secondary pressor effect of this peptide. All substances were administered at least 10 min apart.

In one group of Long Evans rats ($n = 8$) glyceryl trinitrate, acetylcholine, bradykinin and endothelin-1 were administered and, at least 90 min after endothelin-1, L-NAME (10 mg kg^{-1}, i.v.) was given. Elsewhere (Gardiner *et al.*, 1990h) we have shown there is a relatively stable haemodynamic profile for at least 60 min after this dose of L-NAME. Therefore, starting 10 min after injection of L-NAME, animals were re-challenged at 10 min intervals with glyceryl trinitrate, acetylcholine, bradykinin (randomized) and, finally, endothelin-1 (i.e. about 40 min after L-NAME injection).

In another group ($n = 8$) of Long Evans rats, glyceryl trinitrate, acetylcholine, bradykinin and endothelin-1 were administered in the absence and in the presence of pentolinium (5 mg kg^{-1} bolus followed by 5 mg kg^{-1} h^{-1} infusion), captopril (2 mg kg^{-1} bolus followed by 1 mg kg^{-1} h^{-1} infusion), [(1-β-mercapto-β, β-cyclopentamethylenepropionic acid), 2-(0-ethyl)tyrosine, 8-D-arginine]vasopressin (abbreviated to d(CH$_2$)5[Tyr-(Et)]DAVP; 10 μg kg^{-1} bolus followed by 10 μg kg^{-1} h^{-1} infusion) and L-NAME (10 mg kg^{-1} bolus). Elsewhere we have shown the combination of these doses of pentolinium, captopril and d(CH$_2$)$_5$[Tyr-(Et)]DAVP blocks all endogenous factors contributing to blood pressure recovery in conscious, Long Evans rats (Tomlinson *et al.*, 1990). Hence, these experiments were designed to abolish any possible contributions of baroreflex on neurohumoral mechanisms to the responses evoked by glyceryl trinitrate, acetylcholine, bradykinin and endothelin-1.

In order to simulate the experiments carried out by Whittle *et al.* (1989) we also investigated the effects on blood pressure of glyceryl trinitrate, acetylcholine, bradykinin and endothelin-1 in pentobarbitone-anaesthetized Long Evans ($n = 2$) and Wistar ($n = 3$) rats in the absence and presence of L-NAME (10 mg kg^{-1}) or L-NMMA (50 mg kg^{-1}), respectively.

Cardiac haemodynamic effects of glyceryl trinitrate, acetylcholine, bradykinin and endothelin-1

Long Evans rats ($n = 8$) with thoracic aortic flow probes were connected to a Skalar MDL 1401 flowmeter and data were digitized on-line with a haemodynamics microprocessor (Schoemaker, 1989; Gardiner *et al.*, 1990g,h). This system provided values for cardiac index, peak aortic flow, maximum positive slope of aortic flow ($+dF/dt_{max}$), total peripheral conductance, stroke index, mean central venous pressure, heart rate and mean arterial blood pressure. Changes in these variables were measured in response to administration of glyceryl trinitrate, acetylcholine, bradykinin, and endothelin-1 in the absence and in the presence of L-NAME (10 mg kg^{-1}) as above.

Data analysis

The profiles of change in mean blood pressure and regional haemodynamics differed following administration of glyceryl trinitrate, acetylcholine, bradykinin and endothelin-1, although the nadirs in blood pressure were similar under control conditions. Furthermore the time courses of the changes in regional flows varied under the different experimental conditions, and so, for each substance in each condition, measurements were made at time points selected to represent the full profile of effect on all variables. Since L-NAME caused substantial haemodynamic changes the responses to glyceryl trinitrate were used as an internal standard and all other responses were compared to them. Thus, for example, the difference between the hypotensive response to glyceryl trinitrate and endothelin-1 in the absence of L-NAME was compared to that in the presence of L-NAME as a means of assessing the extent to which the latter caused a relative attenuation of the hypotensive effect of endothelin-1. In the results the effects of substances relative to glyceryl trinitrate are given also as ratios to facilitate comparisons but statistical procedures were carried out on the raw data. Results were analysed by the Kruskal-Wallis test, Mann-Whitney U test or Wilcoxon's ranks sums test, as appropriate; $P < 0.05$ was taken as indicating statistical significance.

Drugs

Acetylcholine chloride (Sigma), N^G-nitro-L-arginine methyl ester hydrochloride (Sigma), N^G-monomethyl-L-arginine acetate (Wellcome Research Laboratories), glyceryl trinitrate (Tridil; Du Pont, U.K.), pentolinium tartrate (Sigma), captopril (Squibb, U.K.), d(CH$_2$)$_5$[Tyr-(Et)]DAVP (Bachem U.K.), bradykinin (Bachem U.K.) and endothelin-1 (Peptide Institute, Japan) were dissolved in isotonic saline. In the case of the latter peptides, the saline contained 1% bovine serum albumin. All i.v. injections were given as 100 μl boluses which

were flushed in with 100 μl isotonic saline (the dead spaces of the catheters).

Results

Regional haemodynamic effects of glyceryl trinitrate, acetylcholine, bradykinin and endothelin-1 before and after administration of L-NAME

Blood pressures In the first group of animals ($n = 8$), under control conditions, the falls in arterial blood pressure elicited by glyceryl trinitrate (-20 ± 3 mmHg), acetylcholine (-24 ± 2 mmHg), bradykinin (-21 ± 3 mmHg) and endothelin-1 (25 ± 3 mmHg) were not different. L-NAME increased mean arterial blood pressure from 110 ± 2 to 153 ± 2 mmHg and then the falls in arterial blood pressure elicited by glyceryl trinitrate (-49 ± 3 mmHg), acetylcholine (-48 ± 2 mmHg), and endothelin-1 (-38 ± 2 mmHg) were significantly larger than in the absence of L-NAME, but the response to bradykinin (-29 ± 4 mmHg) was not. When considered relative to glyceryl trinitrate, the depressor effects of bradykinin and endothelin-1 were attenuated in the presence of L-NAME, whereas the hypotensive response to acetylcholine was not (Figure 1 and Table 1).

In the second group of animals ($n = 8$), the depressor responses to glyceryl trinitrate, acetylcholine, bradykinin and endothelin-1 under control conditions were similar to those seen in the first group (Figure 1 and Table 2). During combined administration of pentolinium, captopril and $d(CH_2)_5$[Tyr-(Et)]DAVP, mean arterial blood pressure was 47 ± 3 mmHg; 10 min after additional administration of L-NAME blood pressure had risen to 84 ± 6 mmHg. Under those circumstances the absolute hypotensive responses to glyceryl trinitrate (-29 ± 4 mmHg), and acetylcholine (-28 ± 5 mmHg), were not different from those under control conditions. However, the depressor effect of bradykinin (-39 ± 4 mmHg) was enhanced, whereas the response to endothelin-1 (-19 ± 2 mmHg) was reduced. Thus, when considered relative to glyceryl trinitrate, the depressor effects of acetylcholine and endothelin-1 were attenuated whereas the response to bradykinin was augmented (Figure 1 and Table 2). In addition, the later pressor effect of endothelin-1 was enhanced although the absolute pressure level reached was not increased (Figure 1).

In anaesthetized, Long Evans rats ($n = 2$) or anaesthetized, Wistar rats ($n = 3$) treatment with L-NAME (10 mg kg^{-1}) or L-NMMA (50 mg kg^{-1}), respectively, augmented hypotensive responses. For example in anaesthetized Wistar rats in the absence of L-NMMA the falls in mean arterial blood pressure evoked by glyceryl trinitrate, acetylcholine, bradykinin and endothelin-1 were -14 ± 2, -25 ± 1, -11 ± 3 and -16 ± 3 mmHg, respectively. The corresponding values in the presence of L-NMMA were -47 ± 10, -49 ± 8, -27 ± 5 and -46 ± 12 mmHg, respectively (Figure 2).

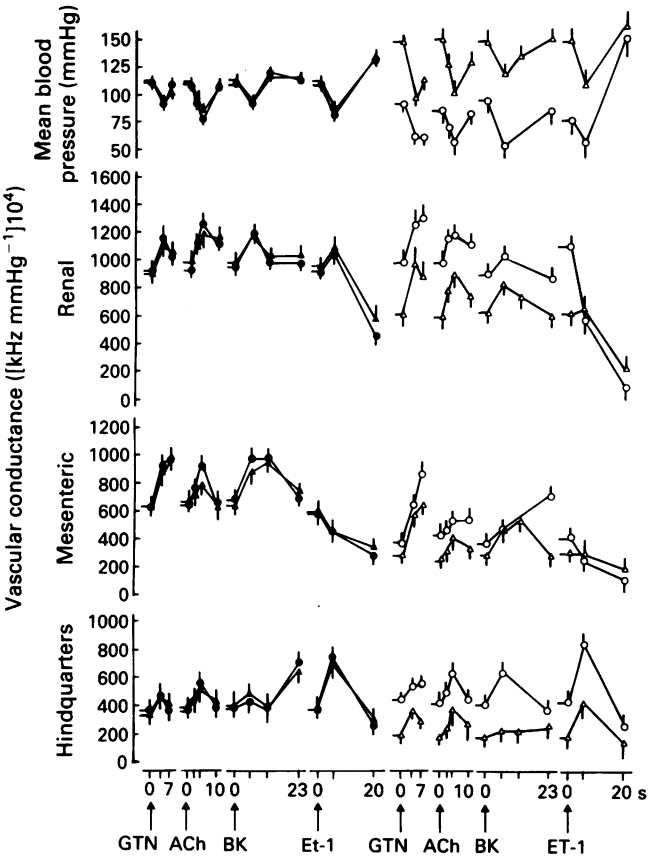

Figure 1 Regional haemodynamic responses to i.v. bolus doses of glyceryl trinitrate (GTN, 40 nmol kg^{-1}), acetylcholine (ACh, 1.2 nmol kg^{-1}), bradykinin (BK, 3.2 nmol kg^{-1}) or endothelin-1 (ET-1, 0.25 nmol kg^{-1}) in conscious Long Evans rats. Left hand panels show responses in two separate groups (●, ▲), $n = 8$ in each) under control conditions. Right hand panels show responses in the same animals either in the presence of N^G-nitro-L-arginine methyl ester (L-NAME, 10 mg kg^{-1}) alone (△) or after administration of L-NAME in the presence of pentolinium (5 mg kg^{-1} bolus, 5 mg kg^{-1} h^{-1} infusion), captopril (2 mg kg^{-1} bolus, 1 mg kg^{-1} h^{-1} infusion) and $d(CH_2)_5$[Tyr-(Et)]DAVP (10 μg kg^{-1} bolus, 10 μg kg^{-1} h^{-1} infusion) (○). Values are the mean and the vertical lines show the s.e.mean.

Renal vascular conductances In the first group of animals, under control conditions, all 4 substances caused initial increases in renal vascular conductance (Figure 1 and Table 1). L-NAME (10 mg kg^{-1}) decreased renal vascular conductance from 952 ± 62 to 583 ± 77 units. Following administration of L-NAME, the increases in renal vascular conductance elicited by glyceryl trinitrate (352 ± 24 units) and acetylcholine (300 ± 18 units) were significantly larger than in the absence of L-NAME (188 ± 34 and 208 ± 21 units, respectively), but the renal vasodilator response to bradykinin

Table 1 Ratios between the depressor and the vasodilator responses to acetylcholine, to bradykinin or to endothelin-1 and those to glyceryl trinitrate in the absence of N^G-nitro-L-arginine methyl ester (control) or in its presence (+ L-NAME)

		Acetylcholine	*Bradykinin*	*Endothelin-1*
Mean blood pressure	Control	1.20	1.05	1.25
	+ L-NAME	0.98	0.59	0.78
Renal conductance	Control	1.11	1.21	0.77
	+ L-NAME	0.85	0.57	0.10
Mesenteric conductance	Control	0.49	0.96	*
	+ L-NAME	0.51	0.86	*
Hindquarters conductance	Control	1.17	2.08	2.48
	+ L-NAME	1.08	0.43	1.43

* Endothelin-1 caused mesenteric vasoconstriction only.
Doses as in Figure 1; ratios were derived from the results shown in Figure 1.

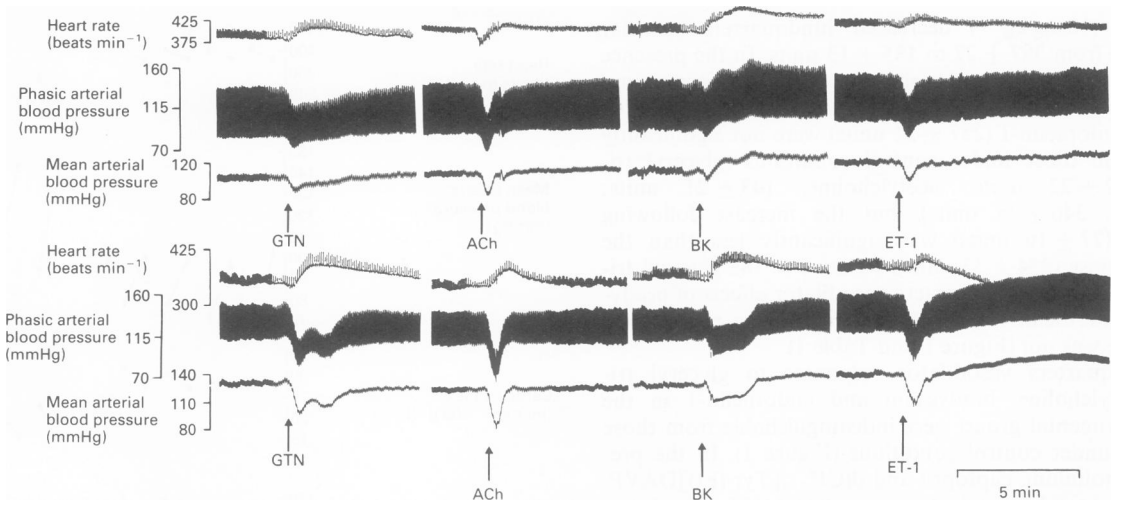

Figure 2 Recordings of blood pressure and heart rate in a Wistar rat anaesthetized with sodium pentobarbitone. The upper traces show the responses to glyceryl trinitrate (GTN, 40 nmol kg^{-1}), acetylcholine (ACh, 1.2 nmol kg^{-1}), bradykinin (BK, 3.2 nmol kg^{-1}) and endothelin-1 (ET-1, 0.25 nmol kg^{-1}). The lower traces show responses to the same 4 substances in the same animal following injection of N^G-monomethyl-L-arginine (50 mg kg^{-1}).

(control, 227 ± 31; plus L-NAME, 199 ± 31 units) was not; there was no significant renal vasodilatation following endothelin-1 in the presence of L-NAME (Figure 1). When considered relative to glyceryl trinitrate, the renal vasodilator effects of bradykinin and endothelin-1 were attenuated in the presence of L-NAME, whereas the response to acetylcholine was not (Figure 1 and Table 1).

In the second group of animals, under control conditions, all 4 substances caused initial increases in renal vascular conductance, similar to those seen in the first group (Figure 1 and Table 2). During combined administration of pentolinium, captopril and $d(CH_2)_5$[Tyr-(Et)]DAVP, renal vascular conductance was 1618 ± 190 units but 10 min after additional administration of L-NAME, renal vascular conductance was 990 ± 16 units. Under these conditions the increases in renal vascular conductance in response to glyceryl trinitrate (327 ± 63 units) and bradykinin (135 ± 84 units) were not significantly different from the responses under control conditions (glyceryl trinitrate, 225 ± 8; bradykinin, 243 ± 29 units). However, the increase in renal vascular conductance in response to acetylcholine (189 ± 68 units) was attenuated (control, 342 ± 55 units) and the renal vasodilatation following endothelin-1 was abolished (Figure 1). Relative to the response to glyceryl trinitrate, the renal vasodilator responses to acetylcholine and bradykinin were both attenuated (Figure 1 and Table 2).

Mesenteric vascular conductances In the first group of animals under control conditions, glyceryl trinitrate, acetylcholine and bradykinin increased mesenteric vascular conductance, whereas endothelin-1 caused mesenteric vasoconstriction (Figure 1).

L-NAME (10 mg kg^{-1}) decreased mesenteric vascular conductance (from 615 ± 76 to 233 ± 35 units) but in the presence of L-NAME, the increases in response to glyceryl trinitrate (309 ± 32 units), acetylcholine (152 ± 17 units) and bradykinin (258 ± 34 units) were not significantly different from those under control conditions (glyceryl trinitrate, 261 ± 63; acetylcholine, 127 ± 51; bradykinin, 250 ± 82 units; Figure 1). Furthermore, relative to glyceryl trinitrate, there was no change in the mesenteric vasodilator responses to acetylcholine or bradykinin (Figure 1 and Table 1).

In the second group of rats the patterns of mesenteric vasodilator responses to glyceryl trinitrate, acetylcholine and bradykinin were similar to those in the first group under control conditions (Figure 1 and Table 2).

During combined administration of pentolinium, captopril and $d(CH_2)_5$[Tyr-(Et)]DAVP, mesenteric vascular conductance was 1222 ± 199 units but, 10 min after the additional administration of L-NAME, mesenteric conductance was 431 ± 68 units (Figure 1). Under those conditions the increases in conductance in response to glyceryl trinitrate (472 ± 98 units) and bradykinin (357 ± 86 units) were not significantly different from those under control conditions (glyceryl trinitrate, 341 ± 32; bradykinin, 285 ± 65 units), but the response to acetylcholine (101 ± 49 units) was reduced (control, 285 ± 57 units). Relative to glyceryl trinitrate, only the response to acetylcholine was attenuated (Figure 1 and Table 2).

Hindquarters vascular conductances In the first group of animals under control conditions all 4 substances caused increases in vascular conductance, although the patterns of change differed, with bradykinin causing a delayed vasodilatation (Figure 1).

Table 2 Ratios between the depressor and the vasodilator responses to acetylcholine, to bradykinin or to endothelin-1 and those to glyceryl trinitrate in the absence of N^G-nitro-L-arginine methyl ester (control) or in the presence of pentolinium, captopril, $d(CH_2)_5$[Tyr-(Et)]DAVP and N^G-nitro-L-arginine (+ PCA, + L-NAME)

		Acetylcholine	*Bradykinin*	*Endothelin-1*
Mean blood pressure	Control	1.52	0.86	1.33
	+ PCA + L-NAME	0.97	1.34	0.66
Renal conductance	Control	1.52	1.08	0.55
	+ PCA + L-NAME	0.58	0.41	*
Mesenteric conductance	Control	0.83	0.84	*
	+ PCA + L-NAME	0.21	0.76	*
Hindquarters conductance	Control	1.61	3.31	3.72
	+ PCA + L-NAME	1.77	2.87	3.57

* No vasodilator responses to endothelin-1.

L-NAME (10 mg kg^{-1}) decreased hindquarters vascular conductance from 387 ± 27 to 185 ± 13 units. In the presence of L-NAME the increases in vascular conductance in response to glyceryl trinitrate (180 ± 30 units), acetylcholine (195 ± 19 units) and endothelin-1 (257 ± 21 units) were not significantly different from those under control conditions (glyceryl trinitrate, 122 ± 22 units; acetylcholine, 143 ± 21 units; endothelin-1, 346 ± 38 units), but the increase following bradykinin (77 ± 16 units) was significantly less than the control response (254 ± 33 units). Relative to the glyceryl trinitrate response, the hindquarters vasodilator effects of bradykinin and endothelin-1 were attenuated but the response to acetylcholine was not (Figure 1 and Table 1).

The hindquarters vasodilator responses to glyceryl trinitrate, acetylcholine, bradykinin and endothelin-1 in the second experimental group were indistinguishable from those in the first, under control conditions (Figure 1). In the presence of pentolinium, captopril and d(CH$_2$)$_5$[Tyr-(Et)]DAVP, hindquarters vascular conductance was 843 ± 126 units, but it fell to 438 ± 67 units 10 min after the additional administration of L-NAME. Under those conditions the increases in hindquarters vascular conductance to glyceryl trinitrate (116 ± 39 units), acetylcholine (205 ± 57 units), bradykinin (333 ± 71 units) and endothelin-1 (414 ± 67 units) were not different from those under control conditions (100 ± 28, 161 ± 39, 331 ± 61, 372 ± 49 units respectively), although the peak response to bradykinin occurred sooner (Figure 1). There were no significant changes in the hindquarters vasodilator responses to acetylcholine, bradykinin or endothelin-1 relative to glyceryl trinitrate (Figure 1 and Table 2).

Cardiac haemodynamic effects of glyceryl trinitrate, acetylcholine, bradykinin and endothelin-1 before and after administration of L-NAME

Under control conditions the hypotensive responses to glyceryl trinitrate (−23 ± 3 mmHg), acetylcholine (−23 ± 3 mmHg), bradykinin (−21 ± 2 mmHg) and endothelin-1 (−21 ± 3 mmHg) were due entirely to reductions in total peripheral conductance since cardiac index did not fall (Figure 3). Indeed, there were significant rises in cardiac index following administration of bradykinin and endothelin-1 (Figure 3). In the presence of L-NAME there were marked reductions in all indices of cardiac function in association with a substantial increase in mean blood pressure and a fall in total peripheral conductance (Figure 3). Under these conditions the hypotensive responses to glyceryl trinitrate (−48 ± 3 mmHg), acetylcholine (−45 ± 4 mmHg), bradykinin (−35 ± 3 mmHg) and endothelin-1 (−37 ± 3 mmHg) were enhanced compared to the responses in the absence of L-NAME (Figure 3). However, relative to the response to glyceryl trinitrate the hypotensive effect of bradykinin was attenuated, but those of acetylcholine and endothelin-1 were not (the latter was on the borderline of significance (Figure 3 and Table 3)).

The rises in total peripheral conductance elicited by bradykinin and endothelin-1 (relative to glyceryl trinitrate) were reduced in the presence of L-NAME, but the relative vasodilator effect of acetylcholine was not (Figure 3 and Table 3).

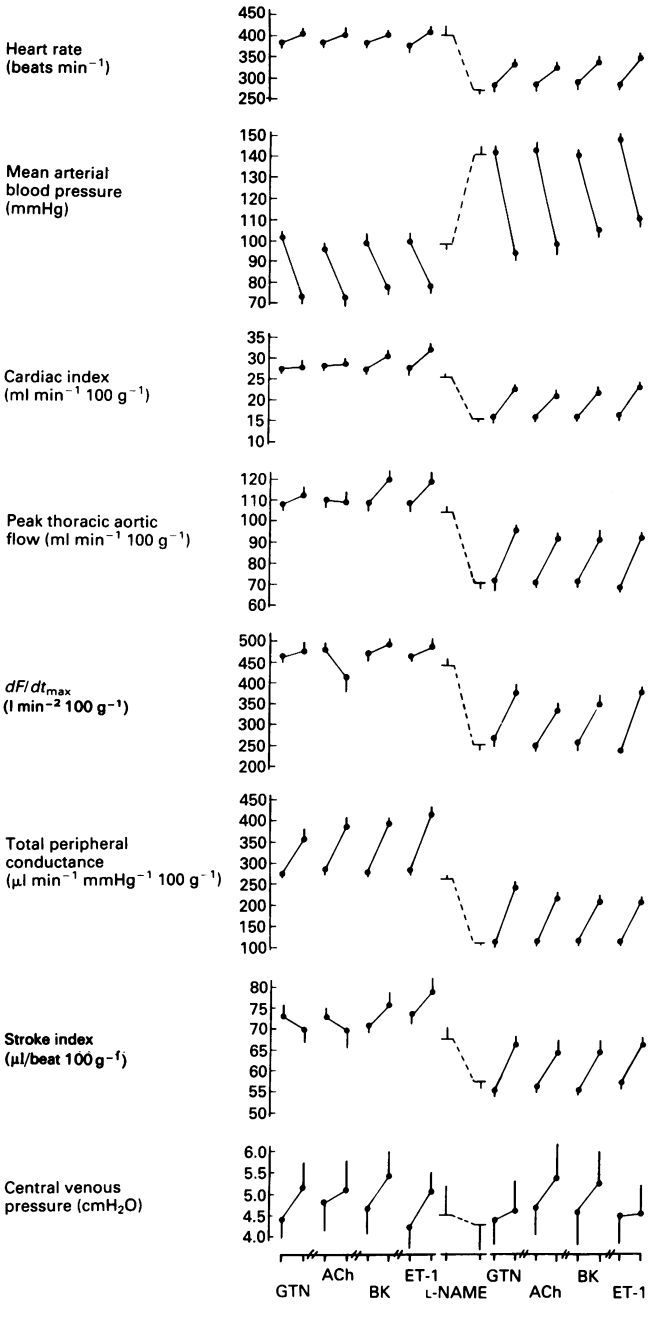

Figure 3 Cardiac haemodynamic responses to i.v. bolus doses of glyceryl trinitrate (GTN, 40 nmol kg^{-1}), acetylcholine (ACh, 1.2 nmol kg^{-1}), bradykinin (BK, 3.2 nmol kg^{-1}) or endothelin-1 (ET-1, 0.25 nmol kg^{-1}) in conscious Long Evans rats (n = 8) in the absence (left hand panels) or in the presence (right hand panels) of N^G-nitro-L-arginine methyl ester (L-NAME, 10 mg kg^{-1}). The steady state changes in cardiovascular variables elicited by L-NAME are shown by the dotted lines in the middle of the figure. The points plotted represent those at the nadir of the hypotensive responses. Values are the mean and the vertical lines show the s.e.mean.

Table 3 Ratios between the depressor and the vasodilator responses to acetylcholine, to bradykinin or to endothelin-1 and those to glyceryl trinitrate in the absence of N^G-nitro-L-arginine methyl ester (control) or in its presence (+ L-NAME)

		Acetylcholine	*Bradykinin*	*Endothelin-1*
Mean blood pressure	Control	1.0	0.91	0.91
	+ L-NAME	0.94	0.73	0.77
Total peripheral conductance	Control	1.21	1.40	1.60
	+ L-NAME	0.82	0.73	0.76

Doses as in Figure 1; ratios were derived from the results shown in Figure 3.

Discussion

The primary objective of the present work was to delineate the effects of L-NAME on the hypotensive and vasodilator effects of acetylcholine, bradykinin and endothelin-1 in conscious rats. Since acetylcholine and bradykinin are considered to be 'classical' endothelium-dependent vasodilators (Furchgott, 1983), and since nitric oxide produced by endothelial cells mediates vasodilatation (Palmer *et al.*, 1987; Moncada *et al.*, 1989; Rees *et al.*, 1989a,b), we reasoned that inhibition of nitric oxide biosynthesis with L-NAME (Moore *et al.*, 1990) ought to affect responses to acetylcholine and bradykinin and, possibly, those to endothelin-1 (Whittle *et al.*, 1989). However, administration of L-NAME had substantial effects on cardiovascular status (see also Gardiner *et al.*, 1990h), so it was necessary to assess the responses to acetylcholine, bradykinin and endothelin-1 relative to a stimulus that acted via the same final common pathway (i.e. cyclic GMP), but not through the production of nitric oxide from L-arginine (Moncada *et al.*, 1988). We chose glyceryl trinitrate for this purpose, matched to give the same hypotensive effects as acetylcholine, bradykinin and endothelin-1 under control conditions in conscious Long Evans rats.

The hypotensive effect of the dose of glyceryl trinitrate used was associated with renal, mesenteric and hindquarters vasodilatations (the effect being most marked in the mesenteric vascular bed). L-NAME caused hypertension and regional vasoconstrictions (Gardiner *et al.*, 1990h) and, under these circumstances, the hypotensive and renal vasodilator effects of glyceryl trinitrate were augmented but the mesenteric and hindquarters vasodilator responses were not changed significantly. In experiments in which L-NAME was administered in the presence of pentolinium, captopril and $d(CH_2)_5$[Tyr-(Et)]DAVP, animals were not hypertensive, and the hypotensive and regional vasodilator effects of glyceryl trinitrate were not augmented, although they were prolonged (Figure 1). These findings indicate that baroreflex and neurohumoral mechanisms may oppose the cardiovascular effects of glyceryl trinitrate under normal conditions. From these results it was clear that the responses to acetylcholine, bradykinin and endothelin-1 could only be considered relative to those of glyceryl trinitrate.

Responses to acetylcholine

Only in the presence of pentolinium, captopril, $d(CH_2)_5$[Tyr-(Et)]DAVP and L-NAME was there any attenuation of the hypotensive and vasodilator (renal and mesenteric) responses to acetylcholine relative to the effects of glyceryl trinitrate. While these results indicate a component of the control response to acetylcholine was probably due to nitric oxide-mediated mechanisms, substantial responses remained under these conditions, and, in particular, the hindquarters vasodilator response was not different from normal. There are several possible explanations for these findings, including: (1) the responses were due to a mechanism not involving nitric oxide (see Long & Berkowitz, 1989); (2) the responses were due to release of nitric oxide from a preformed pool (see Aisaka *et al.*, 1989); (3) the responses were due to nitric oxide, but relatively unaffected by L-NAME due to efficient receptor-effector coupling (Giles *et al.*, 1990). Considering this last possibility we have carried out preliminary experiments to assess the effect of atropine on the sensitivity of acetylcholine-induced vasodilator responses to L-NAME (Giles *et al.*, 1990). However, we have not been able to render the hypotensive or regional vasodilator responses to acetylcholine more sensitive to L-NAME by this intervention (Gardiner, Compton & Bennett unpublished observations).

As indicated above, our present findings do not preclude the possibility that preformed nitric oxide was responsible for the effects of acetylcholine, but they do demonstrate marked differences between *in vitro* (e.g. Moore *et al.*, 1990) and *in vivo* findings. Furthermore, they corroborate previous observations (Gardiner *et al.*, 1989d) showing a basic difference between our results and those of Whittle *et al.* (1989). Thus, under no conditions did we find that the absolute falls in mean (or diastolic) arterial blood pressure elicited by acetylcholine were attenuated in the presence of L-NAME. This result was not peculiar to our main experimental protocol since we obtained similar effects both in anaesthetized rats (Long Evans and Wistar) and when we used L-NMMA rather than L-NAME. It has been suggested (Aisaka *et al.*, 1989) that it is the duration rather than magnitude of hypotensive response to acetylcholine which is affected by inhibiting nitric oxide synthesis, but that did not apply to our results since there was an increase in both the magnitude and duration of the fall in blood pressure following acetylcholine in the presence of L-NAME (Figures 1 and 3).

Initially we thought that the hypotensive responses to glyceryl trinitrate, acetylcholine, bradykinin and endothelin-1 might have been contributed to by differential changes in cardiac function under the different experimental conditions. However, direct measurement showed there was an enhanced increase in cardiac index following administration of all four substances in the presence of L-NAME (Figure 3), and for acetylcholine there was no significant attenuation of the rise in total peripheral conductance (relative to that seen with glyceryl trinitrate) under these conditions. Whatever the explanation of the present findings it is clear that administration of L-NAME (or L-NMMA) *in vivo* does not provide a simple means of quantifying the involvement of nitric oxide in the cardiovascular responses to acetylcholine.

Responses to bradykinin

The comments made above about acetylcholine-mediated responses also pertain, in some respects, to bradykinin, although the detailed picture differed. Thus, there was a relative attenuation of the hypotensive effects of bradykinin in the presence of L-NAME alone. This was associated with renal and hindquarters vasodilatations that were reduced relative to the responses to glyceryl trinitrate, as was the rise in total peripheral conductance in the animals instrumented for measurement of cardiac haemodynamics. However, when L-NAME was administered in the presence of pentolinium, captopril and $d(CH_2)_5$[Tyr-(Et)]DAVP, the hypotensive response to bradykinin was enhanced relative to glyceryl trinitrate and there was no attenuation of the hindquarters vasodilator responses to bradykinin under these conditions (probably due to inhibition of bradykinin catabolism by captopril). These findings indicate that baroreflex or neurohumoral mechanisms, or both, could influence the hindquarters vasodilator responses to bradykinin selectively, since the renal vasodilator effects of bradykinin (relative to glyceryl trinitrate) were attenuated under all experimental conditions, while the peak mesenteric vasodilator effects of bradykinin were not reduced under any conditions (although the profile of change was affected). These results also raise the possibility that nitric oxide-dependent and nitric oxide-independent vasodilator responses to agonists such as bradykinin may be differentially expressed in different vascular beds *in vivo*.

Responses to endothelin-1

The hypotensive effect of endothelin-1 relative to glyceryl trinitrate was attenuated in the presence of L-NAME and the early renal vasodilator effects of endothelin-1 were abolished. In animals in which cardiac haemodynamics were measured there was attenuation of the rise in total peripheral conductance elicted by endothelin-1 relative to glyceryl trinitrate under these conditions. However, the attenuation of the relative hindquarters vasodilator effect of endothelin-1 seen in the presence of L-NAME was not apparent when pentolinium, captopril and $d(CH_2)_5$[Tyr-(Et)]DAVP were also administered, indicating that in the presence of L-NAME alone the hindquarters vasodilator effect of endothelin-1 was probably offset by either baroreflex or neurohumoral mechanisms, or

both. Using a different experimental protocol with a lower dose of endothelin-1, we concluded previously that L-NMMA did not attenuate the hindquarters vasodilator response to this peptide (Gardiner *et al.*, 1989d).

It is of interest that under no conditions did endothelin-1 cause mesenteric vasodilatation, in spite of the finding that release of nitric oxide and cyclo-oxygenase products can cause mesenteric vasodilatation in response to endothelins *in vitro* (De Nucci *et al.*, 1988; Warner *et al.*, 1989a,b; Randall *et al.*, 1989). The present results indicate that our previous observations on the mesenteric haemodynamic effects of endothelin-1 *in vivo* (Gardiner *et al.*, 1989a,d; 1990a,b) were not due to a selective effect in this vascular bed of whatever mechanisms (baroreflex, neurohumoral, or both) activated by the fall in arterial blood pressure.

Conclusions

It appears that in *in vivo* experiments involving L-NAME (or L-NMMA) to determine the possible involvement of nitric oxide in the hypotensive and vasodilator responses to acetylcholine, bradykinin and endothelin-1 an internal standard is required, such as the responses to glyceryl trinitrate, in order to control for the change in cardiovascular variables. Furthermore, it seems that even under those conditions the results obtained may be influenced by baroreflex or neurohumoral mechanisms, or both, in different ways in different vascular beds. In addition, it is likely that the vasodilator responses are contributed to by autoregulatory mechanisms to different extents in different vascular beds, judging by the differential flow patterns seen (Gardiner *et al.*, 1989b; Bennett *et al.*, 1989). One intriguing possibility that arises from the present work is that, *in vivo*, substantial components of some of the active vasodilator responses to acetylcholine, bradykinin and endothelin-1 are nitric oxide-independent, and that this phenomenon is less apparent in studies *in vitro*.

We are grateful to Drs Salvador Moncada and Richard Palmer for useful discussion and suggestions during this study, and also for gifts of L-NMMA.

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(Received May 17, 1990
Revised June 27, 1990
Accepted July 10, 1990)

Br. J. Pharmacol. (1990), **101**, 640–644

Characterization of P_{2x}-receptors in rabbit isolated ear artery

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1 The isolated central ear artery of the rabbit contracts in response to adenosine 5'-triphosphate (ATP) and analogues, effects proposed to be mediated by stimulation of P_{2x}-receptors. We have extended the characterization of the purinoceptor in this tissue by examining the effects of a series of receptor agonists. The study was designed in such a way as to avoid factors which normally limit attempts to classify receptors on the basis of agonist potency orders.

2 D-α,β-methylene ATP (D-α,β-meATP), D-β,γ-methylene ATP (D-β,γ-meATP), L-β,γ-methylene ATP (L-β,γ-meATP), 2-methylthio-D-ATP (2-MeSATP) and ATP produced concentration-related contractions of the ear artery with similar maximum responses, suggesting that they were full agonists. Selective desensitization of P_{2x}-receptors abolished or greatly reduced responses to D-α,β-meATP, L-β,γ-meATP, D-β,γ-meATP and 2-MeSATP. Responses to ATP were inhibited by desensitization but a significant resistant component was still apparent.

3 D-α,β-meATP was the most potent agonist tested (pA_{50} 6.47 ± 0.04) being 2138 times more potent than ATP and approximately 9 times more potent than L-β,γ-meATP. The agonist potency order was: D-α,β-meATP > L-β,γ-meATP > D-β,γ-meATP ≥ 2-MeSATP > ATP. This is generally consistent with the order proposed for P_{2x}-receptors. The relative potencies of P_{2x}-agonists in the rabbit ear artery show both similarities to and differences from data obtained in other smooth muscle preparations.

Introduction

It has been recognized for some time that adenosine 5'-triphosphate (ATP) has pharmacological properties in its own right and these are mediated at P_2-purinoceptors, as distinct from P_1-purinoceptors which are preferentially activated by adenosine (Burnstock, 1978). ATP can, depending upon the chosen experimental conditions, elicit either contractile or relaxant responses from smooth muscle preparations. In 1985, Burnstock & Kennedy proposed that there were sufficient data to support a provisional subclassification of P_2-purinoceptors, designating those receptors mediating contraction of guinea-pig vas deferens, urinary bladder and rabbit ear artery as the P_{2x}-subtype, and those mediating relaxation of guinea-pig taenia coli and various vascular tissues as P_{2y}-receptors; vasorelaxation usually resulted indirectly from the release of endothelial-derived relaxant factors from vascular endothelium (Gordon, 1986).

Given the absence of selective competitive receptor antagonists this sub-classification was based largely on the rank order of agonist potency for a series of structural analogues of ATP, with the order D-α,β-methylene ATP (D-α,β-meATP), D-β,γ-methylene ATP (D-β,γ-meATP) > ATP = 2-methylthio-D-ATP (2-MeSATP) being a general characteristic of the P_{2x}-subtype. The potential pitfalls of attempting to classify receptors using relative agonist potencies alone are well known (e.g. Collis, 1985). Concerns of specific relevance to the investigation of P_{2x}-receptors include the presence of other classes of purinoceptor e.g. P_1, P_{2y} in the same tissue, the use of unstable agonists and the production of poorly-defined agonist concentration-response curves.

Unlike certain visceral smooth muscle preparations, the rabbit isolated central ear artery responds to D-α,β-meATP with a 'classical' sigmoid log agonist concentration-effect curve (Kennedy & Burnstock, 1985), allowing correct definition of a quantitative measure of agonist potency at the 50% response level. This and other factors have led us to choose to characterize the P_2-receptor mediating smooth muscle contraction in this tissue. In doing so we have attempted, where possible, to avoid pitfalls like those listed above which would otherwise tend to reduce the validity of a classification based on relative agonist potencies.

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Spasmogenic effects of series of P_2-purinoceptor agonists, putatively P_{2x}-receptor-mediated, have been described for a number of different tissues e.g. guinea-pig bladder (Cusack & Hourani, 1984), rat portal vein (Reilly & Burnstock, 1987), rabbit mesenteric artery (Burnstock & Warland, 1987) and human pulmonary artery (Liu *et al.*, 1989a). The other purpose of our study was to contribute to the accumulating body of data on these spasmogenic effects, which may allow tentative between-tissue comparisons to be made with respect to the relative potencies of key agonist probes. With this in mind we have included L-β,γ-methylene ATP (L-β,γ-meATP), demonstrated to be a stable, selective P_{2x}-receptor agonist and the most potent agent of this type tested in the guinea-pig bladder (Hourani *et al.*, 1986). A preliminary account of some of this work has been presented to the British Pharmacological Society (O'Connor *et al.*, 1990).

Methods

Tissue preparation

Male New-Zealand White rabbits (2.5–3 kg) were killed by an overdose of pentobarbitone (300 mg i.v.). The ears were removed and the central ear artery dissected out after insertion of a scored polythene cannula (0.75 mm e.d.). The cannula serves as an aid to dissection and as a means of removing the vascular endothelium. The artery was cut into 5–10 mm rings and each ring mounted horizontally on fine tungsten wire hooks in a 20 ml organ bath under isometric conditions. The baths contained Krebs solution of the following composition (mM): NaCl 117.56, NaH_2PO_4 0.89, $NaHCO_3$ 25.0, $MgSO_4$ 1.18, glucose 11.1, KCl 5.36 and $CaCl_2$ 2.55. The tissues were maintained at 37°C and gassed continually with 95% O_2/5% CO_2. Indomethacin (2.8×10^{-6} M) was included in the Krebs to eliminate the influence of products of cyclo-oxygenase. Tissues were set up under an initial tension of 0.5–1 g and allowed to equilibrate for 1 h.

Experimental protocols

Preliminary studies Various preliminary experiments were undertaken for the purpose of establishing the protocol to be used in the study proper.

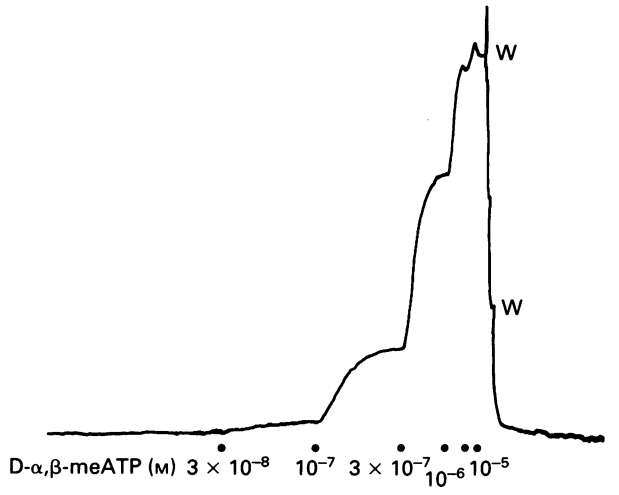

Figure 1 Reproduction of a trace showing a typical cumulative concentration-effect curve to D-α,β-methylene ATP in an isolated, endothelial-denuded, rabbit ear artery. W denotes washing of the tissue.

(a) To determine the validity of using cumulative agonist concentration-effect (E/[A]) curves, D-α,β-meATP and D-β,γ-meATP were examined with a view to comparing E/[A] curves derived from single and cumulative administration of agonist. Single exposure curves were generated using a 40 min dose interval, which had previously been shown to allow stable responses to a submaximal concentration of D-α,β-meATP. Single exposure and cumulative curves were constructed in separate tissue from the same animal.

(b) To establish that agonist sensitivity did not vary significantly throughout the course of the intended protocol, three consecutive cumulative E/[A] curves to D-α,β-meATP were generated at 70 min intervals.

(c) The selective P_1-receptor antagonist 8-sulphophenyltheophylline (8-SPT, Gustafsson, 1984) was included in all experiments in order to eliminate agonist-induced relaxations mediated directly or indirectly at adenosine receptors. A preliminary study was undertaken to confirm that the chosen concentration of 8-SPT (3×10^{-4} M) did not influence responses to a stable selective P_{2x}-receptor agonist (D-α,β-meATP).

(d) An earlier study (Kennedy & Burnstock, 1985) suggested that the smooth muscle of the central ear artery of the rabbit lacks relaxant P_{2y}-receptors. To confirm this we have looked for relaxant properties of the selective P_{2y}-agonist 2-MeSATP in endothelial-denuded preparations contracted with histamine (10^{-6} M).

(e) Exposure to a supramaximal concentration of D-α,β-meATP (3×10^{-5} M, 15 min) was used to desensitize/occupy P_{2x}-receptors and thereby investigate the mechanism by which ATP analogues contract this tissue. The selectivity of this intervention was examined by testing it against cumulative E/[A] curves produced by other spasmogens (potassium chloride, histamine and phenylephrine) in the ear artery.

Comparison of P_{2x}-receptor agonists The relative potencies of a series of selected P_{2x}-agonists and the mechanism by which each contracted the ear artery were investigated using the following protocol.

Tissues were contracted with 80 mM K^+ and 10^{-6} M acetylcholine was added once the contraction had stabilized to confirm functional denudation of endothelium. After washing, the tissues were incubated with 3×10^{-4} M 8-SPT for 45 min and a E/[A] curve was constructed to the agonist internal standard, D-α,β-meATP, by cumulative additions at 0.5 $\log_{10}$ unit increments. After washing, 8-SPT was re-administered and, 70 min after the first curve, a cumulative E/[A] curve was generated for the P_{2x}-agonist under test. The tissues were washed again, 8-SPT re-administered and D-α,β-meATP added at a supramaximal concentration (3×10^{-5} M) to produce desensitization/occupancy of P_{2x}-receptors. Once the contraction had faded (15 min) and in the continued presence of D-α,β-meATP the E/[A] curve to the agonist under test was repeated to confirm mechanism of action.

Data analysis

Contractions were expressed as a percentage of the maximum response (α) produced by the internal standard, D-α,β-meATP, in the first E/[A] curve in each tissue. Negative log molar agonist concentrations producing 50% of the maximum response (pA_{50} values) were used throughout as the index of agonist potency. These were calculated by fitting each E/[A] curve data set to a logistic function of the form;

$$E = \frac{\alpha[A]^m}{[A_{50}]^m + [A]^m}$$

in which α and m are asymptote and slope parameters, respectively, E is effect and [A] is agonist concentration.

Mean ± s.e. pA_{50} values were derived by meaning the values obtained in single tissues each taken from a different animal, with n = number of rabbits. Where appropriate, the statistical significance of differences between group pA_{50} values was determined by one-way analysis of variance with $P < 0.05$ considered to be significant.

Drugs

Drugs were obtained from the following sources; acetylcholine, adenosine 5'-triphosphate, D-α,β-methylene ATP, D-β,γ-methylene ATP, histamine, indomethacin and phenylephrine (Sigma, Poole, U.K.); 2-methylthio-D-ATP and 8-sulphophenyltheophylline (Research Biochemicals Inc., St. Albans, U.K.; L-β,γ-methylene ATP was synthesized by P.A. Cage and S.F. Hunt in the Department of Medicinal Chemistry, Fisons, Loughborough. Indomethacin was dissolved initially in 10% Na_2CO_3, all other drugs were dissolved in distilled water.

Results

Preliminary studies

(a) Agonist potencies derived from cumulative E/[A] curves did not differ significantly from those values obtained from single exposure curves for both of the examples chosen. pA_{50} values were: D-α,β-meATP 6.49 ± 0.07 (cumulative) and 6.57 ± 0.04 (single exposure), $n = 5$; D-β,γ-meATP 4.36 ± 0.12 (cumulative) and 4.26 ± 0.11 (single exposure), $n = 3$. Cumulative E/[A] curves were therefore used throughout the rest of the study.

(b) Cumulative E/[A] curves to D-α,β-meATP repeated at 70 min intervals showed no alteration in responsiveness to the agonist. pA_{50} values for first, second and third curves were; 6.36 ± 0.05, 6.36 ± 0.06 and 6.35 ± 0.07, respectively, $n = 3$.

(c) Inclusion of 8-SPT did not affect the potency of D-α,β-meATP. pA_{50} values were; 6.47 ± 0.04 (vehicle) and 6.44 ± 0.06 (in the presence of 3×10^{-4} M 8-SPT), $n = 4$.

(d) In tissues contracted with histamine, 2-MeSATP (3×10^{-8} M–3×10^{-5} M) did not produce relaxations ($n = 3$). Concentrations above 3×10^{-6} M caused further contraction of the tissues.

(e) Continuous exposure to a desensitizing concentration of D-α,β-meATP did not change the sensitivity of the ear artery to a series of other spasmogens. pA_{50} values obtained for each agent under control and desensitized conditions were as follows: potassium chloride 1.51 ± 0.04, 1.54 ± 0.03, $n = 4$; histamine 6.28 ± 0.09, 6.19 ± 0.04, $n = 4$; phenylephrine 6.99 ± 0.11, 7.02 ± 0.11, $n = 4$.

Comparison of P_{2x}-receptor agonists

A reproduction of an experimental trace showing a typical cumulative response curve to D-α,β-meATP is illustrated in Figure 1. Of the other agonists tested L-β,γ-meATP and 2-MeSATP showed qualitatively similar responses, while those to ATP and D-β,γ-meATP were somewhat less tonic in nature.

Figure 2 shows E/[A] curves for each of the agonists tested in the study (second curves of the protocol). It is apparent that all the compounds tested produced curves which had similar slopes and were sigmoid in nature. In addition, all appeared to be full agonists, as judged by the similarity of their maximum responses (α). Mean α, pA_{50} values and relative potencies are shown in Table 1. D-α,β-meATP was, by a clear margin, the most potent agent tested, showing a potency 2138 times greater than ATP and 9 times greater than the next most potent analogue, L-β,γ-meATP. The relative order of agonist potencies was: D-α,β-meATP > L-β,γ-meATP > D-β,γ-meATP ≥ 2-MeSATP > ATP.

The effect of desensitization/occupancy of P_{2x}-receptors, following exposure to a supramaximal concentration of D-α,β-meATP, on the contractile responses to each agonist is shown in Figure 3. Responses to D-α,β-meATP and L-β,γ-meATP were abolished by this intervention over a wide concentration range. The effects of D-β,γ-meATP and 2-MeSATP were also effectively eliminated, since residual responses amounted to approximately 10% and 19% respectively at the highest concentrations tested. Only ATP showed a significant resistant spasmogenic response, although the E/[A] curve was clearly right-shifted and depressed following desensitization.

Discussion

In this study of P_{2x}-receptors in the rabbit ear artery, we have tried to avoid some of the problems commonly encountered when attempting to classify receptor types by use of agonist potency orders. ATP and analogues may activate P_1-receptors

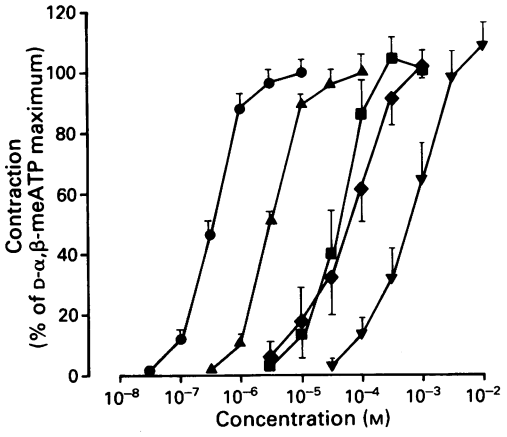

Figure 2 Cumulative log agonist concentration-effect curves for contractions of the rabbit ear artery to D-α,β-methylene ATP (D-α,β-meATP, ●), L-β,γ-meATP (▲), D-β,γ-meATP (■), 2-methylthio-D-ATP (◆) and ATP (▼). Points are means and vertical lines show s.e.mean, $n = 5$, except 2-MeSATP ($n = 3$).

Table 1 Potencies of P_{2x}-receptor agonists to contract the rabbit isolated ear artery

Compound	n	α	pA_{50}	*Relative potency*
D-α,β-meATP	5	100 ± 4	6.47 ± 0.04	2138
L-β,γ-meATP	5	100 ± 4	5.52 ± 0.04	240
D-β,γ-meATP	5	106 ± 6	4.37 ± 0.12	17
2-MeSATP	3	110 ± 7	4.15 ± 0.16	10
ATP	5	116 ± 7	3.14 ± 0.14	1

All data taken from the second E/[A] curve in each tissue.

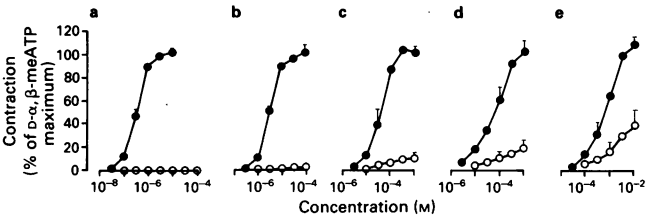

Figure 3 The effect of desensitization with D-α,β-methylene ATP (D-α,β-meATP) on contractile responses of the rabbit ear artery to (a) D-α,β-meATP, (b) L-β,γ-meATP, (c) D-β,γ-meATP, (d) 2-methylthio-D-ATP and (e) ATP. (●) Control responses and (○) responses after desensitization. Points are means and vertical lines show s.e.mean, $n = 5$, except 2-MeSATP ($n = 3$).

resulting in smooth muscle relaxation, as has been shown previously in the ear artery (Kennedy & Burnstock, 1985). This effect, which could interfere with accurate assessment of spasmogenic potency, may occur either directly, or indirectly following degradation to an ADP analogue or adenosine. We have recently illustrated the potential importance of this property by demonstrating that the relaxant effects of the P_{2y}-agonist, ADP-β-F, in the rabbit jugular vein are primarily mediated at P_1-receptors (Wood *et al.*, 1989). In the present study 8-SPT was included throughout to abolish effects of P_1-receptor activation at a concentration which did not affect responsiveness to the stable P_{2x}-analogue D-α,β-meATP. 8-SPT is a potent antagonist at P_1-receptors (pA_2 against N-ethylcarboxamidoadenosine in rabbit jugular vein 6.42, unpublished observation) and is reputed to lack the phosphodiesterase inhibitory properties associated with other compounds of this type, because of poor cell penetration (Gustafsson, 1984). Potential interference from P_{2y}-receptor stimulation was also addressed. Although present in visceral smooth muscle, in the majority of vascular tissues these are exclusively of endothelial location, so denudation is an effective means of eliminating their influence. The rabbit central ear artery appears unusual, in that P_{2y}-receptors have been shown to be absent from both vascular endothelium and smooth muscle (Kennedy & Burnstock, 1985), and we have confirmed that denuded toned preparations do not relax to 2-MeSATP. Of the agonists tested in this study, D-α,β-meATP, L-β,γ-meATP and D-β,γ-meATP have been found to be relatively resistant to degradation by ectonucleotidases (Welford *et al.*, 1987) and therefore are acceptable for receptor classification purposes. Clearly the same does not apply to ATP and 2-MeSATP which are readily dephosphorylated (Welford *et al.*, 1987), although by blocking P_1-receptors in this study we have minimized the consequences of any degradation. Attractive features of the E/[A] curves produced by P_{2x}-agonists in the rabbit ear artery are their sigmoid form, similar slopes and clearly defined maxima. Such characteristics allow agonist potency data to be interpreted with confidence. This contrasts with the biphasic curves observed for contraction of guinea-pig vas deferens (Fedan *et al.*, 1982) and guinea-pig bladder (Cusack & Hourani, 1984) and the poorly defined curves obtained in certain vascular tissues, for example, rat aorta (White *et al.*, 1985), rat pulmonary artery (Liu *et al.*, 1989b) and human small pulmonary vessels (Liu *et al.*, 1989a). Finally, although we have taken measures to increase the validity of the classification of P_{2x}-receptors in this tissue, it should be remembered that the observed potency of an agonist reflects both its affinity for the receptor and its efficacy. Tissue-related changes in receptor density or coupling efficiency can result in significant variation in responsiveness to compounds of different efficacies. Calculation of agonist affinity, as possible amongst a series of partial agonists, would provide the definitive agonist-based classification of P_{2x}-receptors.

Receptor desensitization/occupancy produced by sustained exposure to a supramaximal concentration of D-α,β-meATP was used to establish the extent to which agonist-induced contractions could be attributed to activation of P_{2x}-receptors. In

the absence of a selective receptor antagonist, D-α,β-meATP desensitization was first introduced to investigate the non-adrenergic, non-cholinergic (NANC) component of guinea-pig bladder nerve stimulation (Kasakov & Burnstock, 1983), and has since been used extensively for the purpose of characterizing effects mediated at P_{2x}-receptors (e.g. Liu *et al.*, 1989b). The selectivity of this intervention in our hands was established by the demonstration that it eliminated subsequent contractile responses to D-α,β-meATP over a 1000 fold concentration range, without influencing responses to histamine, phenylephrine and potassium. This desensitization protocol effectively abolished responses to D-α,β-meATP, L-β,γ-meATP, D-β,γ-meATP and 2-MeSATP confirming their mechanism of action. Responses to ATP, although inhibited, did show a component which was resistant to desensitization. This may have introduced a slight over-estimate of its potency at P_{2x}-receptors as based on the calculated pA_{50} value, but this does not significantly alter relative agonist potencies. Other investigators have shown small desensitization-resistant contractions to ATP in the rabbit ear artery (Kennedy *et al.*, 1986; von Kugelgen *et al.*, 1987). The resistant component appears more prominent in our study because we have gone to higher concentrations of ATP in order to define fully its E/[A] curve. The mechanism responsible is not clear, although an effect at pyrimidine-recognising receptors, as shown in the rabbit ear artery for uridine 5'-triphosphate (von Kugelgen *et al.*, 1987), is a possibility.

The absolute potencies of D-α,β-meATP and ATP found in the present study show excellent agreement with those obtained earlier for the rabbit ear artery (Kennedy & Burnstock, 1985). The relative order of agonist potency found in this study; D-α,β-meATP > L-β,γ-meATP > D-β,γ-meATP ≥ 2-MeSATP > ATP, is broadly consistent with the order designated as characteristic of P_{2x}-receptors (Burnstock & Kennedy, 1985, see Introduction). Another criterion of Burnstock's classification, selective desensitization by D-α,β-meATP, has also been fulfilled. A number of points arising from the present data are worthy of note. For example, the Burnstock classification does not distinguish between D-α,β-meATP and D-β,γ-meATP, yet in this tissue they differed in potency by more than 100 fold, a result comparable with that found for rabbit mesenteric artery (Burnstock & Warland 1987), but apparently dissimilar to the guinea-pig bladder where these analogues have similar potency (Cusack *et al.*, 1987; Welford *et al.*, 1987). The activity of L-β,γ-meATP is interesting in two respects. Firstly, because it showed 14 fold greater potency than its D-isomer. This is at odds with the general stereochemical preference for D-ribose forms attributed to P_2-purinoceptors (Burnstock & Kennedy, 1985; Gordon, 1986). Of possibly greater importance is the observation that L-β,γ-meATP was 9 fold less potent than D-α,β-meATP in the ear artery, in contrast to the guinea-pig bladder where it has been described as the most potent P_{2x}-receptor agonist tested (Hourani *et al.*, 1986). Overall, the order of agonist potency observed in the rabbit ear artery is similar to that described for rat portal vein longitudinal muscle (Reilly & Burnstock, 1987), but quite different from that demonstrated in guinea-pig bladder where L-β,γ-meATP is the most potent agonist and D-α,β-meATP and D-β,γ-meATP have similar potency (Cusack & Hourani, 1984; Welford *et al.*, 1987).

In view of the foregoing discussion on the limitations of attempting to classify receptors by use of relative agonist potencies alone, the significance of the apparent differences highlighted between this and other studies investigating P_{2x}-receptor characteristics in various tissues in not easy to assess. It is certainly conceivable that they merely reflect tissue-related factors and variations in experimental protocols, and in particular the extent to which individual studies have managed to eliminate possible sources of error. Alternatively, they may be the first indicators of a genuine difference between the receptors involved. Further quantitative studies of the P_{2x}-receptors mediating spasmogenic effects would appear warranted, particularly those comparing vascular and visceral smooth muscle preparations.

The authors wish to thank Miss N.T. Fear for technical assistance.

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(Received May 21, 1990
Accepted June 27, 1990)

Br. J. Pharmacol. (1990), **101**, 645–649

Suramin is a slowly-equilibrating but competitive antagonist at P_{2x}-receptors in the rabbit isolated ear artery

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1 The antagonist dynamics of suramin were investigated at P_{2x}-receptors in isolated rings of endothelium-denuded ear artery from New Zealand White (NZW) rabbits.

2 α,β-Methylene adenosine 5'-triphosphate (ATP) concentration-effect curves were constructed cumulatively in a paired curve design in the absence and presence of increasing concentrations of suramin, incubated for 45 min. The slope of the resulting Schild plot was significantly greater than unity (1.50 ± 0.08).

3 Assuming that slow equilibration by suramin explains the steep Schild plot, further experiments were conducted using short (15 min) and long (3 h) incubation times. The resulting Schild plot slopes were 1.66 ± 0.36 and 1.06 ± 0.13 respectively confirming the assumption. However, after 3 h incubation, suramin also caused depression of α,β-methylene ATP curves.

4 In an attempt to minimize the depressant effect of suramin, a kinetic study was designed to calculate the minimum incubation times for each concentration of suramin used in the Schild analysis to achieve effectively complete equilibrium. Theoretically fractional occupancy for the antagonist is given by $(r-1)/r$, where r is the dose-ratio. A plot of $(r-1)/r$ against time allowed the apparent 'on' and 'off' rate constants to be calculated.

5 With the resulting rate constant estimates, an optimised antagonism study was carried out in which incubation times were chosen such that >95% occupancy by suramin could be achieved without agonist curve depression at each concentration of suramin used.

6 Under these conditions, suramin fulfilled all criteria for simple competition: parallel rightward displacement of α,β-methylene ATP curves and a Schild plot slope of unity (1.00 ± 0.09). The resulting pK_B estimate was 4.79 ± 0.05. This estimate of affinity was shown to be independent of the agonist used in another experiment in which L-β,γ-methylene ATP was employed ($pK_B = 5.17$).

7 Under the same conditions, suramin was found to have no effect on KCl-induced contractions and only slight effects on phenylephrine- and histamine-induced responses.

8 This analysis provides the first evidence that suramin is a genuine competitive P_{2x}-receptor antagonist.

Introduction

The quantitative classification of purine receptors of the P_{2x}-class and their identification in different tissues is seriously limited by the lack of genuinely competitive and selective antagonists. However, an agent which has been claimed to possess antagonistic properties at these receptors is the trypanocide, suramin (Dunn & Blakely, 1988). This substance has been shown to inhibit P_{2x}-receptor-mediated contractile responses to α,β-methylene adenosine 5'-triphosphate (ATP) in the mouse isolated vas deferens (Dunn & Blakely, 1988) and in guinea-pig bladder strips (Hoyle *et al.*, 1990), although in an earlier study in the latter tissue suramin was apparently without effect (Hourani & Chown, 1989).

However, no study has appeared which establishes whether, according to quantitative pharmacological criteria (Schild, 1973; Black *et al.*, 1983), the actions of suramin accord with simple competition. Indeed, where antagonist action has been demonstrated, there was clear evidence for deviation from competitive dynamics: steepening of agonist concentration effect (E/[A]) curves in one case (Dunn & Blakely, 1988); apparent potentiation followed by E/[A] curve depression in the other (Hoyle *et al.*, 1990). Under these circumstances the utility of suramin as a probe for P_{2x}-receptors must remain questionable. Despite this, a number of other studies have appeared (Schlicker *et al.*, 1989; Mallard *et al.*, 1990) which seem to presume the reliability of suramin as a classification tool.

The purpose of this study, therefore, was to determine whether the designation of suramin as a competitive P_{2x}-receptor antagonist could be substantiated. The tissue used for this study was the rabbit isolated ear artery. This preparation was chosen because it exhibits well-defined and reproducible contractile responses to ATP analogues which, according to agonist potency order information, are subserved by P_{2x}-receptors and are amenable to quantitative pharmacological analysis (O'Connor *et al.*, 1990). A preliminary account of the results of this study were presented to the British Pharmacological Society (Wood *et al.*, 1990).

Methods

Rabbit ear artery

Isolated, endothelium-denuded rings of central ear artery from male NZW rabbits (2.5–3.0 kg) were prepared as described in the preceding paper (O'Connor *et al.*, 1990). Each ring was suspended in a 20 ml organ bath containing Krebs solution of the following composition (mM): NaCl 117.56, NaH_2PO_4 0.89, $NaHCO_3$ 25.0, $MgSO_4$ 1.18, glucose 11.1, KCl 5.36 and $CaCl_2$ 2.55; aerated with 95% O_2: 5% CO_2, pH 7.4, 37°C. The bathing solution also contained indomethacin (2.8 μM). Contractile responses were recorded as changes in isometric force by Ormed Beam transducers and displayed on Advance Bryans flat-bed chart recorders. Each tissue was subjected to an initial force of 1 g which reduced upon subsequent relaxation to between 0.50 and 0.75 g.

Experimental protocols

Each tissue was initially subjected to challenges with KCl (80 mM) followed by acetylcholine (1 μM) in order to establish

tissue viability and to confirm the absence of endothelium, respectively. Following several washes of the tissue with fresh Krebs solution to regain basal tone 8-sulphophenyltheophylline (8-SPT) (0.3 mM) was added (O'Connor et al., 1990) and readministered after all subsequent exchanges of bath fluid.

After an equilibration period of 45 min a cumulative E/[A] curve was constructed to α,β-methylene ATP or another contractile agent (histamine, phenylephrine or KCl). Then, following washout and re-equilibration, suramin or vehicle was added to the bath and after a subsequent incubation period (see Results for details) a second E/[A] curve was constructed. In different studies, differences in experimental design meant that the total interval between the first and second curves varied, but within any particular study the interval was kept the same. Moreover, control experiments were conducted to quantify any systematic differences between first and second curves that may have occurred (see Results). Other than where stated, no significant differences were encountered between first and second control curves.

Drugs and solutions

Stock solutions of α,β-methylene ATP (dilithium salt, Sigma), 8-sulphophenyltheophylline (Research Biochemicals Inc.), phenylephrine hydrochloride (Sigma), L-β,γ-methylene ATP (tetrasodium salt, prepared by P.A. Cage and S.F. Hunt, Medicinal Chemistry Department, Fisons) were made up in distilled water; suramin (Bayer, UK) was prepared in Krebs solution of the same composition given above; indomethacin (Sigma) was prepared as a 28 mM solution in Na_2CO_3 (0.35 M) and subsequently diluted with Krebs solution; histamine dihydrochloride (Sigma) was made up as a 0.2 M solution in distilled water, then neutralised by addition of 10 M NaOH (10 μl to 2 ml).

Analysis of data

E/[A] curve fitting Each E/[A] curve data set was fitted by a logistic function of the form:

$$E = \frac{\alpha[A]^m}{[A_{50}]^m + [A]^m} \quad \text{(i)}$$

in which α, $[A_{50}]$ and m are the asymptote, location and slope parameters respectively. $[A_{50}]$ values were estimated as logarithms and are quoted as $p[A_{50}]$s ($-\log_{10}[A_{50}]$).

Systematic differences between first and second E/[A] curves were tested by one-way analyses of variance on the three parameter estimates. Suramin-induced curve displacements were tested for parallelism by one-way analyses of variance on estimates of α and n.

Analysis of antagonism In experiments where suramin was shown to produce parallel displacements of α,β-methylene ATP curves, dose-ratios (r) were calculated from the $[A_{50}]$ estimates for each pair of curves and fitted to the equation (Arunlakshana & Schild, 1959):

$$\log_{10}(r - 1) = n\log_{10}[B] + pK_B \quad \text{(ii)}$$

Accordance with simple competition was tested by comparing the Schild plot slope parameter, n, with unity by Student's *t* test.

Kinetic analysis In one experiment, the dependence of the antagonistic effects of suramin on incubation time was examined. The following reaction scheme was assumed to apply between antagonist, B and receptors, R,

$$B + \underset{(1-\rho_B)}{R} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \underset{(\rho_B)}{BR} \quad \text{(iii)}$$

in which k_1 and k_{-1} are the association and dissociation rate constants respectively and ρ_B is the fractional occupancy of receptors by B. Paton (1961) showed that the kinetics of this reaction are described by the equation:

$$\rho_B = \rho_{Beq}(1 - \exp\{-(k_1[B] + k_{-1})t\}) \quad \text{(iv)}$$

in which ρ_{Beq} is the equilibrium value of ρ_B. Paton (1961) also showed that to produce the same response in the presence of B as in its absence, the agonist concentration must be raised by $1/(1 - \rho_B)$, that is the dose-ratio, r, is defined by:

$$r = 1/(1 - \rho_B) \quad \text{(v)}$$

Upon rearrangement this gives:

$$\rho_B = (r - 1)/r \quad \text{(vi)}$$

meaning that fractional occupancy values for B can be calculated from the dose-ratios it produces. Therefore, in theory, a plot of $(r - 1)/r$ against time allows the kinetics of equilibration of antagonist occupancy to be characterized according to equation (iv) (Paton, 1961).

In practice, dose-ratios produced by suramin were estimated from pairs of α,β-methylene ATP curves, where second curves were obtained following a range of suramin incubation times. The $(r - 1)/r$ values were calculated from each pair and fitted as a function of time by use of equation (iv). Rate constant estimates were obtained and an estimate of ρ_{Beq}. By definition, ρ_{Beq} is the equilibrium value of occupancy, that is, $[B]/(K_B + [B])$, so an estimate of K_B, the dissociation constant for B can be made from this quantity as well as from the ratio, k_{-1}/k_1.

The rate constant estimates made by this analysis were subsequently used to calculate (using equation (iv)) the incubation times required to achieve at least 95% occupancy by suramin at different concentrations.

In all experiments, the times stated refer to the periods of incubation before the construction of E/[A] curves.

Errors on parameter estimates are standard errors. All fitting procedures were carried out with a VAX Mainframe computer, employing the BMDP Statistical software package.

Results

Preliminary analysis of suramin antagonism

Figure 1a illustrates the effects of increasing concentrations of suramin, incubated for 45 min, on α,β-methylene ATP E/[A] curves. Although the E/[A] curves appeared to be displaced in parallel, analysis showed that there was a significant steepening with increasing concentrations of suramin. However, more importantly, the slope of the associated Schild plot (Figure 1b) showed marked and significant deviation from unity (1.50 ± 0.08, 18 d.f.).

Effect of incubation time

Figure 2 illustrates the antagonistic effects of 30 μM (a) and 1 mM (b) suramin after 15 min and 3 h incubation. After 15 min incubation, 30 μM suramin produced negligible curve shifts ($\Delta p[A_{50}] = 0.08 \pm 0.11$, 8 d.f.) whereas after 3 h incubation the same concentration produced substantial shift ($\Delta p[A_{50}] = 0.45 \pm 0.09$, 8 d.f.). The higher concentration of suramin produced the same displacement of α,β-methylene ATP curves at 15 min and 3 h ($\Delta p[A_{50}] = 1.84 \pm 0.09$, 8 d.f. and 1.87 ± 0.19, 9 d.f., respectively). This behaviour would be expected with a slowly-equilibrating antagonist (Paton, 1961; Kenakin, 1980). Figure 2b also shows that exposure of the tissue to 1 mM suramin for 3 h caused depression of α,β-methylene ATP E/[A] curves.

Kinetics of equilibration

Figure 3 illustrates the kinetics of equilibration by suramin (30 μM). Fractional occupancy values for suramin were calcu-

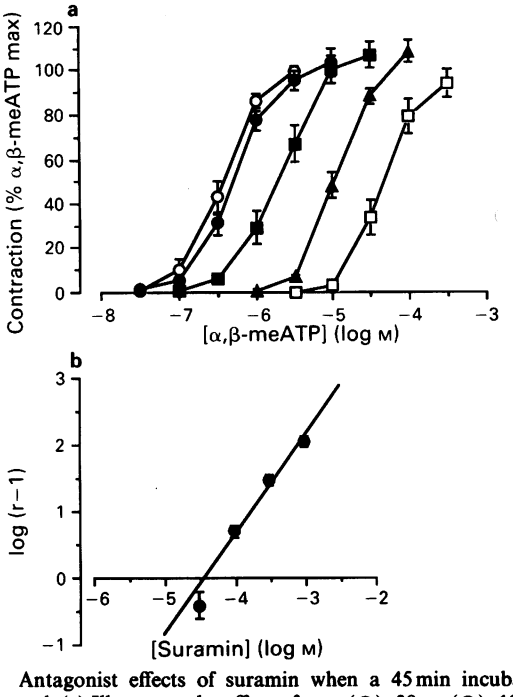

Figure 1 Antagonist effects of suramin when a 45 min incubation time was used. (a) Illustrates the effect of zero (○), 30 μM (●), 100 μM (■), 0.3 mM (▲) and 1 mM (□) suramin on α,β-methylene ATP E/[A] curves in the rabbit ear artery. For illustration purposes only the second curve data are shown. Each point represents the mean and vertical lines show s.e.mean ($n = 5$). Statistical analysis was carried out on paired curve data as described in the text. (b) The Schild plot derived from the associated paired dose-ratio (r) data. The slope of the plot was 1.50 ± 0.08 (18 d.f.) which was significantly greater than unity (*t* test, $P < 0.001$).

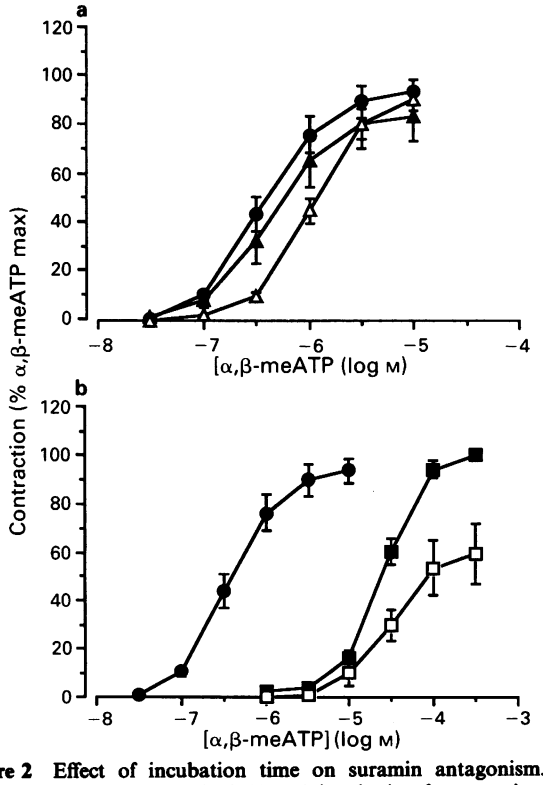

Figure 2 Effect of incubation time on suramin antagonism. The effect of 15 min (▲, ■) and 3 h (△, □) incubation for suramin, 30 μM (a) and 1 mM (b) on α,β-methylene ATP E/[A] curves is shown. Control curve to α,β-methylene ATP represented by (●). Average response data are shown with vertical lines indicating s.e.mean ($n = 4$ or 5). Following 15 min incubation, 30 μM suramin produced negligible curve displacement whereas after 3 h incubation, a 0.45 $\log_{10}$ unit shift was produced. Suramin 1 mM produced effectively the same curve displacement under the two conditions, 1.84 (15 min) and 1.87 $\log_{10}$ units. (b) Also shows how, following a 3 h exposure, 1 mM suramin produced a substantial depression of the α,β-methylene ATP dose-response curve.

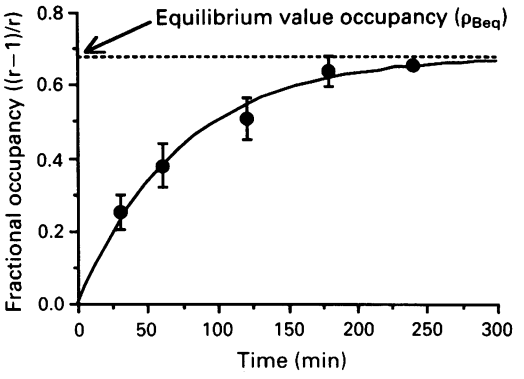

Figure 3 Kinetics of equilibration by suramin. The diagram shows fractional occupancy of suramin (30 μM) plotted against time. Occupancy values were calculated from dose-ratios (r) according to Paton (1961) as described in the text. Average values are shown with vertical lines indicating s.e.mean ($n = 5$). The line drawn through the data was obtained by fitting equation (iv) to the whole set of fractional occupancy values. The estimated association and dissociation rate constants were 306 $min^{-1} M^{-1}$ and 4.4 × 10^{-3} min^{-1}, respectively.

lated from dose-ratios elicited by the antagonist at different times as explained in the Methods section. In these experiments the total time elapsing between the two E/[A] curves was 4 h. Control experiments under these conditions showed that the second curve was slightly but significantly left-shifted compared with the first curve ($\Delta p[A_{50}] = 0.053 \pm 0.017$, 15 replicates). Individual dose-ratios calculated in the kinetic analysis were corrected by this factor. The line drawn through the fractional occupancy (r/(r − 1)) values is the result of fitting equation (iv) to the data. The resulting rate constant estimates were: $k_1 = 306 \pm 45\ min^{-1} M^{-1}$; $k_{-1} = 4.40 \pm 1.52 \times 10^{-3}\ min^{-1}$ equivalent to a $pK_B(-\log_{10}(k_{-1}/k_1))$ of 4.84.

Optimised analysis of suramin antagonism

The above rate constant estimates were used to calculate the minimum times for different concentrations to achieve at least 95% occupancy, that is, values of $\rho_B/\rho_{Beq} > 95\%$, using equation (iv). These were: 30 μM, 220 min; 100 μM, 86 min; 0.3 mM, 31 min; 1 mM, 10 min. With these incubation times another analysis of antagonism was performed. The total time elapsing between first and second curves was again 4 h. The results of this experiment are shown in Figure 4. Analysis of variance indicated that α,β-methylene ATP E/[A] curves were shifted in parallel (Figure 4a) and the Schild plot derived from the paired dose-ratios (corrected by the same factor as above) had a slope of unity (1.00 ± 0.09, 23 d.f.) (Figure 4b). The pK_B estimate (obtained with n in equation (ii) constrained to unity) was 4.79 ± 0.05 (24 d.f.).

Agonist-independence of suramin affinity

With the latter, optimised conditions, suramin was tested as an antagonist against a second agonist, L-β,γ-methylene ATP. The results of this experiment are shown in Figure 5.

Selectivity of suramin

Suramin (1 mM, 15 min incubation) was tested against other constrictor agents. It had no effect on KCl E/[A] curves, but produced slight and in the latter case, significant displacements of phenylephrine ($\Delta p[A_{50}] = 0.22 \pm 0.12$, 10 d.f.) and histamine (0.36 ± 0.06, 8 d.f.) curves.

Discussion

This study describes an attempt to characterize the antagonistic properties of suramin at P_{2x}-receptors in the rabbit ear

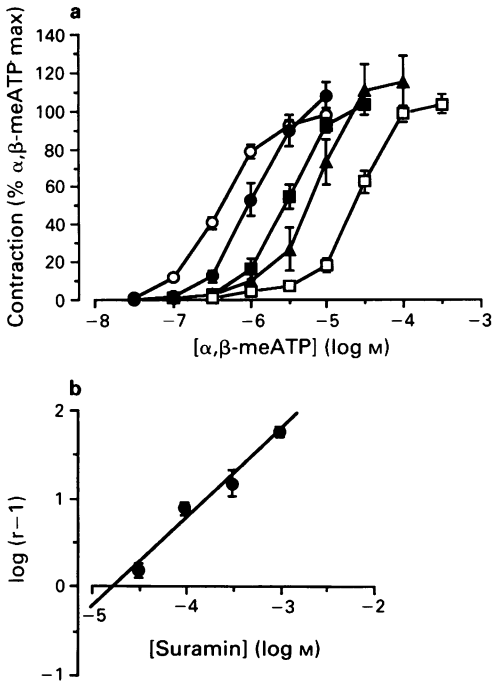

Figure 4 Optimised analysis of suramin antagonism. Minimum incubation times to achieve 95% occupancy were calculated for different concentrations of suramin by use of the following estimated rate constants (Figure 3 and analysis): 30 μM (●), 220 min; 100 μM (■), 86 min; 0.3 mM (▲), 31 min; 1 mM (□), 10 min. (a) Average second α,β-methylene ATP E/[A] curves. Vertical lines show s.e.mean (n = 6–9). Analysis of variance of the paired curves indicated no significant deviation from parallelism, consistent with simple competition. Control curve to α,β-methylene ATP is represented by (○). (b) Illustrates the associated Schild analysis of the paired dose-ratio (r) data. The slope of the plot was 1.00 ± 0.09, consistent with simple competition, and the estimated pK_B was 4.79 ± 0.05.

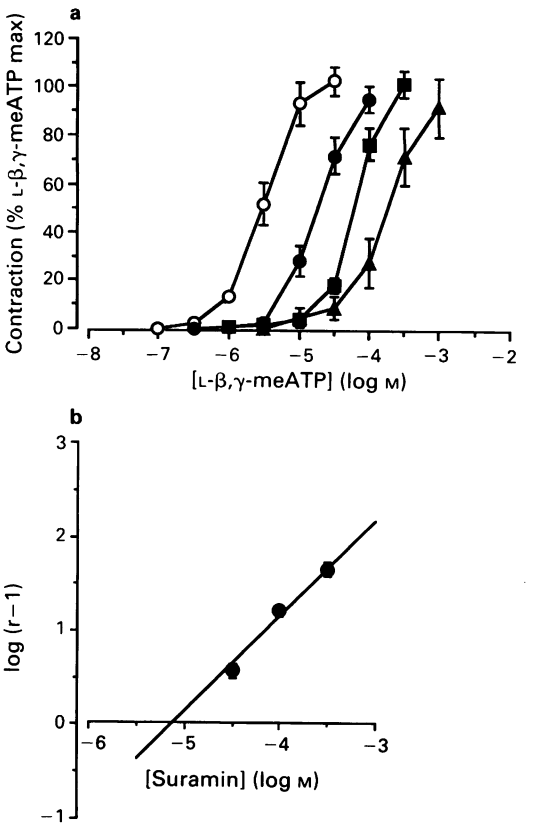

Figure 5 Agonist-independence of suramin affinity. (a) Shows the effects of zero (○), 30 μM (●), 100 μM (■) and 0.3 mM (▲) suramin on L-β,γ-methylene ATP E/[A] curves. Average second curves (n = 6) are illustrated, with vertical lines indicating s.e.mean. Analysis of variance of the paired curves showed no significant deviation from parallelism. (b) The corresponding Schild analysis of the paired dose-ratios (r). The slope of the plot was not significantly different from unity (1.08 ± 0.10) and the estimated pK_B was 5.17 ± 0.04 (17 d.f.).

artery (O'Connor *et al.*, 1990), in particular to subject suramin to analysis according to the well-established, quantitative pharmacological criteria for the classification of competitive antagonists (Schild, 1979; Black *et al.*, 1983).

The first experiment carried out (see Figure 1 and analysis) utilized an incubation time for suramin of 45 min. Under this condition suramin failed two criteria for simple competition, in that it produced non-parallel displacement of α,β-methylene ATP E/[A] curves and more notably, a steep Schild plot slope. A possible explanation for the latter finding was that 45 min was an insufficient period for lower concentrations of suramin to achieve equilibrium with the receptors (Kenakin, 1980). In order to test this possibility, an experiment was performed in which any inadequacy in incubation time would be exaggerated. Thus, the effects of the two extreme concentrations used in the first experiment, 30 μM (Figure 2a) and 1 mM (Figure 2b) were studied using 15 min and 3 h incubation periods. The results of this experiment clearly established the time-dependence of the antagonism of suramin, the lower concentration produced negligible displacement of E/[A] curves at 15 min but substantial displacement at 3 h, and the higher concentration elicited the same degree of shift in both cases. This analysis indicated that 3 h may have been a sufficient incubation time for suramin to come to equilibrium with receptors. However, the evident depression of E/[A] curves produced by the higher concentration precluded further analysis of suramin as a competitive antagonist.

As the depressive property of suramin appeared to be associated only with extended exposure times, we attempted to minimize the time that suramin spent in contact with the tissues. This was done by analysing the kinetics of suramin with a low concentration of suramin using theoretical principles described by Paton (1961), which allowed estimation of the apparent rate constants determining the reaction between suramin and receptors (see Figure 3 and analysis). Then, with these estimates it was possible to calculate the periods of time required for different concentrations of suramin to achieve effective equilibrium which, for practical purposes, we took to be at least 95% fractional occupancy. It is worth mentioning that we attach no fundamental significance to the rate constant estimates. For reasons discussed elsewhere relating to tissue thickness and diffusion, it cannot be assumed that such estimates represent the true microscopic association and dissociation rate constants at the receptor (Colquhoun, 1981). The purpose of estimating them here was to calculate the minimum periods of time required for different concentrations of suramin to achieve effective equilibrium. For practical purposes, 'effective equilibrium' was considered to be at least 95% fractional occupancy.

When the resulting incubation times were used, suramin exhibited antagonist properties which were indistinguishable from simple competition (Figure 4 and analysis); it produced parallel rightward displacements of α,β-methylene ATP E/[A] curves which accorded with a unit Schild plot slope. Under these conditions, a valid estimate of the dissociation constant for suramin could be estimated which, given as pK_B, was 4.79. This value is incidentally supported by that calculated from the ratio of the rate constant estimates made in the kinetic analysis (4.84). Furthermore, a very similar estimate of affinity was obtained with L-β,γ-methylene ATP as the agonist (5.17).

In experiments in which the selectivity of suramin was addressed, significant effects were found only against histamine. The small degree of shift involved corresponded to a pA_2 of 3.11. According to these studies, therefore, suramin is selective for P_{2x}-receptors over other contractile mechanisms by some 50 fold. However, it is important to point out that this figure does not represent the selectivity of suramin

amongst P_2-receptor subtypes. Indeed, there is evidence that suramin is as effective an antagonist of P_{2y}-receptors as it is of P_{2x}-receptors (Hoyle *et al.*, 1990).

The present study has shown that suramin fulfils pharmacological criteria for simple competitive antagonism at P_{2x}-receptors. This appears to be the first demonstration of genuine competitive antagonism by suramin and, as such, supports the use of this agent in the quantitative classification of purine receptors. Evidently, in other, visceral smooth muscle systems containing P_{2x}-receptors, suramin appears to produce complex effects which are not readily amenable to quantitative analysis (Dunn & Blakely, 1988; Hoyle *et al.*, 1990). Whether, as in the present study, conditions can be sought in those systems in which simple competition can be revealed is not immediately clear. However, if the P_{2x}-receptors in vascular and visceral smooth muscle are assumed to be similar, if not identical (O'Connor *et al.*, 1990), it seems unlikely that slow kinetics of equilibration by suramin should be a property only of the vascular receptor. Clearly, further studies along these lines are necessary.

In conclusion, suramin is a slowly-equilibrating, but competitive antagonist at P_{2x}-receptors in rabbit vascular smooth muscle.

The authors wish to thank Miss N.T. Fear for technical assistance.

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(Received May 21, 1990
Accepted June 27, 1990)

Br. J. Pharmacol. (1990), 101, 650-654

Reduced high-affinity α_1-adrenoceptors in liver of senescent rats: implications of assessment at various temperatures

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1 We investigated the changes occurring as a result of aging in α_1-adrenoceptors in the livers of Fisher 344 female rats. For comparison, we also measured β-adrenoceptors in this tissue. Three age groups were studied, including young adults (aged 6 months), mature adults (aged 16 months) and senescent animals (aged 25 months).

2 The density of α_1-receptors was measured by use of [^{3}H]-prazosin and was found to be reduced 39% ($P < 0.01$) at 25 months compared with 6 months. The percentage of α_1-receptors displaying high affinity for adrenaline was also reduced from 85.6% at 6 months to 51.6% at 25 months ($P < 0.02$).

3 In contrast, the density of β-receptors, which was measured with [^{125}I]-iodocyanopindolol, was increased 104% between 6 months and 25 months. The affinity of both α_1- and β-adrenoceptors for antagonists was unchanged with age.

4 We found that receptor affinity for agonists may be measured accurately in binding studies conducted at 4°C or 25°C, but that the apparent affinity for agonist was artifactually reduced in studies conducted at 37°C. This effect is poorly reversible, in that reduced agonist-affinity is also observed in tissue which has been incubated at 37°C and then cooled to 4°C before performing the binding studies.

5 It is concluded that liver α_1-adrenoceptor function is reduced and β-adrenoceptor function increased in senescence.

Introduction

Both α_1- and β-adrenoceptors stimulate glycogenolysis in the liver (Morgan *et al.*, 1983). This response is mediated by adenosine 3':5'-cyclic monophosphate (cyclic AMP), in the case of β-receptors; and by phospholipase C, in the case of α_1-receptors. Activation of phospholipase C results in the hydrolysis of phosphoinositides (PI) and the formation of the dual second messengers diacyl glycerol and inositol phosphates. Diacyl glycerol subsequently activates protein kinase C. Inositol phosphates, especially IP_3, mobilize intracellular stores of calcium. There is emerging evidence that the liver α_1-receptor is linked to phospholipase C by a guanine nucleotide binding protein (G_p) in a manner analogous to the way G_s and G_i link β- and α_2-receptors to adenylate cyclase (Goodhardt *et al.*, 1982; Uhing *et al.*, 1986). There is also evidence that the α_1-receptor can stimulate cyclic AMP formation in the liver of mature rats (Exton, 1988).

β-Adrenergic responses are reduced in senescence in numerous tissues (Scarpace & Armbrecht, 1987). In contrast, β-adrenergic stimulation of glycogenolysis in the liver is dramatically increased in senescent rats compared with mature adults (Katz *et al.*, 1987). α_1-Adrenergic responses are reduced with age in some tissues (Partilla *et al.*, 1982; Hamilton *et al.*, 1985), but not in others (Docherty & O'Malley, 1985). The present studies were undertaken to assess the changes that occur as a result of aging in the liver α_1-adrenoceptor and to compare those changes with changes in the β-adrenoceptor.

Methods

Female Fisher 344 NNia rats of 6, 15 and 25 months of age were obtained from Harlan Industries (Indianapolis, IN, U.S.A.) under contract with the National Institute of Aging. Upon arrival, animals were examined and remained in quarantine for 1 week. Rats were randomly selected within age groups and housed 3 to a cage. Rats were fed Purina rat chow *ad libitum* and maintained on a 12-h, light-dark cycle. Ill animals were not used in the experiments. [^{3}H]-prozosin (specific activity = 83 Ci mmol^{-1}) was obtained from Amersham Corp., Arlington Heights, IL, U.S.A. [^{125}I]-iodocyanopindolol ([^{125}I]-ICYP) (specific activity = 2200 Ci mmol^{-1}) was obtained from New England Nuclear, Wilmington DE, U.S.A.

Tissue preparation

Under nembutal anesthesia, livers were excised from rats and prepared on ice. For studies of receptor affinity for agonists, a purified membrane preparation was employed. For studies of receptor density, crude homogenates were employed to avoid any artifacts which might occur if recovery of receptors during purification were different among the age groups employed.

The tissue was finely minced in 40 volumes of 0.25 M sucrose, 1 mM $MgCl_2$, 5 mM Tris HCl, 0.4 mM phenylmethanesulphonyl fluoride (PMSF), pH 7.4 (Suspension Buffer). Following disruption with a Tekmar Tissuemizer for 60 s, tissue was homogenized with 10 strokes of a motor-driven Teflon pestle. After passage through 2 layers of cheesecloth, the homogenate was centrifuged at 48,000*g* for 15 min and the pellet resuspended with a Dounce glass homogenizer in 50 mM HEPES, 18 mM $MgCl_2$, 1 mM EGTA, 0.08 mM ascorbic acid, pH 7.4 (HEPES Assay Buffer) containing a protease-inhibitor mixture (1 μM leupeptin, 100 μM benzamnidine and 100 μM PMSF). This preparation was used for studies of receptor density and is subsequently referred to as 'liver homogenates'.

Because the affinity of the liver α_1-receptor for agonists is labile at 37°C, a purified membrane preparation was used in order to exclude any possible effects due to the presence of other cellular components. In addition, we observed lower nonspecific binding (less than 20%) with purified membranes than with homogenates. Homogenates were purified as follows. The 48,000*g* pellet was resuspended in 120 mM NaCl, 4 mM KCl, 1 mM $MgCl_2$, 10 mM dextrose, 2 mM NaH_2PO_4, 0.1% heat-denatured bovine serum albumin, protease-inhibitor mixture and 25 mM HEPES, Ph 7.4. The mixture was layered on a bed of 2 M sucrose and centrifuged for 20 min at 5000*g*. The resulting interface was diluted 8 fold in the above buffer, and centrifuged for 15 min at 48,000*g*. Pellets were then resuspended in HEPES Assay Buffer containing protease inhibitors. This preparation is referred to as 'purified

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liver membranes'. Protein content was measured by the method of Bradford (1976).

[^3H]-prazosin binding studies

For α_1-receptor density studies, liver homogenates (400 μg) were suspended in 1.0 ml HEPES Assay Buffer containing [^{3}H]-prazosin in concentrations ranging from 20 to 500 pM in the presence and absence of 200 μM naphazoline. After a 60 min incubation at 37°C, the assay was terminated by vacuum filtration over Whatman GF/C filters. Filters were then washed rapidly (< 1 s) with 20 ml ice-cold HEPES Assay Buffer and counted. Specific binding was defined as that inhibited by naphazoline and was expressed as fmol mg^{-1} protein.

For α-agonist-affinity studies, purified liver membranes (400 μg) were suspended in 1.0 ml of HEPES Assay Buffer containing [^{3}H]-prazosin and (−)-adrenaline in concentrations ranging from 100 pM to 1 mM. Ascorbic acid (0.3 mM) was present to prevent oxidation of catecholamines. Incubations were terminated as described above. EGTA (1 mM) was included in the reaction mixture for α_1-receptor affinity studies because calcium ions have been shown to uncouple the liver α_1-receptor, resulting in the conversion of high-affinity receptors to the low affinity state (Lynch *et al.*, 1987).

[^{125}I]-iodocyanopindolol binding studies

For studies of β-adrenoceptor density, liver homogenates (400 μg) were suspended in 250 μl of HEPES Assay Buffer containing [^{125}I]-ICYP in concentrations ranging from 20 to 500 pM in the presence and in the absence of 10 μM propranolol. After a 60 min incubation at 37°C, the assay was terminated as described above. Specific binding was defined as that inhibited by propranolol and was expressed as fmol mg^{-1} protein.

Data analysis

Receptor density and the K_D for [^{3}H]-prazosin and [^{125}I]-ICYP were calculated by Scatchard analysis. IC_{50} values for high- and low-affinity binding of adrenaline and the fraction of α_1-receptors in high- and low-affinity states were calculated by two-site nonlinear analysis using a least squares computer modelling programme. The inhibition constants (K_i) for adrenaline (K_{DH} and K_{DL}) were calculated from the equation: $K_i = IC_{50} \times K_D/(K_D + D)$ where D is the added concentration of [^{3}H]-prazosin and K_D is the dissociation constant for [^{3}H]-prazosin as calculated from receptor density assays. Inhibition constants for adrenaline and [^{3}H]-prazosin are given as geometric means ± s.e.mean. For this purpose, the arithmetic mean of the logarithmic transforms of individual estimates of the affinity constants was calculated and its antilogarithmic transform used as the geometric mean. The standard error of the geometric mean was calculated by multiplying the geometric mean by the standard error of the logarithmically-transformed data (De Lean *et al.*, 1982). Receptor density and percentages of receptors displaying high- and low-affinity for agonists are given as arithmetic means ± s.e.mean. The effects of age were determined by one-way ANOVA and pairwise comparison among means was made by Student's t test, with $P < 0.05$ defined as the level of significance.

Results

Scatchard analysis indicated that [^{3}H]-prazosin binds with high affinity to a single class of α_1-receptors on rat liver homogenates. Specific and nonspecific binding were distinguished by competition between [^{3}H]-prazosin and unlabelled naphazoline. Inhibition of [^{3}H]-prazosin binding by naphazoline was unchanged with age with IC_{50} values of 1.68 μM at 5 months, 1.80 μM at 14 months and 1.75 μM at 26 months (Figure 1, $n = 2$). Concentrations of naphazoline between

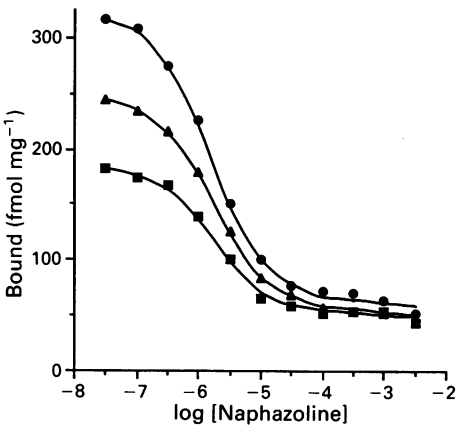

Figure 1 Inhibition of [^{3}H]-prazosin binding by naphazoline: liver homogenates (400 μg) prepared from rats of the following ages were incubated for 30 min at 25°C with 1.25 nM [^{3}H]-prazosin in the presence of the indicated concentrations of naphazoline: (●) 5 months; (▲) 14 months; (■) 26 months. Similar results were obtained in another such experiment. At all three ages, specific binding was almost completely inhibited by concentrations of naphazoline greater than 10 μM. Inhibition of nonspecific binding did not occur at concentrations of less than 1 mM.

10 μM and 1 mM fully inhibited specific binding without reducing nonspecific binding. A concentration of 200 μM naphazoline was chosen as the competing ligand for α_1-receptor density studies. Nonspecific binding was less than 40% of total binding at the highest concentration of [^{3}H]-prazosin. A maximum of 13% of added radioactivity was bound at the highest concentration of [^{3}H]-prazosin. The density of liver α_1-receptors decreased with age (Figure 2, Table 1, ANOVA,

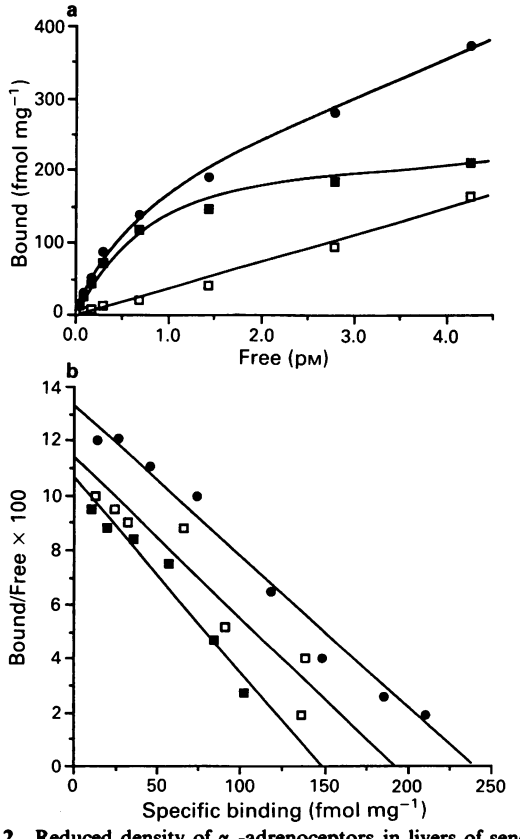

Figure 2 Reduced density of α_1-adrenoceptors in livers of senescent rats: (a) binding of the indicated concentrations of [^{3}H]-prazosin to liver homogenates (400 μg) prepared from a rat aged 6 months was measured in the absence (total binding, ●) and in the presence of 200 μM naphazoline (nonspecific binding, □); (■) indicates specific binding. (b) Scatchard plots of the same specific binding data from the animal aged 6 months (●) and of similar data obtained from animals aged 14 (□) and 26 months (■). See also Table 1.

Table 1 Reduction in the density of liver α_1-adrenoceptors with age

Age	B_{max} (fmol mg^{-1})	K_D(nM)
6 months	221.5 ± 16.6	0.66 ± 0.04
15 months	196.1 ± 17.7	0.64 ± 0.05
25 months	136.2 ± 12.1	0.53 ± 0.05

Receptors were quantitated by Scatchard analysis of [^{3}H]-prazosin binding to liver homogenates. Receptor density was reduced 39% at 25 months compared with 5 months ($P < 0.01$). The K_D for [^{3}H]-prazosin was unchanged with age. Values are means of determinations from six separate animals ± s.e.mean.

$F(2,15) = 7.83$, $P < 0.005$, $n = 6$). Receptor density was reduced 39% at 25 months compared with 6 months ($P < 0.01$ unpaired t test, $n = 6$). There was also a non-significant trend toward a reduction at 16 months compared with 6 months ($P = 0.052$, unpaired t test, $n = 6$). No change was observed in the K_D for [^{3}H]-prazosin (ANOVA, $F(2,14) = 1.88$, $P = 0.18$).

The inhibition of [^{3}H]-prazosin binding to purified liver membranes by unlabelled (−)-adrenaline was used to discriminate between two classes of receptors, one displaying high affinity and one low affinity for agonists. Before studying the effect of age on agonist-affinity, studies were performed to identify appropriate assay conditions. Ratios of high- and low-affinity receptors were determined at 4°C, 25°C and 37°C. At 4°C, 88% of α_1-receptors displayed high affinity for (−)-adrenaline ($K_{DH} = 1.98 \times 10^{-7}$ M) and 12% displayed low affinity ($K_{DL} = 6.57 \times 10^{-5}$ M, Figure 3, ■). Similarly, at 25°C, 86% of α_1-receptors displayed high affinity for (−)-adrenaline ($K_{DH} = 2.57 \times 10^{-7}$ M) and 14% displayed low affinity ($K_{DL} = 4.89 \times 10^{-5}$ M, Table 2). However, at 37°C (Figure 3, ▲), there was no high-affinity binding of (−)-adrenaline. There was an approximate 100 fold rightward shift of the inhibition curve, which was resolved into a single class of binding sites with a K_D for (−)-adrenaline of 2.56×10^{-5} M. The curve for binding at 37°C is nearly superimposable on the curve for 37°C binding in the presence of 2.5 mM Gpp(NH)p (Figure 3, ○), a treatment that converts high-affinity receptors to the low affinity state.

Binding studies were performed to determine whether the conversion of high- to low-affinity receptors occurring at 37°C is reversible (Figure 4). Assay tubes, containing aliquots of purified membranes and a range of concentrations of (−)-adrenaline, were incubated at 37°C for 30 min and then cooled to 4°C prior to the initiation of [^{3}H]-prazosin binding studies (Figure 4, ▲). In these studies, a greatly reduced affinity was observed compared with studies where 4°C binding studies were performed without preincubation at 37°C (Figure 4, ■). A partial reduction in affinity was also observed when tissue was preincubated at 37°C in the absence of agonist prior to binding studies performed at 4°C (Figure 4, ●). No reduction in affinity was observed following preincubation of the membrane preparations at 25°C (data not shown). The equivalence of 4°C and 25°C binding studies was demonstrated by obtaining identical (−)-adrenaline inhibition curves in 4°C binding studies, 25°C binding studies and 4°C binding studies following a preincubation of the membranes at 25°C (data not shown).

As a result of the aforementioned studies, all subsequent binding assays were conducted at 25°C for 30 min when examining the effects of age on the affinity of the α_1-receptor for agonists (Figure 5, Table 2). The percentage of receptors in the high-affinity state was significantly reduced with age (ANOVA, $F(2,14) = 5.93$, $P < 0.02$, $n = 6$). In purified membranes derived from animals aged 6 months, 85.6% of the

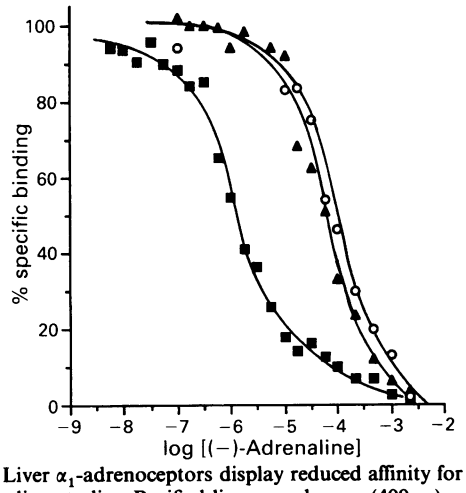

Figure 3 Liver α_1-adrenoceptors display reduced affinity for agonists in 37°C binding studies. Purified liver membranes (400 μg) were incubated with 0.35 nM [^{3}H]-prazosin in the presence of 1 mM EGTA and the indicated concentrations of (−)-adrenaline. Binding studies were conducted at 4°C for 4 h (■); at 37°C for 30 min (▲); or 37°C for 30 min in the presence of Gpp(NH)p (○) ($n = 3$).

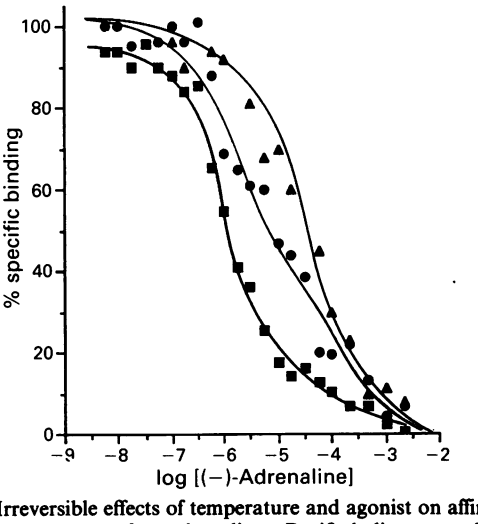

Figure 4 Irreversible effects of temperature and agonist on affinity of liver α_1-adrenoceptors for adrenaline. Purified liver membranes (400 μg) were incubated with 0.35 nM [^{3}H]-prazosin in the presence of 1 mM EGTA and the indicated concentrations of (−)-adrenaline. Binding studies were conducted at 4°C for 4 h after preincubation of the tissue for 30 min under the following conditions: 4°C (■); 37°C (●); and 37°C in the presence of the indicated concentrations of (−)-adrenaline (▲) ($n = 3$).

Table 2 Reduced high-affinity binding of agonists to α_1-adrenoceptor of senescent rat livers

	High-affinity binding			*Low-affinity binding*		
Age	% R_H	pK_{DH}	K_{DH} (10^{-7} M)	%R_L	pK_{DL}	K_{DL} (10^{-5} M)
6 months	85.6 ± 2.82	−6.59 ± 0.07	2.57 ± 0.18	14.4 ± 2.87	−4.31 ± 0.16	4.89 ± 0.78
15 months	80.8 ± 4.88	−6.59 ± 0.13	2.51 ± 0.33	19.2 ± 4.88	−4.42 ± 0.20	3.80 ± 0.76
25 months	51.6 ± 10.0*	−7.1 ± 0.23	7.80 ± 1.79	48.4 ± 10.0*	−4.74 ± 0.22	1.82 ± 0.40

Binding of [^{3}H]-prazosin to purified liver membranes was measured in the presence of a range of concentrations of (−)-adrenaline. The percentage of high- (R_H) and low-affinity (R_L) receptors, and inhibition constants for high- (K_{DH}) and low-affinity (K_{DL}) binding were calculated by nonlinear regression. The percentage of α_1-receptors in the high affinity state was significantly reduced with age ($P < 0.02$). Values for K_{DH} and K_{DL} were unchanged with age. Values are means of determinations from 6 separate animals ± s.e.mean.
* $P < 0.05$ vs. 6 months.

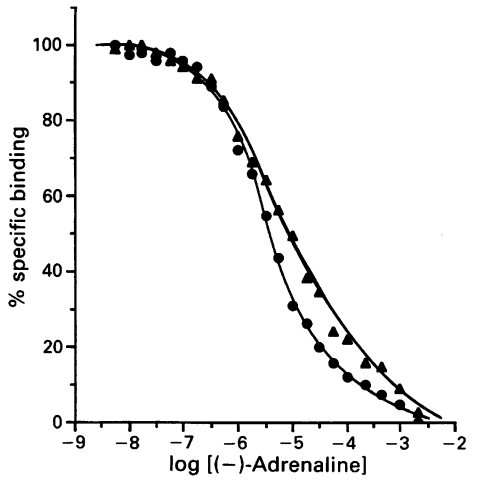

Figure 5 High-affinity binding of adrenaline to liver α_1-adrenoceptors is reduced in senescence. Purified liver membranes (400 μg) were incubated with 2.5 nM [^{3}H]-prazosin for 30 min at 25°C in the presence of 1 mM EGTA and the indicated concentrations of (−)-adrenaline. Binding inhibition curves were analyzed by nonlinear regression of a 2-site model. The percentage of α_1-receptors in the high-affinity state was significantly reduced with age ($P < 0.02$): (●) 6 months; (▲) 25 months. Values for K_{DH} and K_{DL} were unchanged with age ($n = 6$). Values for animals aged 15 months are similar to those aged 6 months and have been omitted for the sake of clarity.

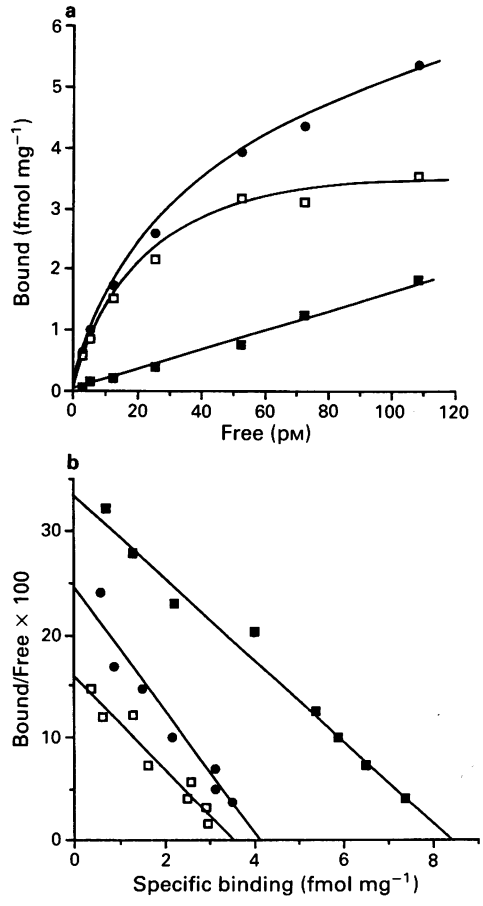

Figure 6 Increased density of β-adrenoceptors in livers of senescent rats. (a) Binding of the indicated concentrations of [^{125}I]-iodocyanopindolol ([^{125}I]-ICYP) to liver homogenates (400 μg) prepared from a rat aged 5 months. Binding was measured in the absence (total binding, ●) and in the presence of 10 μM propranolol (nonspecific binding, ■); (□) specific binding. (b) Scatchard plots of the same specific binding data from the animal aged 5 months (●) and of similar data obtained from animals aged 14 (□) and 26 months (■). The above is representative of 6 experiments. Receptor density was unchanged between 6 months and 15 months, but was increased at 25 months ($P < 0.01$). The K_D for [^{125}I]-ICYP was unchanged with age.

receptors were in the high-affinity state, compared with 80.8% at 15 months and 51.6% at 25 months. The percentage of receptors in the high-affinity state was significantly reduced between 6 and 25 months ($P < 0.05$, unpaired t test). No changes with age in the inhibition constants for either high-affinity (K_{DH}, ANOVA, $F(2,14) = 3.07$, $0.1 > P > 0.05$) or low-affinity (K_{DL}, ANOVA, $F(2,15) = 1.08$, $P > 0.3$) binding of (−)-adrenaline were observed.

The density of β-adrenoceptors in liver homogenates was quantitated by Scatchard analysis of [^{125}I]-ICYP binding data (Figure 6). Nonspecific binding was measured in the presence of 10 μM propranolol and was never more than 30% of total binding. A maximum of 32% of the added radioligand was bound at the highest concentration of [^{125}I]-ICYP. The density of β-receptors was low compared with that of α_1-receptors. β-Receptor density was unchanged between 6 months (2.91 ± 0.38 fmol mg^{-1}) and 15 months (2.84 ± 0.39 fmol mg^{-1}, $P > 0.8$, unpaired t test), but was increased by 104% at 25 months (5.93 ± 0.77 fmol mg^{-1}, $P < 0.01$, unpaired t test). The K_D for [^{125}I]-ICYP (25.36 ± 2.61 pM at 6 months, 27.70 ± 3.27 pM at 15 months and 23.54 ± 3.36 pM at 25 months) was unchanged with age ($P > 0.5$, unpaired t test).

Discussion

The mechanism by which α_1-adrenoceptors are coupled to phospholipase C is not fully understood. Numerous analogies exist with the β-adrenergic system. Stable guanosine 5'-triphosphate (GTP) analogues stimulate adenylate cyclase and destabilize the high-affinity tertiary complex of agonist/β-receptor/G protein, resulting in the conversion of a mixed population of β-receptors displaying high and low affinity for agonists to a homogeneous population of low-affinity receptors. Analogously, stable GTP analogues also stimulate phospholipase C and convert α_1-receptors to the low-affinity form (Minneman, 1988). Prior exposure to agonist converts β-receptors to the low-affinity form, resulting in a reduced agonist-stimulation of adenylate cyclase. Agonist treatment also reduces the affinity of α_1-receptors for agonist (Schwarz *et al.*, 1986), and subsequent α_1-stimulation of PI hydrolysis has been reported to be reduced by some groups (Gonzales *et al.*, 1987) but not by others (Rosenbaum *et al.*, 1987).

In the present studies, we examined the effect of age on the ability of α_1-receptors in liver of female F344 rats to form high-affinity complexes with agonist. We observed a 39% reduction in receptor density. We also observed, in 25°C binding assays, a reduction of the proportion of receptors displaying high affinity for agonist from 85.6% at 6 months to 51.6% at 25 months. Thus the total formation of high affinity complexes was reduced by 63%. There was no difference in the ratio of high- and low-affinity receptors measured either at 4°C or 25°C, with all receptors converting to the low-affinity state at 37°C. Our results differ somewhat from another report published by Lynch *et al.* (1988). Their 25°C [^{3}H]-prazosin binding studies with liver membranes prepared from male Sprague-Dawley rats showed an increase in the proportion of high-affinity α_1-receptors in senescence. The reason for this discrepancy is not clear; however, gender differences have been reported in adrenergic control of liver glucose metabolism. Studer & Borle (1982) found that both α_1- and β-receptors mediate catecholamine-stimulation of glycogenolysis in hepatocytes isolated from female Sprague-Dawley rats, but that in males the response is mediated solely by α_1-receptors.

We observed, as did Lynch *et al.* (1985), a mixed population of high- and low-affinity receptors in binding studies conducted at 25°C. We measured the same mixed population at 4°C. However, Lynch *et al.* (1985) measured a homogeneous population of high affinity receptors at 2°C (Lynch *et al.*, 1985). We observed that the curve for adrenaline-inhibition of [^{3}H]-prazosin binding is shifted to the right 100 fold in 37°C binding assays, compared with those performed at 4°C or

25°C. A mixed population of receptors displaying high- and low-affinity binding of (−)-adrenaline are converted to a homogeneous population displaying low affinity. At 37°C, no further rightward shift of the curve occurs if Gpp(NH)p is added to the binding assay, again indicating that all receptors are in the low-affinity state. Schwarz *et al.* (1986) have reported a similar finding in that the affinity for adrenaline of α_1-receptors in membranes prepared from rat isolated hepatocytes is 100 fold lower in 37°C binding assays compered to 4°C assays. The reduced affinity observed in 37°C studies is probably the result of desensitization. However, an artifactual partial reduction in affinity is also seen when tissue is incubated at 37°C in the absence of agonist. Because of these findings, we prepared purified liver membranes at 4°C and kept them on ice prior to 25°C binding studies. Our results point out a potential pitfall in that agonist-affinity studies should not be performed at 37°C.

In the liver, the β-adrenergic stimulation of glycogenolysis is mediated by sympathetically innervated receptors and α_1-stimulation by non-innervated receptors. Studies with perfused liver have shown that sympathetic nerve stimulation and noradrenaline administration stimulate glucose and lactate output to the same degree, but that PI hydrolysis is stimulated appreciably only by administered catecholamine (Puschel & Jungerman, 1988). Changes with age in α_1 and β-receptors may be adaptive responses to the changing availability of hormone. α_1-Receptors may be down-regulated in response to higher circulating noradrenaline levels (Lakatta *et al.*, 1975; Zeigler *et al.*, 1976). β-Receptors may be up-regulated in response to decreased noradrenaline release from sympathetic nerve terminals (Daly *et al.*, 1988).

Our finding of increased β-receptor density in the livers of senescent animals is in agreement with two other reports (Graham *et al.*, 1987; Dax *et al.*, 1987). The increase in β-receptor density is accompanied by an increase in isoprenaline-stimulated glycogenolysis in isolated hepatocytes (Graham *et al.*, 1987; Katz *et al.*, 1987). In contrast, changes with age in the α_1-receptor may or may not result in altered α_1 control of glycogenolysis. Lynch *et al.* (1988) reported that noradrenaline-stimulated phosphorylase activity in isolated hepatocytes is unchanged with age. This is consistent with our finding that adrenaline-stimulated PI hydrolysis in liver slices prelabelled with [^{3}H]-myoinositol is also unchanged with age (unpublished observations).

In conclusion, we found that age is accompanied by a combined reduction in α_1-adrenoceptor density and the fraction of high-affinity receptors, resulting in a marked reduction in the ability to form stable receptor-agonist complexes. One of the most serious consequences of the aging process is a loss of plasticity. The physiological consequences of these changes in the liver α_1-receptor are unknown, but may reflect a reduced adaptive capacity.

The authors appreciate the excellent technical assistance of Michael Matheny and Parke Lewis. These studies were supported by the Medical Research Service of the Department of Veteran Affairs.

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(Received October 3, 1989
Revised April 12, 1990
Accepted June 11, 1990)

Br. J. Pharmacol. (1990), **101**, 655–661

Different profile of electrocortical power spectrum changes after micro-infusion into the locus coeruleus of selective agonists at various opioid receptor subtypes in rats

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1 The effects of various opioid receptor agonists given directly by means of a chronically implanted cannula into the locus coeruleus (LC) on behaviour and ECoG activity, continuously analysed, and quantified as total power spectrum (0–16 Hz) and in preselected frequency bands (0–3; 3–6; 6–9; 9–12 and 12–16 Hz), were studied in rats.

2 Dermorphin (0.05, 0.5, 1, 2 and 5 pmol) and Tyr-D-Ala-Gly-N-Me-Phe-Gly-ol (DAMGO; 1, 10, 30, 100 pmol and 1 nmol), two typical μ-receptor agonists, applied unilaterally or bilaterally directly into the LC, produced a typical dose-dependent ECoG synchronization with a significant increase in total power spectrum as well as in the lower frequency bands. Dermorphin was found to be approximately 30 times more powerful than DAMGO in producing similar quantitative ECoG changes.

3 D-Ala-D-Leu-Thr-Gly-Gly-Phe-Leu (DADLE; 1, 10, 50 and 100 pmol), a selective δ-receptor agonist, micro-infused into the LC produced dose-dependent behavioural soporific effects and ECoG increase in total power spectrum as well as in 3–6, 6–9, 9–12 Hz frequency bands. In comparison to dermorphin, the ECoG power spectrum effects of DADLE were 10 fold less potent, whereas in comparison to DAMGO it was approximately 3 times more potent. A lower dose (0.1 pmol) was ineffective in changing behaviour and ECoG power spectrum.

4 The microinfusion into the LC of U 50, 488H, a selective κ-opioid receptor agonist, (0.25, 1, 2.5, 5 and 10 pmol) produced a typical pattern characterized by a first short-lasting (3–25 min) phase of behavioural arousal and ECoG desynchronization, followed by a longer lasting (20–130 min according to the dose) phase of behavioural sleep and ECoG synchronization. A lower dose (0.1 pmol) was ineffective in changing behaviour and ECoG power spectrum.

5 Dextromethorphan and ketamine, two selective agonists at σ-receptors given into the LC (1, 5 and 10 pmol) induce behavioural arousal, increase in locomotor activity and an intense pattern of stereotyped movements. However, by increasing the dose of ketamine (50 and 100 pmol), marked sedation, postural changes and an increase in low frequency ECoG bands, sometimes associated with high amplitude fast frequency potentials, were observed.

6 Naloxone applied directly into the LC (1 and 2 pmol 15 min before) was able to prevent the behavioural and ECoG effects induced by dermorphin, DAMGO and DADLE. Higher doses of naloxone (10 pmol into the LC) were however, required to antagonize the behavioural and ECoG soporific effects induced by the κ-receptor agonist U 50,488H. In contrast, naloxone (10 pmol into the LC) was unable to prevent or reduce the behavioural and ECoG effects induced by subsequent administration into the same site of dextromethorphan and ketamine.

7 In conclusion, the present experiments confirm that behavioural and ECoG effects elicited following stimulation of μ-, δ-, κ- and σ-opioid receptors located in the LC are quite different. Activation of μ-, δ- and κ-receptors induced sedative effects whereas dextromethorphan and ketamine, two σ-receptor agonists, induced behavioural arousal and ECoG desynchronization. In addition, the present results strongly support the crucial role played by opioid mechanisms, in the locus coeruleus, in the mediation of the soporific effects of drugs acting as agonists at opioid receptors.

Introduction

In the last 20 years a large body of evidence has accumulated showing that in several animal species and man, morphine and its congeners given systemically produce not only analgesia but also concomitant electrocortical (ECoG) and behavioural alterations characterized by synchronization and associated behavioural stupor (Bradley, 1968; Domino, 1968; Khazan, 1975). In addition, classical experiments have shown that in rats pentazocine and cyclazocine (Colasanti & Khazan, 1973; Kareti *et al.*, 1979) and in dogs ketocyclazocine and ethylketocyclazocine (Pickworth & Sharpe, 1979) produce similar EEG effects. However, it has also been shown that low doses of pentazocine and cyclazocine produce initially ECoG desynchronization in rats accompanied by behavioural excitation (Carruyo *et al.*, 1968). More recently, Tortella *et al.* (1980) have shown that in rats morphine and ethylketocyclazocine produce an initial phase of ECoG synchronization and stupor followed by a period of ECoG activation and behavioural arousal. In contrast, the profile of cyclazocine was not biphasic, but was characterized by intermittent periods of behavioural excitation and ECoG desynchronization before the onset of slow wave sleep. Naloxone produced a dose-related antagonism of behavioural stupor and ECoG synchronization. However, unlike morphine and ethylketocyclazocine, cyclazocine was not sensitive to naloxone (Tortella *et al.*, 1980). These findings can be explained on the basis of the existence in the brain of multiple opioid receptors (Martin *et al.*, 1976; Pert, 1981; Goldstein & James, 1984; Zukin & Zukin, 1984); specific effects can be induced by compounds acting at specific subtypes of opioid receptors. The classical experiments of Martin *et al.* (1976) indicated the existence of three types of

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opioid receptor: μ for morphine-like compounds, κ for ketocyclazocine-like drugs and δ for drugs like cyclazocine and N-allylnorcyclazocine. In addition, by use of peripheral organ systems the existence has been demonstrated of another receptor type, referred to as epsilon, associated with the mouse vas deferens bioassay, where enkephalin peptides were found to be particularly potent. Actually, the σ-receptor is now considered to be a non-opioid one (Chang, 1984). There is a large body of evidence that the 3 distinct opioid receptors (μ, κ and δ) are present in the brain and their anatomical distribution in different areas of the brain has been demonstrated (Mansour *et al.*, 1988). The recent discovery of new drugs acting as selective agonists at the different opioid receptors is a useful tool to understand better the role of a single receptor population in the control of different functions in the brain. Thus the aim of the present experiments was to characterize the electrocortical profile of specific agonists at the 3 types of opioid receptor after their direct microinfusion into the locus coeruleus, where noradrenaline cell bodies are located. In addition there is evidence that on these neurones there is a dense projection of terminals containing β-endorphin and μ- and κ-opioid receptors are situated there (Cuello, 1983; Mansour *et al.*, 1988). The locus coeruleus represents the site of origin of the noradrenergic diffuse ascending and descending projecting systems which send terminals to the cortex, hippocampus, thalamus, hypothalamus as well as to other subcortical, cerebellar and spinal areas (Dählstrom & Fuxe, 1964; Moore & Card, 1984).

In previous experiments it was shown, in the rat, that following the local microinfusion of very small amounts of α_2-adrenoceptor agonists, such as clonidine, a dose-dependent behavioural and electrocortical slow wave sleep occurs (De Sarro *et al.*, 1987; 1988a). Similarly in preliminary experiments we have shown dermorphin, a heptapeptide (H-Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH) extracted from the skin of the South American frog, *Phyllomedusa Sauvagei* (Broccardo *et al.*, 1981), to be a potent μ-agonist (Amiche *et al.*, 1987), which when given directly into the LC produced dose-dependent behavioural sleep and ECoG synchronization (De Sarro *et al.*, 1988b).

In the present experiments we planned to characterize the role of the μ-, δ-, κ- and σ-receptors in the locus coeruleus further and to reveal possible dose-related differences, by microinjecting very tiny and progressively increasing doses of agonists at specific receptor types.

We followed changes in gross behaviour as well as in the ECoG activity, continuously analysed and quantified in its total voltage power and in single frequency bands. As agonists at opioid μ-receptors we used dermorphin (Amiche *et al.*, 1987) and Tyr-D-Ala-Gly-N-Me-Phe-Gly-ol (DAMGO, Handa *et al.*, 1981; Zajac *et al.*, 1983), as an agonist at δ-receptors we used the low selective agonist D-Ala-D-Leu-Thr-Gly-Gly-Phe-Leu (DADLE, Kosterlitz *et al.*, 1981; Chang *et al.*, 1981; Pfeiffer & Hertz, 1981), as an agonist at κ-receptors we used U 50,488H (Lahti *et al.*, 1982; VonVoigtlander *et al.*, 1982; Tang & Collins, 1985) and as agonists at σ-receptors we used ketamine (Zajac *et al.*, 1983; Chaillet *et al.*, 1984; Zukin & Zukin, 1984) and dextromethorphan (Craviso & Musacchio, 1983; Musacchio *et al.*, 1988).

Methods

Adult male Sprague-Dawley rats (200 ± 25 g; 60–90 days old) were purchased from Charles River Italia (Calco, Como, Italy) and housed under stable conditions of humidity (60 ± 5%) and temperature (22 ± 2°C). They were fed on a standard diet, had water *ad libitum* and maintained on a 12 h light-dark cycle (lights on 6 h 00 min–18 h 00 min, off 18 h 00 min–6 h 00 min). Animals were stereotaxically implanted with permanent stainless steel guide cannulae (25 gauge) under chloral hydrate anaesthesia (400 mg kg^{-1} i.p.; Carlo Erba, Milan, Italy) to permit a unilateral or a bilateral microinjection into the locus coeruleus. The cannulae had an angle of 5° from the vertical and tips were 2 mm away from the LC. All coordinates were modified from the atlas of Paxinos & Watson (1982) (AP = 1.1 mm posterior to lambda, L = 0.5 lateral to the midline, H = 5.7 mm ventral to the skull surface). After surgery a minimum of 48 h was allowed for recovery before experiments were carried out. All experiments were performed beginning at approximately 10 h 00 min. Freely moving rats were microinjected via an injector cannula (28 gauge) which extended approx 2 mm below the tip of the guide cannula. The injector cannula, placed in the implanted guide cannula before the beginning of experiments, was connected by a polyethylene tube to a swivel system and to a 5 μl Hamilton syringe, in order to let the animals move freely into the box before and during the infusion.

The same animals were used for both behavioural and ECoG studies.

The animal was considered catatonic when it maintained the same position for 3 min.

Electrocortical activity was recorded (8 channel ECoG machine OTE, Biomedica, Florence) via 4 chronically implanted steel screw electrodes inserted bilaterally onto the fronto-parietal and the fronto-occipital area by a Stoelting stereotaxic frame. The ground electrode was implanted epidurally over the nasal bone. All electrodes and the injection guide cannulae were anchored to the skull by acrylic dental cement. For statistical purposes, the bipolar signals from each cortical area were integrated as total voltage power (0–16 Hz) and as individual frequency bands (0–3, 3–6, 6–9, 9–12 and 12–16 Hz) by means of a Berg-Fourier analyser (OTE Biomedica, Florence, Italy) according to Bricolo *et al.* (1978).

ECoG spectra power was obtained by averaging spectra 5 min ECoG epochs and the integrated energy signals were computerized as previously described (De Sarro *et al.*, 1987; 1988a) and expressed as μV s^{-1}; the time constant (0.03) was short enough to reduce the number of artifacts (HF cut-off was 5.3 Hz). The animals, placed individually in transparent cages (35 × 35 × 25 cm) were allowed 30 min before drug administration to acclimatize to the new environment.

The behavioural changes and their onset and duration were recorded after drug injection. In particular, two independent observers followed gross behavioural changes consisting of eyes open or closed, locomotor activity, possible stereotyped movements and squatting posture. Also, they noted whether the rats concomitant to ECoG changes were alert, drowsy or sleepy. An additional group of rats (at least 3 for each dose and compounds used) that had not been used for behavioural and electrocortical studies were tested for a possible analgesic effect on a hot plate (Technilab Instruments, Pegnameck, U.S.A.). The surface of the hot plate was maintained at 55 ± 1°C. A 14 cm tall rectangular hollow Plexiglas enclosure with 25 × 18 cm base was placed on the hot plate in order to prevent rats from escaping the hot plate surface during test trials. Hot-plate responding was tested in rats 30 min after vehicle or drug injection. Each trial consisted of placing the rat on the hot plate surface and timing latency to emit either one of two analgesic responses: hind paw licking or vertical jumping with all feet leaving the surface of the plate. This latency was recorded but not statistically analysed.

Post-mortem histological examination confirmed the location of the guide cannula. Only animals in which the location of the injection site was confirmed histologically were used in the analysis of behavioural and ECoG data.

To quantify changes of total voltage power and of preselected bands of frequency induced by compounds acting on opioid receptors, the area (expressed in mm^2) under the curve corresponding to plotted total voltage values during 30 min periods after each compound was integrated by means of Commodore Computer and percentage changes of the integrated area, in comparison to the same interval during the pretreatment period, were calculated according to the 'trapezoidal rule' (Tallarida & Murray, 1981). In addition, in order to reduce the possible inter-animal variations of baseline elec-

trocortical activity and of a single frequency band that existed in the same group, the percentage changes following drug-treatment were compared to the values of the corresponding period before treatment by paired Student's t test. In addition, statistical analysis between groups treated with various opioid receptor subtypes agonists or vehicle (in the same volume) was performed by the Mann-Whitney U-test.

Microinfusion of the same volume of the vehicle (0.5 μl) lacked effects on behaviour and electrocortical activity. The number of experiments is shown in parentheses.

Drugs

The following drugs were used: DAMGO (Tyr-D-Ala-Gly-N-Me-Phe-Gly-ol) (Peninsula Labs., Belmont, U.S.A.), DADLE (D-Ala-D-Leu-Thr-Gly-Gly-Phe-Leu) (Peptide Institute, Osaka, Japan), dermorphin (H-Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2) (Farmitalia Carlo Erba Research Labs., Milan), naloxone hydrochloride (Sigma, St. Louis, MO, U.S.A.), U 50,488H (trans-3,4-dichloro-N-methyl-N-[2-(1-pyrolidinyl)-cyclohexyl]-benzeneacetamide; Upjohn, Kalamazoo, Michigan, U.S.A.), dextromethorphan hydrobromide (Sigma, St. Louis, MO, U.S.A.), ketamine hydrochloride (Parke Davis, Milan, Italy). All drugs were easily dissolved in Ringer solution (NaCl 8.6 g, KCl 0.3 g and CaCl 0.33 g dissolved in 1000 ml pyrogen-free bidistilled water). The drugs were infused into the LC in a volume of 0.5 μl, at a constant rate of 0.2 μl min^{-1} the injector cannula was left *in situ* for a further 1 min and then gently removed.

Results

Effects of agonists at μ-receptors

Dermorphin The unilateral microinjection of dermorphin (0.5, 1, 2 and 5 pmol) (at least 4 rats for each dose), into the locus coeruleus induced dose-dependent behavioural and soporific effects, as well as electrocortical slow-wave sleep, which were evident within 1–15 min after the microinfusion and lasted approximately 45–160 min depending on the dose. During this period, the animals showed a significant ($P < 0.05$) increase in total voltage power and in 0–3, 3–6, 6–9 and 9–12 Hz frequency bands. In addition, a significant increase in 12–16 Hz frequency band was observed after dermorphin 2 and 5 pmol, while the slight increase in 12–16 Hz frequency band did not reach significance after the lower doses of dermorphin (Table 1). In comparison to the unilateral microinjection, much lower doses of dermorphin (0.05, 0.1 and 0.2 pmol), given bilaterally ($n = 5$ for each dose) into the locus coeruleus, were required to produce behavioural and electrocortical synchronization. In particular, a dose-dependent increase in total voltage power and in the same frequency bands stated above was observed. The ECoG spectral analysis showed a significant ($P < 0.05$) increase in total voltage power and in 0–3, 3–6, 6–9 and 9–12 Hz frequency bands. The dose-dependent duration of ECoG synchronization after dermorphin is shown in Figure 1 and Table 1. A slight analgesia and catatonia occurred concomitantly with the sedative behavioural correlates of dermorphin (2 and 5 pmol) in rats.

The most effective dose of dermorphin (0.2 pmol), injected bilaterally into the LC, induced a maximal increase in total voltage power and in some frequency bands this lasted for approximately 150 min after the microinjection, with diminishing effectiveness after about 180 min, producing slow-wave sleep with spindle lasting approximately 120 min. The effects of the lower dose used (0.05 pmol) were quite slight; the animals were drowsy with minor exploratory or locomotor activity compared to vehicle-treated animals. Such symptoms lasted about 30 min, whereas the increase in total voltage power and in 0–3 and 9–12 Hz frequency bands was not significantly different in comparison to the pretreatment period except during the first 30 min post injection. The lowest dose of dermorphin (0.01 pmol) was unable to affect behaviour and ECoG activity as compared with the pretreatment control period.

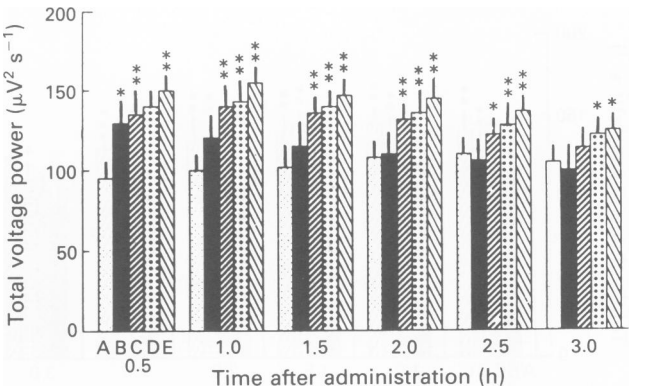

Figure 1 Effects of dermorphin on total voltage power. The drug was injected after 30 min of ECoG control recordings. Significant differences between dermorphin and vehicle group are denoted: * $P < 0.05$, ** $P < 0.01$ by Mann-Whitney U-test. Columns A vehicle; B 0.5 pmol; C 1.0 pmol, D 2.0 pmol and E 5.0 pmol dermorphin.

Table 1 Effects of microinfusion of various μ- and δ-opioid agonists into the locus coeruleus on changes in baseline voltage power of frequency bands 30 min after drug administration

Compound	Dose (pmol)	Changes in baseline voltage power frequency bands					Number of animals
		0–3 Hz	3–6 Hz	6–9 Hz	9–12 Hz	12–16 Hz	
Vehicle		3.5 ± 0.5	24.2 ± 2.8	41.8 ± 5.1	27.4 ± 3.2	23.1 ± 3.4	10
DAMGO	1	3.4 ± 0.6	23.7 ± 2.9	42.4 ± 5.8	26.2 ± 4.1	23.2 ± 4.1	5
	10	2.9 ± 0.4	24.6 ± 3.1	44.2 ± 6.1	25.6 ± 4.6	22.7 ± 3.8	4
	30	3.2 ± 0.3	43.2 ± 4.5*	68.3 ± 7.9*	30.7 ± 7.2	22.5 ± 3.9	6
	100	3.6 ± 0.4	49.5 ± 5.1**	82.6 ± 10.5*	34.5 ± 8.9	21.9 ± 4.2	5
	1000	3.9 ± 0.4	58.9 ± 6.1**	96.4 ± 9.6**	39.7 ± 10.9	24.1 ± 3.7	4
Dermorphin	0.5	3.9 ± 1.0	38.4 ± 4.2	64.5 ± 9.8*	39.2 ± 6.4	24.2 ± 3.9	6
	1.0	5.2 ± 2.1*	44.3 ± 5.1*	71.3 ± 10.2**	41.2 ± 7.3	28.7 ± 4.3	4
	2.0	6.1 ± 2.4*	50.7 ± 5.4**	78.9 ± 12.4**	45.3 ± 7.8*	37.2 ± 4.1*	7
	5.0	6.4 ± 2.7**	59.8 ± 6.3**	86.8 ± 11.7**	52.6 ± 8.2**	42.2 ± 4.3*	6
DADLE	0.1	3.7 ± 0.8	23.2 ± 3.1	40.9 ± 5.5	30.2 ± 4.1	23.1 ± 3.2	4
	1	3.9 ± 0.7	34.1 ± 4.3	41.7 ± 6.1	32.4 ± 5.5	25.2 ± 3.7	6
	10	3.6 ± 0.5	42.4 ± 5.2**	44.3 ± 7.3	33.2 ± 5.8	24.5 ± 4.2	5
	50	3.8 ± 0.6	48.7 ± 5.9**	52.7 ± 8.1	47.1 ± 6.2*	26.7 ± 5.1	6
	100	4.1 ± 0.7	53.5 ± 6.3**	58.8 ± 9.4	58.3 ± 7.1**	25.9 ± 5.3	7

The results are presented as mean values ± s.e.mean of values of single frequency bands evaluated 30 min after microinfusion of different μ- and δ-opioid agonists in comparison to baseline. Significant differences are denoted: * $P < 0.05$ and ** $P < 0.01$ (paired Student's t test).

DAMGO DAMGO (1, 10, 30, 100 pmol and 1 nmol) was administered unilaterally into the LC of rats (at least 4 rats for each dose). At the lowest dose studied (1 pmol) DAMGO produced no significant behavioural and ECoG changes, whereas at the higher doses it induced marked and dose-dependent sedative effects. Analgesia and slight catatonia were also observed after the highest doses used (100 pmol and 1 nmol). The ECoG background activity of DAMGO was characterized by an increase in total voltage power (Figure 2 and Table 1) with a predominant significant ($P < 0.05$) increase in 3–6 and 6–9 frequency bands. Auditory or tactile stimuli were able to induce the behavioural and phasic EEG activity in the animals.

Effects of DADLE, an agonist at δ-receptors

Over the dose range tested (0.1 pmol–100 pmol) DADLE microinfused into the LC induced changes in behaviour and ECoG background activity. In particular, DADLE (1 pmol) injected unilaterally elicited initially stereotyped movements which were followed within 5–10 min following infusion by a slight behavioural sedation. The ECoG background activity revealed a significant increase in the theta rhythm in the frontoparietal cortical recordings. Spectral analysis showed an increase in total voltage power (Figure 3) and a sharp peak lying within the 3–6 Hz range. DADLE (10 and 50 pmol) induced behavioural and ECoG slow wave sleep lasting from 60 to 135 min according to the dose. Sensory stimuli were able to induce behavioural and phasic ECoG activity in the animals. After DADLE (100 pmol) the rats showed a marked soporific effect lasting approximately 180 min, associated with an ECoG activity characterized by a significant ($P < 0.01$) increase in total voltage power as well as in some bands of frequency (Table 1). The spectral analysis of the ECoG activity was in fact characterized by two distinct spectral peaks, one lying within the 3–6 Hz and the other within the 9–12 Hz range. Sometimes spindles occurred within the 12–16 Hz range. With such a dose a slight analgesia on the hot-plate test and slight catatonia were also evident. The lowest dose of DADLE (0.1 pmol) was unable to affect significantly the ECoG activity compared with the pretreatment control period or with vehicle-treated animals.

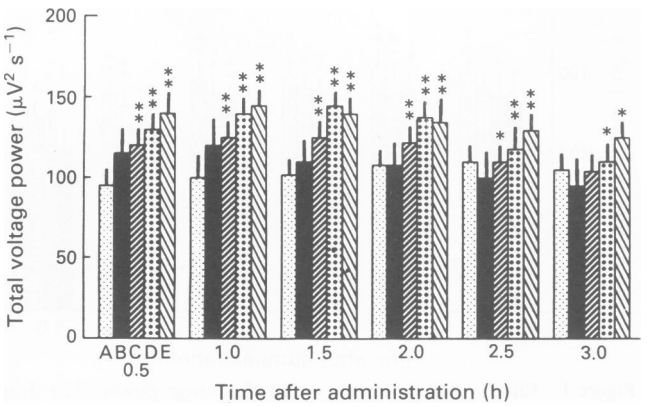

Figure 2 Effects of Tyr-D-Ala-Gly-N-Me-Phe-Gly-ol (DAMGO) on total voltage power. The drug was injected after 30 min of ECoG control recordings. Significant differences between DAMGO and vehicle group are denoted: * $P < 0.05$, ** $P < 0.01$ by Mann-Whitney U-test. Columns A, vehicle; B, 10 pmol; C, 30 pmol; D, 100 pmol; E, 1 nmol DAMGO.

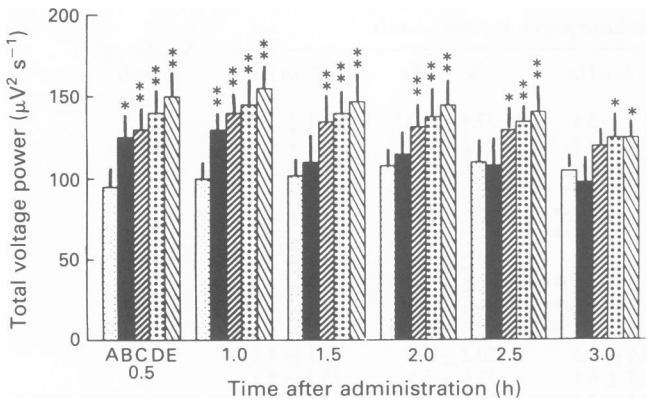

Figure 3 Effects of D-Ala-D-Leu-Thr-Gly-Gly-Phe-Leu (DADLE) on total voltage power. The drug was injected after 30 min of ECoG control recordings. Significant differences between DADLE and vehicle group are denoted: * $P < 0.05$, ** $P < 0.01$ by Mann-Whitney U-test. Columns A, vehicle; B, 1 pmol; C, 10 pmol; D, 50 pmol and E, 100 pmol DADLE.

Effects of U 50,488H, an agonist at κ-receptors

The unilateral microinjection of U 50,488H (0.1, 0.25, 1.0, 2.5, 5.0 and 10.0 pmol) into the locus coeruleus induced, after a preliminary phase of arousal and ECoG desynchronization, behavioural and electrocortical soporific effects which appeared within 3 to 25 min after the start of the infusion and lasted approximately 35–130 min depending on the dose (at least 6 experiments for each dose). During this period, the rats showed a periodic increase in total voltage power and in 0–3, 3–6, 6–9 and 9–12 Hz frequency bands (Table 2). The duration of the initial arousal period, characterized by some stereotyped movements (sniffing, grooming, licking) without an increase in locomotor and explorative activity, appeared to be dose-dependent and lasted from 3–25 min. In addition, during the soporific effects slight muscular hypotonia was observed; sensory stimuli were able to produce behavioural and phasic electrocortical arousal in the rats. A lower dose of U 50,488H (0.1 pmol) given into the LC did not significantly affect behavioural and electrocortical activity. In particular, after U 50,488H (0.25 pmol), a significant ($P < 0.01$) increase in total voltage power and in 3–6 and 6–9 Hz frequency bands was observed, while a less significant ($P < 0.05$) increase in 0–3 and 9–12 Hz frequency bands was obtained (Table 2). A slight and short-lasting increase in 12–16 Hz frequency band was seen after the largest dose of U 50,488H (10 pmol). Interestingly, the increase in total voltage power and in preselected frequency bands occurred initially in the ipsilateral hemisphere and then within 1–3 min diffused to the contralateral one.

Bilateral microinjection of U 50,488H (0.25 pmol) into the locus coeruleus was as effective as unilateral injection of 2.5 pmol (8 experiments) in producing behavioural and ECoG slow-wave sleep. When the injection of U 50,488H was more than 2 mm from the locus coeruleus no behavioural and electrocortical sedation occurred.

Effects of dextromethorphan and ketamine, two agonists at σ-receptors

Dextromethorphan The unilateral microinjection of 1, 5 and 10 pmol of dextromethorphan induced a mild increase of locomotor and exploratory activity. Stereotyped behaviour such as rearing, sniffing, licking and grooming was usually observed. During this state a decrease of total voltage power (Table 2), associated with a mild increase in the theta rhythm in the frontoparietal cortical recordings ($n = 4$ animals for each dose), was observed. This state of behavioural arousal lasted approx 19–43 min depending on the dose.

Bilateral microinfusion of dextromethorphan, 0.1 and 1 pmol, into the locus coeruleus was as effective as unilateral injection of 1 and 10 pmol, respectively, in producing behavioural and ECoG changes.

Ketamine Ketamine produced characteristic behavioural and ECoG effects after microinfusion of 10, 50 and 100 pmol into the locus coeruleus. After the microinfusion of 10 pmol, the animals ($n = 5$ rats) exhibited a state of behavioural arousal with a mild increase of locomotor and exploratory

Table 2 Effects of microinfusion of various κ and σ-opioid agonists into the locus coeruleus on changes in baseline voltage power of frequency bands 30 min after drug administration

Compound	Dose (pmol)	Changes in baseline voltage power frequency bands 0–3 Hz	3–6 Hz	6–9 Hz	9–12 Hz	12–16 Hz	Number of animals
Vehicle		3.5 ± 0.5	24.2 ± 2.8	41.8 ± 5.1	27.4 ± 3.2	22.1 ± 3.4	10
U 50,488H	0.1	3.3 ± 0.6	26.7 ± 3.3	46.2 ± 5.4	29.2 ± 3.4	23.4 ± 4.1	6
	0.25	3.6 ± 0.4	38.1 ± 4.1*	64.3 ± 7.7*	30.1 ± 4.1	24.7 ± 3.7	8
	1.0	4.1 ± 0.3	41.2 ± 5.2*	69.5 ± 8.3**	31.7 ± 5.2	26.3 ± 4.8	6
	2.5	4.9 ± 0.4	47.4 ± 5.5**	73.4 ± 8.9**	33.1 ± 4.4	29.2 ± 5.1	7
	5.0	5.3 ± 0.9*	49.8 ± 5.4**	77.6 ± 9.2**	37.2 ± 4.8*	31.7 ± 5.6	6
	10.0	5.5 ± 1.3*	55.1 ± 6.2**	74.5 ± 10.6**	42.5 ± 5.2*	39.2 ± 4.7*	6
Dextromethorphan	1	3.4 ± 0.6	39.4 ± 4.3*	44.2 ± 6.4	26.4 ± 2.9	24.5 ± 4.1	4
	5	3.9 ± 0.7	36.5 ± 4.2	49.3 ± 6.2	24.2 ± 3.7	23.9 ± 4.3	4
	10	3.2 ± 0.8	37.8 ± 4.4*	51.6 ± 7.5	23.9 ± 4.1	24.2 ± 3.8	4
Ketamine	10	3.7 ± 0.7	41.4 ± 4.8*	49.5 ± 6.4	25.2 ± 2.7	23.2 ± 3.7	5
	50	4.9 ± 0.6*	42.2 ± 5.1*	55.2 ± 6.8	23.4 ± 4.2	24.1 ± 4.1	5
	100	5.3 ± 0.8*	47.3 ± 5.6*	60.7 ± 7.6*	26.2 ± 5.1	22.8 ± 4.4	5

The results are presented as mean values ± s.e.mean of values of single frequency bands evaluated 30 min after microinfusion of different σ- and κ-opioid agonists in comparison to baseline. Significant differences are denoted: * $P < 0.05$ and ** $P < 0.01$ (paired Student's t test).

activity. Stereotyped behaviour such as rearing, sniffing, licking and grooming was also evident. This state of behavioural arousal did not affect the total spectrum power (Table 2), but was associated with a significant ($P < 0.05$) increase in the theta rhythm in the frontoparietal cortical recordings. After 50 and 100 pmol of ketamine, the rats ($n = 5$ for each dose) were lying on their sides sedated with their fore and hind limbs adducted. Other significant changes such as stereotyped behaviour and analgesia were not observed. The ECoG activity was characterized by an increase of total voltage power as well as of the lower frequency bands (Table 2). The spectral analysis of the ECoG activity revealed two distinct spectral peaks lying within the 0–3, 3–6 Hz and another within the 6–9 Hz frequency bands. It was also noted that the predominant frequency band of ECoG activity tended to shift to the lower values when the dose of ketamine was increased. Touch or tactile stimuli were able to elicit a behavioural and ECoG arousal state.

Effects of naloxone on overt sedation and on ECoG spectrum power changes induced by DAMGO, dermorphin, DADLE, U 50,488H and ketamine

Pretreatment (15 min before) with naloxone (1 and 2 pmol into the locus coeruleus) was able to antagonize or to reduce significantly the behavioural and ECoG effects induced by dermorphin, DAMGO and DADLE (at least 4 animals for each dose and compound studied). Similarly pretreatment (15 min before) with naloxone, given into the locus coeruleus, was able to affect behavioural and ECoG spectrum power changes induced by U 50,488H microinjected into the same site (at least 5 experiments for each dose). In particular, naloxone (10 pmol) was able to prevent behavioural and electrocortical soporific effects induced by U 50,488H. In contrast, lower doses of naloxone (1 and 2 pmol) were unable to antagonize the behavioural and electrocortical changes induced by U 50,488H (2.5 and 10 pmol). Naloxone (10 pmol into the LC) was unable to prevent or reduce the behavioural and ECoG effects induced by ketamine (10, 50 and 100 pmol) or dextromethorphan (1 and 10 pmol).

Discussion

The present experiments show that stimulation of specific subtypes of opioid receptors at the level of the locus coeruleus produces dose-dependent increase in ECoG total voltage power with a predominant increase in the lower frequency bands. This suggests that soporific effects induced by morphine, its congeners as well as endogenous opioid peptides are mediated by the locus coeruleus (Wilson & Dovosz, 1984). Several experiments have demonstrated that intracerebroventricular administration of β-endorphin and other opioid peptides produces in different animal species behavioural and electrocortical sleep (Freye & Arndt, 1979; Aloisi *et al.*, 1980; Mitra *et al.*, 1981; Nisticò *et al.*, 1980; 1981; 1985).

The present results confirm that the locus coeruleus is a crucial location involved in sleep regulation. Previous experiments have in fact documented that during sleep-awake stages there is a change in the bioelectric activity of single units in the locus coeruleus (Steriade & Hobson, 1976; Clark, 1979; Ramm, 1979; Aston-Jones & Bloom, 1981a,b; Cespuglio *et al.*, 1982). Furthermore, in previous experiments we have shown that the direct microinfusion of agonists at α_2-adrenoceptors into the LC produces dose-dependent behavioural sleep and ECoG synchronization (De Sarro *et al.*, 1987; 1988a).

In preliminary experiments we have also shown that the direct microinfusion of dermorphin, an agonist at μ-opioid receptors (Amiche *et al.*, 1987), was able to produce marked behavioural and ECoG soporific effects (Nisticò *et al.*, 1981; 1985). Actually, from the present experiments it was confirmed that dermorphin was the most potent hypnogenic compound after its microinfusion into the LC. In fact, comparatively, DAMGO, another selective agonist for μ-receptors, was found to be approximately 30 fold less potent in increasing ECoG total voltage power. Similar ECoG spectrum power effects were observed after microinfusion into the LC of DADLE, a selective agonist at δ-opioid receptors, although in comparison to dermorphin much higher doses (10 times) were required to elicit similar electrocortical changes (Figure 4). The common effects on ECoG spectrum power shared by μ- and δ-agonists are not surprising as DADLE possesses an affinity for δ-receptors only approx 4.2 times higher than for μ-receptors (Kosterlitz *et al.*, 1981; Chang *et al.*, 1981; Pfeiffer &

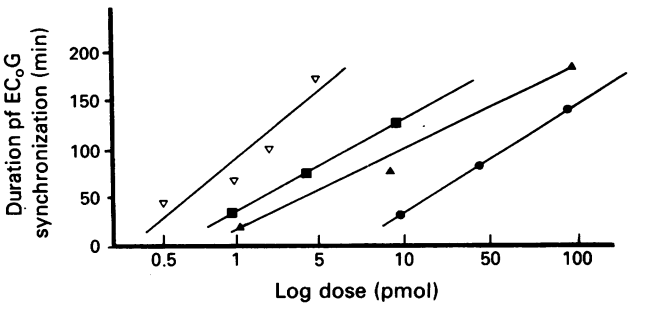

Figure 4 Duration of ECoG synchronization induced by several doses of dermorphin (▽), DADLE (■), DAMGO (▲) and U 50,488H (●). It is evident that dermorphin was the most potent compound studied. For key to abbreviations used see legends of Figures 2 and 3.

Hertz, 1981). In addition, μ- and δ-receptors seem to share common transducing mechanisms since they act through a G_i protein leading to inhibition of adenylate cyclase as well as the same ionic mechanism (increase in K^+ conductance) at the membrane level (see Di Chiara & Morelli, 1987).

On the other hand, at the level of the LC stimulation of opioid receptors inhibits the neuronal firing of noradrenergic cell bodies (Pepper & Henderson, 1980; Williams & North, 1984; Andrade & Aghajanian, 1985). A different behavioural and electrocortical pattern was instead observed after microinfusion of U 50,488H, a selective agonist at κ-opioid receptors. In fact, a typical biphasic response characterized by an initial phase of behavioural arousal and ECoG desynchronization and followed by a longer-lasting second phase of behavioural and electrocortical sleep was observed. Also in this case, the doses required to produce similar electrocortical synchronization during the second phase were higher (approx 15 times) in comparison to those of dermorphin (Figure 4). Thus, it seems that with κ-agonists the predominant effect is also behavioural and electrocortical sleep, confirming the sedative effects described by Gilbert & Martin (1976). However, these effects were preceded by a short-lasting stage of behavioural stimulation, which could be due to a non-specific effect on other receptor subtypes. The soporific effects induced by U 50,488H are very likely due to inhibition of the neuronal firing of the noradrenergic LC neurones, since *in vitro* this compound has been shown to produce at the level of the LC a dose-dependent depression of excitatory post-synaptic potentials (Henderson *et al.*, 1986).

The κ-agonist U 50,488H had specific and different effects on behaviour and ECoG activity after LC microinfusion, suggesting that it is not active at μ-receptors. This hypothesis is further supported by the finding that a 5–10 times higher dose of naloxone was necessary to antagonize the behavioural and ECoG changes induced by U 50,488H. The approximately 10 fold higher potency of naloxone in antagonizing behavioural and ECoG responses of dermorphin than those of U 50,488H is in good agreement with binding assay data obtained by several authors (VonVoigtlander *et al.*, 1982; Lahti *et al.*, 1982; Chang, 1984; Goldstein & James, 1984; Cotton *et al.*, 1985; Tang & Collins, 1985).

Walker *et al.* (1982) showed that direct administration of dynorphin, a κ-agonist, into the hippocampus inhibits the discharge rate of hippocampal pyramidal neurones and intracerebroventricular administration of dynorphin produces large amplitude, slow wave activity. Thus our data support these previous findings and are also in agreement with earlier results, suggesting a sedative role for κ-opioid receptors (Tilson *et al.*, 1986; VonVoigtlander *et al.*, 1982).

In contrast to the soporific effect and to the increase in total voltage ECoG power elicited by μ-, δ- and κ-opioid receptor agonists, the stimulation of σ-receptors by ketamine and dextromethorphan induced a sustained behavioural arousal, ECoG desynchronization and an intense pattern of stereotyped movements. Thus it is conceivable that the behavioural stimulation initially observed with U 50,488H may be due to a non-specific activation of σ-receptors or other excitatory transmitter receptors.

Overall, the present experiments seem to be in good agreement with the anatomical distribution of the different opioid receptor types in the brain, since at the level of the LC μ- and κ-receptors are present in a parallel distribution, whereas little or no δ-binding sites occur in the brain-stem (Mansour *et al.*, 1988). In addition, the present experiments are also in agreement with the microiontophoretic study carried out, with specific agonists at μ-, δ- and κ-opioid receptors, on the activity of single neurones in the brain stem and other areas of the rat brain. The predominant effect of all the opioid agonists was depression of neuronal activity in the brain stem which was antagonized by naloxone (Bradley & Brookes, 1984).

These results illustrate the usefulness in studying *in vivo* changes in gross behaviour and ECoG spectrum induced by pharmacological agents. The qualitative differences in ECoG response profiles of DAMGO, dermorphin, DADLE, dextromethorphan, ketamine and U 50,488H provided a helpful comparison of activity between these compounds acting at different opioid receptors.

In conclusion, the present experiments show that stimulation of μ- and κ-opioid receptors at the locus coeruleus level produces dose-dependent ECoG sleep, although with a different electrocortical profile. The most powerful soporific substance was represented by dermorphin, a μ-receptor agonist. Hence, the LC may represent one of the predominant sites through which morphine, its congeners and opioid peptides exert their marked effects on electrocortical activity.

Partial support from the Italian Council for Research (CNR, Rome) and Italian Ministry of Public Education (MPI, Rome) is gratefully acknowledged. Dermorphin was kindly supplied by Professor V. Erspamer (Rome). Our thanks to Mrs Adriana Mastroeni for excellent typing of the manuscript, to Dr M.G. Audino, Mrs Marika Nisticò Mr F. Aceto and G. Marra for their collaboration in the experimental work, in the statistical evaluation and in the preparation of the graphs.

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(Received June 5, 1989
Revised April 20, 1990
Accepted June 21, 1990)

Br. J. Pharmacol. (1990), **101**, 662–666

Two distinct α_1-adrenoceptor subtypes involved in noradrenaline contraction of the rabbit thoracic aorta

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1 Recently, α_1-adrenoceptors in blood vessels have been classified into three subtypes (α_{1H}, α_{1L} and α_{1N}). We examined which subtype (or subtypes) is involved in the noradrenaline-induced contraction of rabbit thoracic aorta.

2 Noradrenaline produced a concentration-dependent contraction in the rabbit isolated thoracic aorta. Prazosin antagonized the contractions to noradrenaline, resulting in a rightward displacement of the concentration-response curve. However, the shift was not proportional to the concentration of prazosin; Schild plots showed that the inhibition by prazosin was biphasic, implying that noradrenaline acted through two receptor populations. Two affinity constants (pK_B values of 10.02 and 8.83) were determined for prazosin at these sites.

3 However, under continuous treatment with 1 nM prazosin, or in strips pretreated with chlorethylclonidine (CEC; an α_{1H} inactivating agent) to remove the contribution of one receptor population, prazosin showed a single pK_B or pA_2 value of approximately 8.3.

4 Yohimbine also produced biphasic antagonism of noradrenaline-induced contractions, resulting in two affinity constants (pK_B = 6.52 and 6.17). However, a monophasic Schild plot was obtained for yohimbine either in the presence of 1 nM prazosin (pA_2 = 6.08) or in strips pretreated with CEC (pA_2 = 6.03).

5 The Schild plot for HV723 (a selective α_{1N}-antagonist) yielded a monophasic slope (pK_B = 8.47) and the inhibition was not affected by 1 nM prazosin or CEC-pretreatment.

6 [^{3}H]-prazosin bound to α_1-adrenoceptors of the aortic membrane preparations with two different affinities (pK_D = 9.94 and 8.37). The high but not the low affinity site was completely masked by 1 nM prazosin and inactivated by pretreatment with CEC.

7 These results strongly suggest that noradrenaline-induced contraction of the rabbit thoracic aorta is mediated through two distinct α_1-adrenoceptor subtypes, designated α_{1H} and α_{1L}.

Introduction

α_1-Adrenoceptors are not homogeneous in all tissues and their heterogeneity has been suggested to be related in part to the presence of α_1-adrenoceptor subtypes (Bülbring & Tomita, 1987; McGrath & Wilson 1988; Minneman, 1988). Recently, Flavahan & Vanhoutte (1986) and Muramatsu *et al.* (1990) have demonstrated that α_1-adrenoceptors of blood vessels can be classified into two (α_{1H} and α_{1L}) or three (α_{1H}, α_{1L} and α_{1N}) subtypes. However, it is uncertain whether one or more subtypes contribute to noradrenaline-induced contraction in an individual blood vessel. In the pulmonary arteries of rabbits and dogs, Holck *et al.* (1983) and Flavahan *et al.* (1987) showed that prazosin antagonized the contractile responses to methoxamine and clonidine or phenylephrine with two different affinities, which suggested the existence of two subtypes of α_1-adrenoceptor in a single tissue. However, Docherty (1988) and Docherty & Ruffolo (1989) could not find any evidence for heterogeneity in α_1-adrenoceptors in the same arteries. Single or multiple affinity sites of α_1-adrenoceptor have also been demonstrated in binding studies with vascular tissues, including the rabbit thoracic aorta (Awad *et al.*, 1983; Babich *et al.*, 1987; Jagadeesh & Deth, 1987; Piascik *et al.*, 1988). In the present study, we examined which subtype (or subtypes) contributes to the noradrenaline-induced contraction of rabbit thoracic aorta, in the light of the recently proposed criteria for α_1-adrenoceptor subclassification (Muramatsu *et al.*, 1990). The results obtained show that noradrenaline-induced contraction of the rabbit thoracic aorta is mediated through both α_{1H} and α_{1L} subtypes.

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Methods

Functional study

Rabbits of either sex, weighing 2.5–4 kg, were killed under pentobarbitone anaesthesia, and the thoracic aorta was rapidly removed. The aorta was cleaned of adhering connective tissue and cut helically under a dissecting microscope. In order to avoid the possible involvement of endothelium-derived relaxing factor in the mechanical response (Furchgott, 1981a), the endothelial cells were removed by rubbing them with filter paper, and the functional loss of endothelial cells was confirmed by the loss of the relaxation response to acetylcholine (1 μM) in noradrenaline-precontracted aorta (Muramatsu *et al.*, 1987). The strip was mounted vertically in an organ bath containing 20 ml Krebs-Henseleit solution of the following composition (mM): NaCl 112, KCl 5.9, $MgCl_2$ 1.2, $CaCl_2$ 2, $NaHCO_3$ 25, NaH_2PO_4 1.2 and glucose 11.5. Desmethylimipramine (0.1 μM), deoxycorticosterone (5 μM) and propranolol (3 μM) were added to the bathing solution to block neuronal and extraneuronal uptake of noradrenaline and to block β-adrenoceptors, respectively. The bath medium was maintained at 37°C, pH 7.4, and was equilibrated with a gas mixture consisting of 95% O_2 and 5% CO_2. A resting tension of 1.5 g was applied and the responses were recorded isometrically through force-displacement transducers. All preparations were equilibrated for 90 min before the experiments were begun.

Concentration-response curves for noradrenaline were obtained by adding the drug directly to the bathing media in cumulative concentrations. The curves were obtained 6 times in the same strip at 90 min intervals and the third

concentration-response curve was used as control. In preliminary experiments, reproducibility of the concentration-response curves obtained by third to sixth trials in the absence of antagonist was confirmed (Muramatsu *et al.*, 1990). Preparations were treated with α-adrenoceptor antagonists for 30 min before, and during, the construction by concentration-response curves to noradrenaline. With chlorethylclonidine (CEC) treatment, the preparations were treated once for 20 min with 50 μM CEC and then washed with the drug-free solution for 30 min, before the second concentration-response curve for noradrenaline. However, in the experiments shown in Figure 2, CEC was applied for 20 min between the third and fourth concentration-response curves for noradrenaline.

The antagonist potency was represented as the pK_B value or pA_2 value. Briefly, the concentration of noradrenaline necessary to elicit a half-maximal contraction in the presence of each α-adrenoceptor antagonist was divided by the concentration giving a half-maximal response in the control, to determine the agonist concentration-ratio (CR). Data were plotted as the −log antagonist concentration (M) vs the log (agonist CR − 1). pA_2 values were calculated from Schild plots along mean slope and 95% confidence limits (95% CL) and straight lines were drawn by least square linear regression. When the straight line yielded a slope with unity, the pA_2 value estimated was represented as the pK_B (Arunlakshana & Schild, 1959). The pK_B value was also determined for single concentrations of antagonist by the concentration-ratio method (Furchgott, 1972).

[^3H]-prazosin binding study

Rabbit thoracic aorta was homogenized in 50 volumes of ice-cold 10 mM HEPES buffer containing 0.32 M sucrose (pH 7.2) and centrifuged at 700 *g* for 10 min. The supernatant was filtered through cheese cloth and subjected to further centrifugation at 10,000 *g* for 20 min and at 100,000 *g* for 30 min, consecutively. The resulting pellet from 100,000 *g* spinning was suspended in assay buffer (50 mM Tris-HCl containing 5 mM $MgCl_2$, pH 7.7 at 25°C), recentrifuged at 100,000 *g* for 30 min and the final pellet used as the membrane preparation. [^{3}H]-prazosin (30–6000 pM) was incubated with the membrane preparation (about 50 μg protein ml^{-1}) for 30 min at 25°C. Reactions were terminated by rapid filtration with a Brandel cell harvester onto Whatman GF/B filters presoaked in 0.3% polyethylenimine for 5 min. The filters were then washed 4 times with 4 ml of ice-cold 50 mM Tris-HCl buffer (pH 7.7) and dried; the filter-bound radioactivity was determined. Non-specific binding was defined as binding in the presence of 10 μM phentolamine. Assays were conducted in duplicate or triplicate.

CEC treatment The membrane preparation was incubated for 20 min at 37°C with 50 μM CEC and then centrifuged at 100,000 *g* for 30 min. The pellet was washed once with assay buffer before the binding assay.

Data were analysed by the weighted least-squared iterative curve fitting programme LIGAND (Munson & Rodbard, 1980). The data were first fitted to a one- and then a two-site model, and if the residual sums of squares was statistically less for a two-site fit of the data than for a one-site, as determined by an F-test comparison, then the two-site model was accepted. *P* values less than 0.05 were considered significant. Negative log equilibrium dissociation constants at the high and low affinity sites were expressed as pK_{Dhigh} and pK_{Dlow}, respectively. The proportion binding at the low affinity site was denoted as $\%R_L$ of total binding sites.

Statistical analyses

Experimental values are given as a mean ± s.e.mean or means with 95% confidence limits. Results were analysed by Student's *t* test (unpaired or paired comparison) and a probability of less than 0.05 was considered significant.

Drugs

The following drugs were used: (−)-noradrenaline bitartrate, desipramine hydrochloride (Sigma, St. Louis, U.S.A.), deoxycorticosterone acetate, (±)-propranolol hydrochloride, yohimbine hydrochloride (Nacalai, Kyoto, Japan), chlorethylclonidine dihydrochloride (CEC: Funakoshi, Tokyo, Japan), HV723 (α-ethyl-3,4,5-trimethoxy-α-(3-((2-(2-methoxyphenoxy) ethyl))-amino)propyl) benzeneacetonitrile fumarate, Hokuriku Seiyaku, Katsuyama, Fukui Japan), [^{3}H]-prazosin (specific activity 82 Ci $mmol^{-1}$, NEN, Boston, U.S.A.).

Results

Functional study

Noradrenaline, at concentrations in excess of 1 nM, produced a concentration-dependent contraction in the rabbit isolated thoracic aorta ($pD_2 = 7.16 \pm 0.10$, 12 experiments) (Figure 1a). Treatment with prazosin antagonized the noradrenaline-induced contraction, resulting in a rightward displacement of the concentration-response curves. However, the extent of shift was not proportional to the concentrations of prazosin; the shift elicited by a 10 fold increase in prazosin concentration was smaller at 0.1 to 10 nM than those at 100 to 1000 nM. The Schild plot for the antagonistic effect of prazosin clearly showed that the slope of the regression line was significantly different from unity (slope = 0.608, 95% CL = 0.533–0.683, broken line on left in Figure 1b) and rather suggested that the antagonism of noradrenaline by prazosin was biphasic (Figure 1b). Therefore, it is likely that prazosin distinguished between two components in the concentration-response curve to noradrenaline: one component was selectively activated by low concentrations of noradrenaline (1–

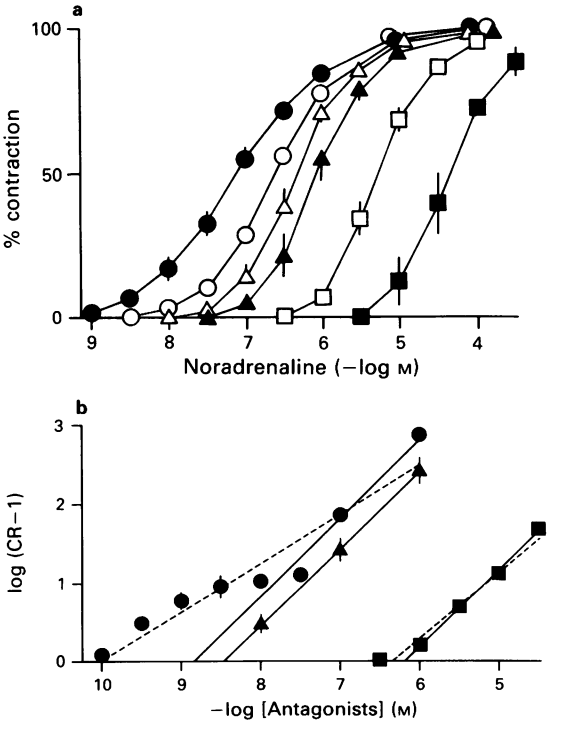

Figure 1 (a) Effects of prazosin on the concentration-response curves to noradrenaline in the rabbit thoracic aorta. (●) Control responses; responses in presence of prazosin (○) 0.1 nM, (△) 1 nM, (▲) 10 nM, (□) 100 nM and (■) 1000 nM. (b) Schild plots for competitive inhibition of noradrenaline contractions by prazosin (●), HV723 (▲) and yohimbine (■). Each point is the mean of data from six to eight experiments and vertical lines show s.e.mean. Broken lines: regression lines obtained from all data for prazosin (left) or yohimbine (right). For pA_2 values and slope of solid lines see Table 1.

Table 1 α_1-Adrenoceptor affinities for prazosin, HV723 and yohimbine in the rabbit thoracic aorta after various treatments

Antagonist	*Treatment*	*pK_B or pA_2*	*Slope (95% CL)*
Prazosin	None	10.02 ± 0.05[a]	
		8.83 ± 0.12	0.997 (0.860–1.135)
	Prazosin 1 nM	8.34 ± 0.11	0.951 (0.802–1.099)
	CEC 50 μM	8.29 ± 0.07	0.903 (0.818–0.988)[b]
HV723	None	8.47 ± 0.16	0.978 (0.756–1.199)
	Prazosin 1 nM	8.21 ± 0.11	0.899 (0.748–1.051)
	CEC 50 μM	8.31 ± 0.10	0.876 (0.755 ± 0.997)[b]
Yohimbine	None	6.52 ± 0.09[a]	
		6.17 ± 0.06	1.000 (0.874–1.127)
	Prazosin 1 nM	6.08 ± 0.04	0.939 (0.841–1.036)
	CEC 50 μM	6.03 ± 0.04	0.820 (0.744–0.897)[b]

[a] The pK_B values were estimated from the inhibitory effects of 100 and 300 pM prazosin or 0.3 μM yohimbine.
[b] The slope deviates slightly from unity ($0.05 < P < 0.1$). CEC = chlorethylclonidine.

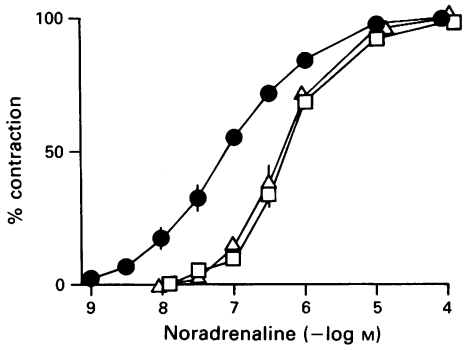

Figure 2 Concentration-response curves to noradrenaline in the rabbit thoracic aorta. (●) Control responses, (△) responses in the presence of 1 nM prazosin, (□) after pretreatment with 50 μM chlorethylclonidine for 20 min. Each value is the mean of 6–8 experiments with s.e.mean shown by vertical lines.

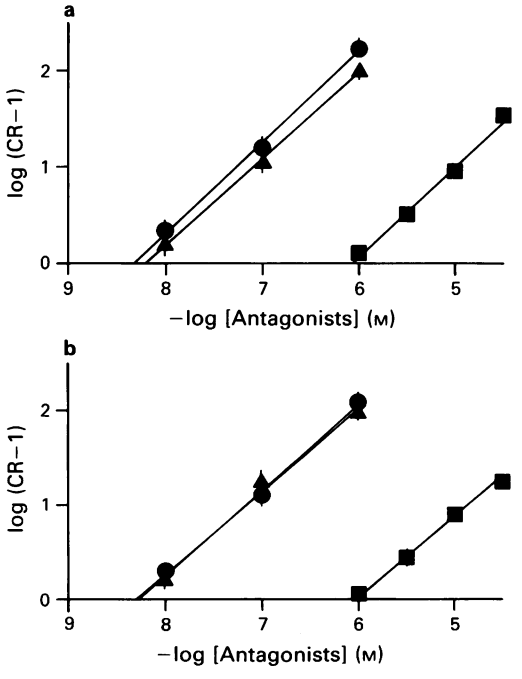

Figure 3 Schild plots for competitive inhibition of noradrenaline contractions by prazosin (●), HV723 (▲) and yohimbine (■) in (a) presence of 1 nM prazosin and (b) strips pretreated with 50 μM chlorethylclonidine. Each value is the mean of 6–8 experiments with s.e.mean shown by vertical lines. For pA_2 values and slopes of Schild plots see Table 1.

Table 2 [^{3}H]-prazosin binding of rabbit aortic membrane preparations

Treatment	n	*pK_{Dhigh}*	*pK_{Dlow}*	*$\%R_L^a$*
None	5	9.94 ± 0.07	8.37 ± 0.13	66
Prazosin 1 nM	4	—	8.75 ± 0.06	100
CEC 50 μM	4	—	8.97 ± 0.11	100

Data shown are means ± s.e.mean, n = number of experiments
[a] The proportion binding at the low affinity site compared to total binding sites. pK_{Dhigh} and pK_{Dlow} are negative log of the equilibrium dissociation constants at the high and low affinity sites, respectively.

30 nM) and inhibited by low concentrations of prazosin (0.1–1 nM), while the second component was activated by higher concentrations of noradrenaline and inhibited by higher concentrations of prazosin. Two apparent affinity constants for prazosin ($pK_B = 10.02 \pm 0.05$ and 8.83 ± 0.12) were estimated from concentration-ratios in the presence of 0.1–0.3 nM and 10–1000 nM prazosin, respectively (Figure 1b and Table 1).

Yohimbine and HV723 also antagonized the response to noradrenaline. The slope of Schild regression for the inhibitory effect of yohimbine was different from unity (slope = 0.858, 95% CL = 0.745–0.971, broken line on right in Figure 1b), supporting the existence of two components (Table 1). However, HV723 could not distinguish the two components, producing a linear Schild plot (Figure 1b) and a pK_B value of 8.47 (Table 1).

If one of two subtypes was selectively masked by a given drug, a competitive antagonist should yield a linear Schild plot from which can be derived a single affinity. Prazosin at 1 nM was first used for this purpose, as there was a 15 fold difference between its affinity constants for the two putative receptors. In the presence of 1 nM prazosin, noradrenaline at concentrations over 30 nM produced a concentration-dependent contraction ($pD_2 = 6.35 \pm 0.09$, $P < 0.05$ compared with the pD_2 in the absence of prazosin, 6 experiments, Figure 2). Prazosin and yohimbine antagonized the contraction and yielded a straight line with a Schild plot slope not different from unity (Figure 3a). Since the log (agonist CR − 1) at each concentration of antagonist was less than that under prazosin-unmasking, slightly smaller pK_B values were estimated compared with the low values obtained under unmasked conditions (Table 1). In another series of experiments, an irreversible alkylating agent chlorethylclonidine (CEC) was used because this drug had been shown to inactivate selectively the prazosin-high sensitivity site (Muramatsu *et al.*, 1990). CEC (50 μM) itself produced no contractile action in the rabbit aorta. In the preparations pretreated with 50 μM CEC, noradrenaline produced a concentration-response curve ($EC_{50} = 6.30 \pm 0.06$, $P < 0.05$ compared with the pD_2 in the absence of prazosin, 6 experiments) similar to that in the presence of 1 nM prazosin (Figure 2), and the competition by prazosin or yohimbine each yielded a monophasic slope and a low pA_2 value from the Schild plots (Figure 3b and Table 1). On the other hand, the pK_B or pA_2 value for HV723 was not significantly changed by such pretreatments.

[^3H]-prazosin binding study

[^{3}H]-prazosin in concentrations ranging from 30 to 6000 pM was used to label α_1-adrenoceptors in the membrane preparation of rabbit thoracic aorta. The saturation isotherm for specific [^{3}H]-prazosin binding showed that [^{3}H]-prazosin binding was not monophasic, as found in other studies (Babich *et al.*, 1987; Piascik *et al.*, 1988). LIGAND analysis fitted the data to a two-site model. The high affinity site had a B_{max} of 94.2 ± 12.1 fmol mg^{-1} protein and an equilibrium dissociation constant (expressed as pK_D) of 9.94 ± 0.07, while the low affinity site had a B_{max} of 184.2 ± 41.4 fmol mg^{-1} protein and a pK_D of 8.37 ± 0.13 (5 experiments). In the presence of

1 nM prazosin or in the membrane fraction pretreated with 50 μM CEC, the high affinity site was completely masked. Thus, [^{3}H]-prazosin recognized only a single site after such treatments, with a pK_D value close to the value of the low affinity site observed in untreated membrane preparations (Table 2).

Discussion

Noradrenaline-induced contraction of the rabbit thoracic aorta was biphasically inhibited by prazosin and yohimbine. In the binding study, also, [^{3}H]-prazosin bound to α_1-adrenoceptors with two different affinities, the values of which were in good agreement with the pK_B and pA_2 values for prazosin obtained in the functional study. These results suggest that prazosin and yohimbine can recognize two distinct α_1-adrenoceptor sites.

Recently, α_1-adrenoceptors of blood vessels have been subclassified into three subtypes (α_{1H}, α_{1L} and α_{1N}) according to different affinities for prazosin, yohimbine and HV723 (Muramatsu *et al.*, 1990). The α_{1H} subtype has high affinity for prazosin (pA_2: >9.5), and yohimbine (>6.5), while the α_{1L} subtype has lower affinities for both antagonists. The α_{1N} subtype is highly selective for HV773 (>9.0) and yohimbine (>6.5) but shows relatively low affinity for prazosin (<9.0). CEC, an alkylating agent (LeClerc *et al.*, 1980), also produces a selective inactivation or persistent activation of the α_{1H} subtype.

According to these criteria, it is likely that the two α_1-adrenoceptor sites of rabbit thoracic aorta correspond to α_{1H} and α_{1L} subtypes. Because, (1) the absolute pK_B and pA_2 or pK_D values for prazosin or yohimbine accorded with the affinity of α_{1H} or α_{1L} subtype; (2) HV723, a selective α_{1N} antagonist (Muramatsu *et al.*, 1990), could not discriminate between the high and low affinity sites for prazosin or yohimbine; and (3) under the conditions in which the α_{1H} subtype was selectively inhibited by prazosin (a low concentration) or masked by CEC pretreatment, prazosin and yohimbine recognized only a single site with an affinity equal to that of α_{1L}.

Two distinct [^{3}H]-prazosin binding sites were previously obtained in the rabbit thoracic aorta (Babich *et al.*, 1987; Piascik *et al.*, 1988). However, there has been no functional study demonstrating two affinity constants for prazosin in the aorta. Since narrow ranges of prazosin concentration have been used for Schild analysis in most previous studies, the high affinity site might have been missed. Kenakin (1984) emphasized the need to use a wide range of concentrations of an antagonist for Schild analysis.

The coexistence of two α_1-adrenoceptor subtypes in a single tissue would complicate Schild analysis (Furchgott, 1981b; Kenakin, 1984; Milnor, 1986). A reliable pK_B value for each subtype can be estimated when the response is caused through only one of two subtypes at a given concentration of agonist, or if both subtypes have the same affinity for an antagonist. Since noradrenaline-induced contractions of the rabbit thoracic aorta are likely to be produced through two distinct α_1-adrenoceptor populations, the pK_B and pA_2 values obtained in the present study would be regarded as approximate estimates for each antagonist. Indeed, pD_2 values for noradrenaline were significantly reduced by masking one of the two subtypes by prazosin (1 nM) or CEC, resulting in a reduction of pK_B or pA_2 values for prazosin (Table 1).

Milnor (1986) has analysed the Schild plots for a two-receptor system where both receptor subtypes produces the same qualitative response to an agonist, and has suggested that the proportion of one subtype in the total number of receptors influences the validity of Schild analysis. In the rabbit thoracic aorta, the high affinity site for [^{3}H]-prazosin comprised approximately 34% of the total binding sites. This proportion, together with a large difference in sensitivity to prazosin between α_{1H} and α_{1L} subtypes, should cause a clear biphasic inhibition of prazosin in Schild plots and relatively reliable pA_2 values. In contrast, biphasic inhibition by yohimbine in Schild plots was not as evident compared with the results with prazosin. This may be due to the small difference of selectively to yohimbine between both subtypes. However, a more precise analysis method for a two-receptor system must be established in order to estimate two distinct and true pK_B values.

Selective inhibition of the α_{1H} subtype by prazosin (low concentrations) or CEC suggests that the residual concentration-response curves to noradrenaline are predominantly mediated through the α_{1L} subtype (Figure 2). The curves further reveal that the α_{1L} subtype alone can elicit the maximum contraction in the rabbit aorta, as noradrenaline elicited the maximum contraction even after inhibition of the α_{1H} subtype. On the other hand, the contribution of the α_{1H} subtype should be determined after selective inhibition of the α_{1L} subtype.

Previously, we have shown that noradrenaline-induced contractions of the dog carotid artery and of the rat thoracic aorta are inhibited by low concentrations of prazosin, suggesting that the responses are also mediated through the α_{1H} subtype (Muramatsu *et al.*, 1990). However, CEC produced opposite actions on the α_{1H} subtype of both arteries; an inactivation in the dog carotid artery and a persistent activation in the rat thoracic aorta. Similar to the dog carotid artery, in the rabbit thoracic aorta CEC caused an inactivation of the α_{1H} subtype and failed to produce any contractile response. These results suggest that the α_{1H} subtype may not be homogeneous and indicate that the α_{1H} subtype of the rabbit thoracic aorta has similar properties to those of dog carotid artery.

In contrast to the α_{1H}, α_{1L}, α_{1N} subclassification, another subclassification has been proposed (α_{1A} and α_{1B} or α_{1a} and α_{1b}: Morrow & Creese, 1986; Han *et al.*, 1987; Minneman, 1988; Hanft & Gross, 1989). In their subclassifications, however, prazosin cannot distinguish between the proposed subtypes. Therefore, it is unlikely that the two affinity sites observed in the present study fit their subclassifications.

In conclusion, the present results indicate that the noradrenaline-induced contraction of rabbit thoracic aorta is mediated through two distinct α_1-adrenoceptor subtypes, presumably α_{1H} and α_{1L}.

We thank N. Aoki for secretarial assistance. This work was supported in part by a grant from the Ministry of Education, Science and Culture, Japan.

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(Received December 1, 1989
Revised June 13, 1990
Accepted June 25, 1990)

Br. J. Pharmacol. (1990), **101**, 667–673

Behavioural evidence for functional interactions between 5-HT-receptor subtypes in rats and mice

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1 Different 5-hydroxytryptamine (5-HT) receptor subtypes mediate different behavioural responses. Compounds acting at more than one 5-HT receptor exert behavioural effects which may be the result of response competition or a specific interaction between pathways within the CNS. Therefore the mutual interaction between different 5-HT receptor subtypes was studied.

2 Hypothermia and hypoactivity in mice induced by the 5-HT_{1A}-agonist 8-hydroxy-dipropylamino-tetralin (8-OH-DPAT) could be attenuated by the preferential 5-HT_{1C}-agonists MK 212, 1-(meta-chlorophenyl)-piperazine (mCPP) and m-trifluoromethyl phenyl piperazine (TFMPP), and by the mixed 5-$HT_{2/1C}$-agonist 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI). The mixed 5-$HT_{1A/1B}$-agonist CGS 12066B at 10 mg kg^{-1} potentiated hypothermia and had no effect on hypoactivity.

3 Forepaw treading in rats induced by the 5-HT_{1A}-agonist 8-OH-DPAT was attenuated by the 5-HT_{1C}-agonists MK 212 and mCPP. The 5-HT_{1C}-agonist TFMPP had a bimodal effect: at low doses (< 1 mg kg^{-1}) it potentiated, and at higher doses (> 2.2 mg kg^{-1}) it attenuated forepaw treading. the mixed 5-$HT_{2/1C}$-agonist DOI produced 5-HT_2-related behaviours and potentiated 8-OH-DPAT-induced forepaw treading. This indicates an attenuating effect of 5-HT_{1C}-receptor activation and a potentiating effect of 5-HT_2-receptor activation. CGS 12066B had no effect in this respect.

4 Head shakes in rats induced by DOI could be attenuated by 8-OH-DPAT, TFMPP, mCPP and MK 212. The ID_{50}s were 0.03, 0.7, 0.1 and 2 mg kg^{-1}, respectively. This suggests that a 5-HT_2-receptor-mediated effect may be attenuated by activation of 5-HT_{1A}- or 5-HT_{1C}-receptors. CGS 12066B attenuated the head shake response but only at 10 mg kg^{-1}.

5 The results suggest that interactions exist between the different 5-HT receptor subtype-mediated events. Therefore, care is needed in drawing conclusions from functional measurements when compounds have more or less equal affinities for more than one 5-HT-receptor subtype.

Introduction

Since Hess & Doepfner (1961) and later Grahame-Smith (1971) described the 5-hydroxytryptamine (5-HT) syndrome, consisting of forepaw treading, head weaving, flat body posture, head shakes, hind limb abduction and tremors, following injections of 5-hydroxytryptamine plus a monoamine oxidase inhibitor, this syndrome and elements of it have been seen after injection of a variety of 5-HT receptor agonists. The synthesis of 5-HT receptor subtype-selective agonists and/or antagonists has made it possible to study the functions of the different 5-HT receptors, and to ascribe some components of the 5-HT syndrome to selective activation of one of these 5-HT receptor subtypes.

At present there is evidence to suggest that drug-induced head shakes are the result of 5-HT_2-receptor activation (Yap & Taylor, 1983) and that stimulation of the presynaptic 5-HT_{1A}-receptor results in hypothermia (Goodwin *et al.*, 1985; Gudelsky *et al.*, 1986). Activation of the postsynaptic 5-HT_{1A}-receptor induces forepaw treading (Tricklebank *et al.*, 1984). Recently, we suggested that selective activation of the 5-HT_{1A}-receptors, possibly presynaptic, induces lower lip retraction and that the induction of penile erections is the result of 5-HT_{1C}-receptor activation (Berendsen *et al.*, 1989a; 1990).

However, compounds with activity at more than one subtype of 5-HT receptor do not always induce the behaviour thought to be the result of activation of those receptors. For example, the compound RU 24969, (5-methoxy-3(1,2,3,6-tetrahydropiridin-4-yl) 1H indole) which binds to 5-HT_{1A}- and 5-HT_{1B}-receptors (Hoyer, 1988a) only induces the 5-HT_{1A}-related lower lip retraction. 5-Methoxy-N,N-dimethyl tryptamine (5-MeODMT), a compound that binds with high affinity to 5-HT_{1A}-, 5-HT_{1C}- and, with lower affinity to 5-HT_2-receptors (Hoyer, 1988b; Titeler *et al.*, 1988) only induces lower lip retraction after blockade of the 5-HT_{1C} receptors (Berendsen *et al.*, 1989a). (±)-1-(2,5-Dimethoxy-4-iodophenyl)-2-aminopropane HCl (DOI), which binds to both 5-HT_2- and 5-HT_{1C}-receptors (Hoyer, 1988b; Titeler *et al.*, 1988), induces the 5-HT_2-related head shakes, but not 5-HT_{1C}-related penile erections. However, in rats pretreated with a 5-HT_2 antagonist, this compound induces penile erections while the head shakes disappear. It has also been shown that the potency to induce penile erections by compounds that have affinity for 5-HT_{1C}- and 5-HT_2-receptors, is dependent on their 5-HT_{1C}/5-HT_2 affinity ratio (Berendsen *et al.*, 1990). All this points to a functional interaction between the different receptor subtype-induced behaviours, in such a way that induction of a particular behaviour by activation of a certain receptor subtype can prevent or mask the behaviour induced by activation of another 5-HT-receptor subtype. Functional interactions in the penile erection test (5-HT_{1C}-receptor-induced) and in the lower lip retraction test (5-HT_{1A}-receptor-induced) have been described before (Berendsen *et al.*, 1989a; 1990). We have now studied more systematically how activation of one 5-HT-receptor subtype influences the expression of the behaviour induced by activation of another.

In this study, we have measured the 5-HT_{1A}-receptor- mediated forepaw treading and the 5-HT_2-receptor mediated head shake response in rats. Locomotor activity and body temperature in mice were included to show that the functional interaction of 5-HT-receptor subtypes also takes place in this species.

Animals

Mice Naïve male mice (Crl: CD-1(ICR)BR, from Charles River, Germany) weighing 24–26 g were used. The mice were housed in macrolon cages (40 × 23 × 15 cm) 20 animals per cage, under a controlled 12 h light-dark cycle (light on 6 h 00 min) and were allowed free access to standard food pellets and tap water.

Rats Naïve male Wistar rats (Cpb:WU, Harlan Sprague-Dawley, Zeist, The Netherlands) weighing 200–250 g were used. The animals were housed in white PVC cages (40 × 40 × 18 cm) with a wire mesh lid, 5 animals per cage, under controlled 12 h light-dark cycle (lights on 6 h 00 min) and were allowed free access to standard food pellets and tap water.

Procedures

Locomotor activity in mice The locomotor activity of the mice was measured in 20 small photocell cages (11 × 11 × 16 cm) (four rows of 5 connected cages). The lid and both side walls of the cages were made of grey PVC, the front and back walls of clear perspex and the base was a stainless steel grid floor. Each cage was supplied with 8 infra-red light beams connected to a counter for recording the light beam interruptions. Tests were done in blocks, consisting of 20 mice, with treatments randomized between all animals within the experiment. At least 10 animals per treatment were used. Immediately following administration of the compounds the mice were placed individually in the activity cages and the number of light beam interruptions was measured for 30 min.

Body temperature in mice The hypothermia tests were performed and replicated 3 times, each consisted of 3 mice from each treatment group. Within a block the various treatments were randomized between the cages, mice receiving the same treatment were placed within one cage. Ten min after treatment with a compound the rectal temperature of the mice was measured with an electrothermometer (Ellab TE3, Electrolaboriet, Copenhagen, Denmark). The lubricated probe was inserted about 2.5 cm. 8-Hydroxy-dipropylaminotetralin (8-OH-DPAT), 0.25 mg kg^{-1}, or placebo was then injected immediately and 10, 20, 30 and 40 min later rectal temperature was measured again. The room temperature was 21 ± 21 ± 1°1°C.

Forepaw treading Forepaw treading in rats was also measured three times. Within each block all treatments were randomized between animals. After the animals had been treated with the compound to be investigated, immediately followed by an injection of 8-OH-DPAT, 0.22 mg kg^{-1}, the rats were placed in small observation cages (7.5 × 18 × 30 cm) and forepaw treading was measured from 15 to 30 min after treatment by scoring the presence or absence of forepaw treading every 30 s. By use of this time sampling method a maximal score of 31 could be reached. Eight rats per treatment group were used.

Head shakes The head shake tests were run with blocks of eight animals, with each treatment present at least once in every block. Head shakes in rats were induced by DOI 0.22 mg kg^{-1}. Immediately after treatment with DOI and the agonist under study the rats were placed in small observation cages (7.5 × 18 × 30 cm) and the number of head shakes was counted during 30 min.

Drugs and solutions

The following drugs were used: 2 chloro-6-(1-piperazinyl) pyrazine monohydrochloride (MK 212; Merck, Sharpe and Dome); 1-(meta-chlorophenyl)-piperazine 2HCl (mCPP; EGA-chemie); (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane HCl (DOI), (±)-8-hydroxy-dipropylaminotetralin HBr (8-OH-DPAT) and 7-trifluoromethyl-4-(4-methyl-1-piperazinyl)-pyrolo[1,2-a] quinoxaline dimaleate (CGS 12066B) all from Research Biochemicals Inc. (RBI) and m-trifluoromethyl phenyl piperazine HCl (TFMPP; Duphar). All compounds were dissolved in sterile saline solution and solutions were freshly prepared. Injections were made subcutaneously into the loose skin at the back of the neck. In rats a dose volume of 5 ml per kg body weight was used and in mice this dose volume was 10 ml per kg body weight. Control animals were injected with an equivalent volume of vehicle.

Statistics

Locomotor activity test The total test period of 30 min was divided into 3 periods of 10 min each. The results of the first 10 min period are presented as mean photocell interruptions ± s.e.mean. Statistical comparisons were made by the Mann-Whitney U test.

Hypothermia test The temperature changes are given in degrees centigrade compared to control groups and expressed as means per treatment group ± s.e. Statistical comparisons were made by use of an analysis of variance for a completely randomized design.

Forepaw treading and head shakes tests The results are expressed as the mean number of scores ± s.e.mean. Statistical comparisons were made by comparing the results of each group with the results of the control group by use of the Mann-Whitney U test.

Results

Locomotor activity in mice

Locomotor activity in mice was measured for 30 min which was divided into periods of 10 min each. The activity score of the placebo-treated mice was highest during the first 10 min and decreased during the following 10 min periods. Suppression of locomotor activity by 8-OH-DPAT (0.5 mg kg^{-1}) was strong and highly significant during the first 10 min. In the next two 10 min periods this effect weakened. In the third period the effect was no longer different from placebo (Figure 1). For the interaction studies of 8-OH-DPAT with other agonists only the first 10 min periods are presented (Figure 2).

When given alone the compunds DOI (0.046–0.22 mg kg^{-1}) and TFMPP (0.22–1.0 mg kg^{-1}) did not significantly change activity in any of the 3 periods. In the third period these compounds tended to increase locomotor activity, but this effect was never significant. The lowest and highest doses of mCPP (0.22 and 1.0 mg kg^{-1}) and MK 212 (0.22 and 1.0 mg kg^{-1}) significantly inhibited locomotor activity of the mice but only in the first 10 min period. CGS 12066B (2.2 and 10 mg kg^{-1}) significantly inhibited the locomotor activity in the same period. But in the second and third period there was no difference compared with placebo.

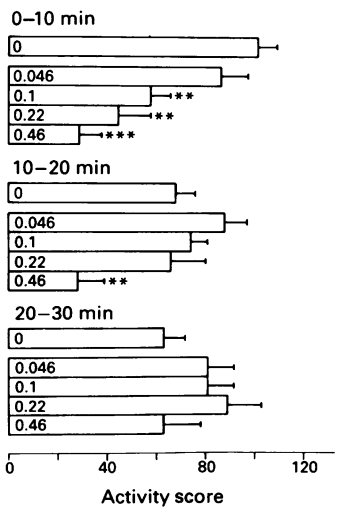

Figure 1 Effect of 8-hydroxy-dipropylaminotetralin (8-OH-DPAT) on locomotor activity in mice. Measurement started immediately after s.c. injection of 8-OH-DPAT. Columns represent the mean activity score of at least 10 animals per group. Horizontal bars show the s.e.mean. ** $P < 0.01$; *** $P < 0.001$ when compared with the placebo group.

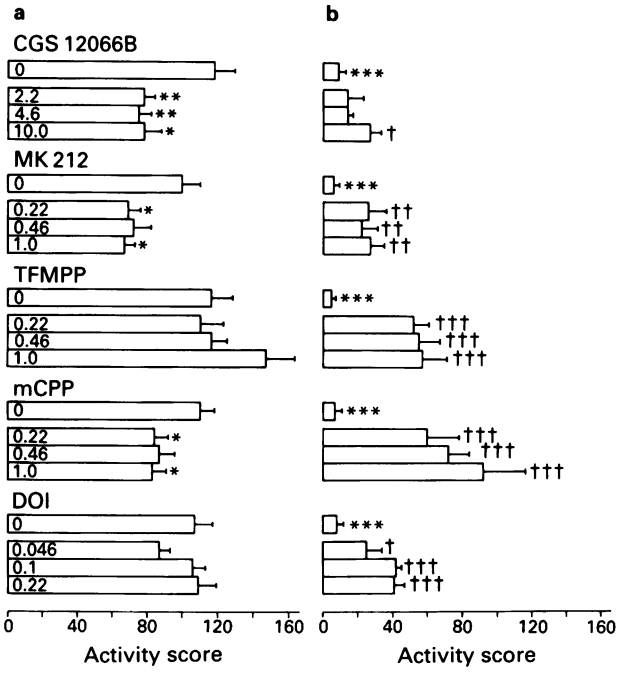

Figure 2 Effects of drug interactions on locomotor activity during the first 10 min after treatment. 8-Hydroxy-dipropylaminotetralin (8-OH-DPAT, 0.5 mg kg^{-1}) was injected immediately after an injection with one of the other compounds or placebo. In (a) the columns represent the mean activity scores after placebo + placebo or 5-hydroxytryptamine (5-HT)-agonists + placebo treatment. In (b) the columns represent the mean activities after 8-OH-DPAT + placebo or 8-OH-DPAT + 5-HT-agonist pretreatment. Horizontal bars show s.e.mean. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ when compared with placebo + placebo-treated group. † $P < 0.05$; †† $P < 0.01$; ††† $P < 0.001$ when compared with placebo + 8-OH-DPAT-treated group. TFMPP = m-trifluoromethyl phenyl piperazine, mCPP = 1-(meta-chlorophenyl)-piperazine, DOI = 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane.

When combined treatments were given, DOI partly attenuated the 8-OH-DPAT-induced hypoactivity in the first 10 min. In the second period the activity of the animals, given the combined treatment, was no longer significantly different from the placebo group and in the third period their activity was significantly higher than that of the placebo group. TFMPP and mCPP at all doses tested markedly attenuated 8-OH-DPAT-induced hypoactivity. In the third period the activity of the combined treatment groups was significantly higher than that of the control groups. MK 212 attenuated 8-OH-DPAT-induced hypoactivity in the first and second period. In the third period no significant differences existed between the groups. Only a dose of 10 mg kg^{-1} CGS 12066B slightly attenuated 8-OH-DPAT-induced hypoactivity in the first 10 min period.

Body temperature in mice

8-OH-DPAT, 0.25 mg kg^{-1}, consistently induced a hypothermic response which was maximal at 10 min after treatment. Therefore, in Figure 3 the responses at this time point only are given. DOI, TFMPP, mCPP and MK 212 all dose-dependently attenuated 8-OH-DPAT-induced hypothermia. When given alone, none of these compounds significantly changed the body temperature compared with the placebo groups. CGS 12066B 10 mg kg^{-1} induced a weak but not significant hypothermia by itself. Given in combination with 8-OH-DPAT this compound at 10 mg kg^{-1} potentiated 8-OH-DPAT-induced hypothermia significantly.

Forepaw treading in rats

8-OH-DPAT, 0.22 mg kg^{-1}, induced forepaw treading with a mean score varying from 13.5 to 19.5 (maximal possible score was 31) (Figure 4). Forepaw treading induced by 8-OH-DPAT was dose-dependently potentiated by DOI (ED_{150} =

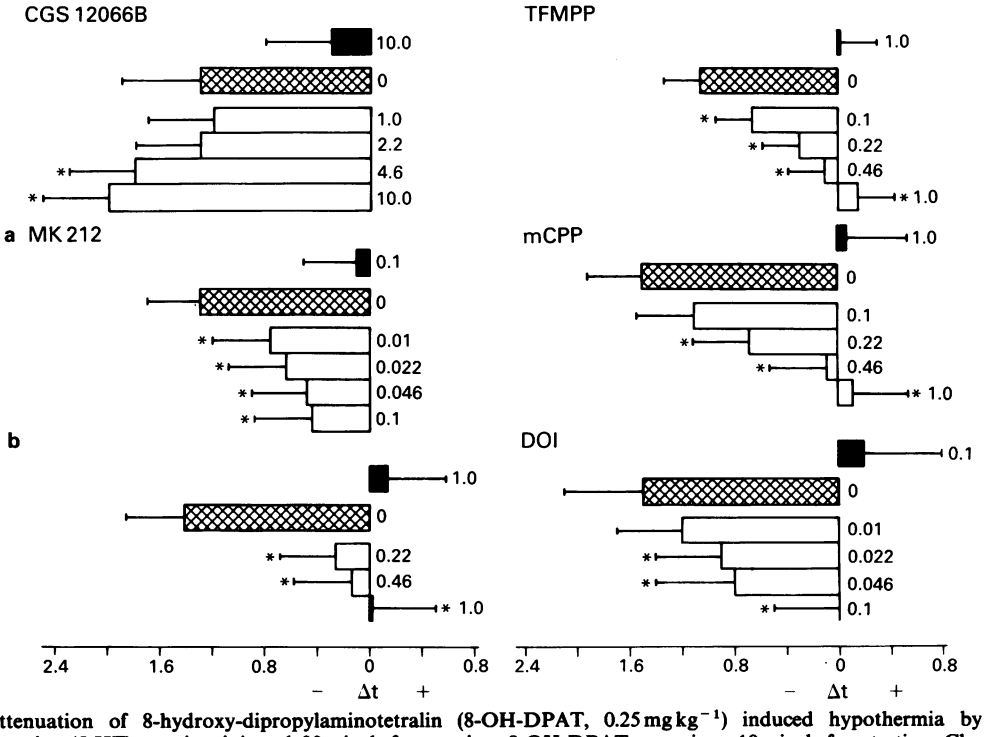

Figure 3 Attenuation of 8-hydroxy-dipropylaminotetralin (8-OH-DPAT, 0.25 mg kg^{-1}) induced hypothermia by various 5-hydroxytryptamine (5-HT)-agonists injected 20 min before testing. 8-OH-DPAT was given 10 min before testing. Changes in temperature (Δt) compared with the placebo + placebo-treated group are shown. The solid columns represent the 5-HT-agonist at the highest dose + placebo treated groups; the hatched columns represent the placebo + 8-OH-DPAT-treated groups; the open columns represent the 5-HT-agonist + 8-OH-DPAT-treated groups. Horizontal bars show s.e.mean. All values are the mean of 9 animals. (a) and (b) are data from 2 separate experiments with MK 212. * $P < 0.05$ when compared with placebo + 8-OH-DPAT group. The effect of 8-OH-DPAT was significantly different from placebo treatment in each test. For key to abbreviations used see legend of Figure 2

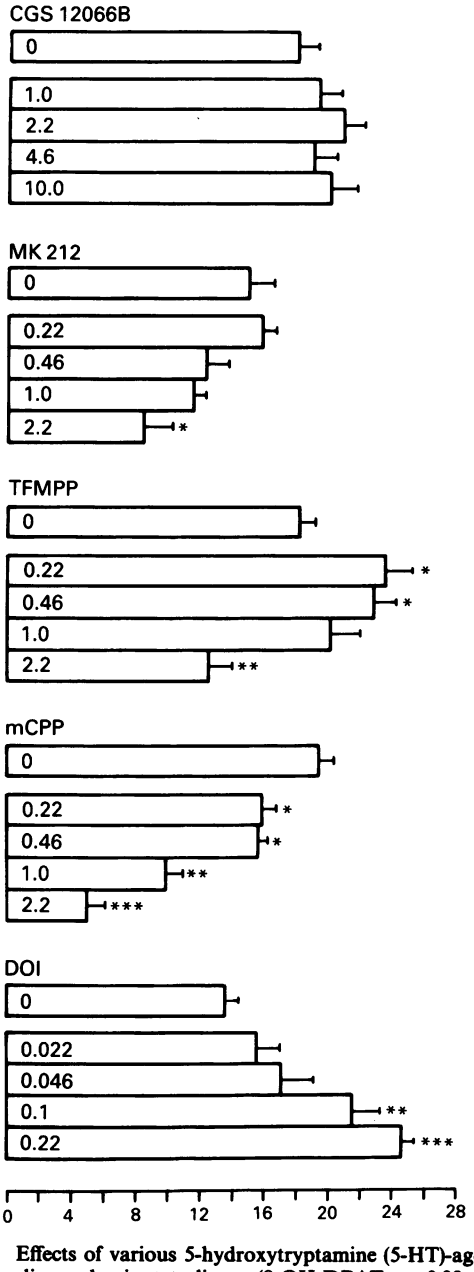

Figure 4 Effects of various 5-hydroxytryptamine (5-HT)-agonists on 8-hydroxy-dipropylaminotetralin (8-OH-DPAT, 0.22 mg kg^{-1})-induced forepaw treading in rats. The 5-HT-agonists were injected immediately before 8-OH-DPAT. The columns represent the mean forepaw treading scores for groups of 8 animals from 15–30 min after 8-OH-DPAT. The horizontal bars show the s.e.mean. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ when compared with the control groups. For key to abbreviations used see legend of Figure 2.

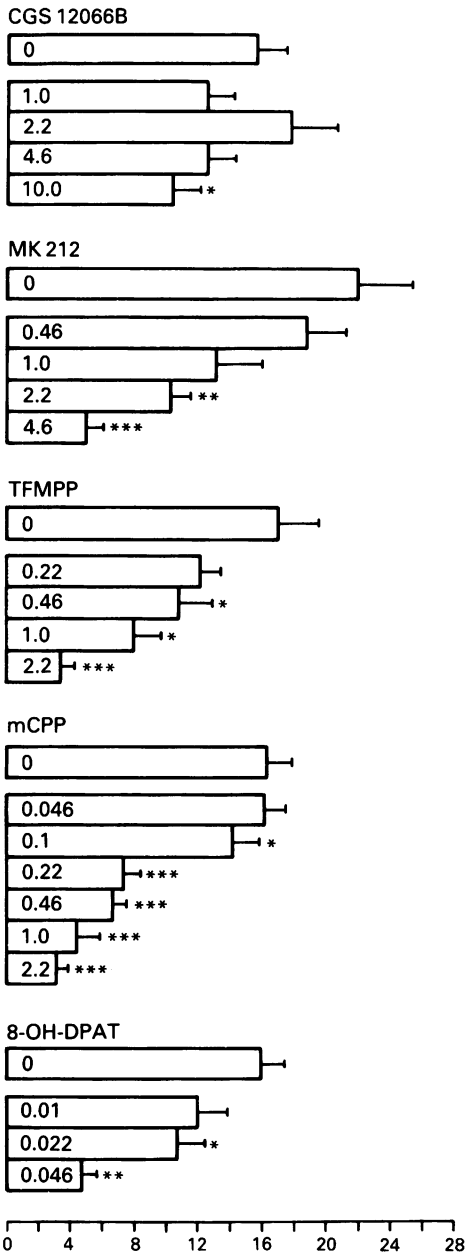

Figure 5 Effect of various 5-hydroxytryptamine (5-HT)-agonists on 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI, 0.22 mg kg^{-1})-induced head shakes in rats. The 5-HT-agonists were injected immediately before DOI. The columns represent the mean number of head shakes of at least 8 animals measured for 30 min after DOI. The horizontal bars show the s.e.mean. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ when compared with control group. For key to abbreviations used see legend of Figure 2.

0.08 mg kg^{-1}). TFMPP, at lower doses potentiated and at higher doses inhibited forepaw treading. mCPP and MK 212 dose-dependently inhibited forepaw treading (ID_{50} = 1 mg kg^{-1} for mCPP). However, MK 212 had less inhibitory activity than mCPP, at 2.2 mg kg^{-1} the inhibition was 43%. CGS 12066B had no effect.

Head shakes in rats

The results of these experiments are given in Figure 5. Head shakes induced by DOI 0.22 mg kg^{-1} were dose-dependently inhibited by 8-OH-DPAT, mCPP, TFMP and MK 212. Their ID_{50} values were 0.03, 0.1, 0.7 and 2 mg kg^{-1} respectively. CGS 12066B significantly inhibited DOI-induced head shakes only at the highest dose tested (10 mg kg^{-1}).

Discussion

The main finding of the present experiments was that behavioural responses elicited by various 5-HT-receptor subtypes can be modified by co-activation of other 5-HT-receptor subtypes. A key element in interpreting these findings is the affinities of each agonist for the different receptor subtypes. These are summarized in Table 1. The table also contains the selectivity-ratios of these compounds for 5-HT_{1C} over 5-HT_2 receptors. These data show that very selective agonists or antagonists for these receptors do not exist. We realize that the lack of selectivity of these compounds for the 5-HT-receptor subtypes seriously hinders the interpretation of the data obtained in the present study. Also, binding data, on which we have to rely extensively, do not distinguish agonist and antagonist properties of the compounds. Nevertheless, our

Table 1 Affinity values of 5-hydroxytryptamine (5-HT)-agonists for the various 5-HT receptor subtypes

Compound	*Affinity values (pK_d) for* $5\text{-}HT_{1A}$-receptor[1]	$5\text{-}HT_{1B}$-receptor[1]	$5\text{-}HT_{1C}$-receptor[1]	$5\text{-}HT_2$-receptor[1]	*Preference ratio for* $5\text{-}HT_{1C}$-receptor[5]
8-OH-DPAT	8.74	4.22	5.24	5.94	0.2
5MeODMT	7.9[3]	6.2[3]	7.06[2]	6.21[2]	7.1
CGS 12066B	7.19[4]	6.94[4]	4.89[4]	—	—
MK 212	5.32	5.03	6.16	4.76	25
mCPP-	6.49	6.58	7.68	6.70	10
TFMPP	6.34	6.36	7.21	6.57	4.4
DOI	5.6[3]	5.9[3]	7.73[2]	7.84[2]	0.8

[1] The data are from several sources: pKd values from Hoyer 1988a; [2] from Hoyer (1988b); [3] calculated from Titeler *et al.* (1988); [4] from Schoeffter & Hoyer (1989); [5] the $5\text{-}HT_{1C}$ preference ratio represents the ratio of dissociation constants for $5\text{-}HT_2$- and $5\text{-}HT_{1C}$-receptors. Dissociation constants were the antilogs of the pK_d values as given in the table.

Table 2 Proposed effects of activation of 5-hydroxytryptamine (5-HT) receptor subtype on the effect induced by activation of another 5-HT-receptor subtype

	Response	*Effect of activation of* $5\text{-}HT_{1A}$	$5\text{-}HT_{1B}$	$5\text{-}HT_{1C}$	$5\text{-}HT_2$ *receptors*
$5\text{-}HT_{1A}$-induced	Hypoactivity in mice		0	–	–
	Hypothermia in mice		(+)	–	–
	Lower lip retraction in rats[a]		0	–	–
	Forepaw treading in rats		0	–	+
$5\text{-}HT_{1C}$-induced	Penile erections in rats[b]	–	0		–
$5\text{-}HT_2$-induced	Head shakes in rats	–	(–)	–	

0 = no effect; – = inhibition; + = potentiation; (–) = inhibition only at a high dose; (+) = potentiation only at a high dose; [a] Berendsen *et al.* (1989a); [b] Berendsen *et al.* (1990).

data require a tentative interpretation in order to provide a working hypothesis for future experiments. Furthermore, the interactions that we have observed should be taken into account when interpreting behavioural changes induced by drugs acting on 5-HT receptors. The proposed interactions between the 5-HT receptor subtype responses are summarized in Table 2.

The effect of 8-OH-DPAT on locomotor activity in rats is controversial, increased locomotor activity was found by Tricklebank *et al.* (1984) and Broekkamp *et al.* (1989) whereas Hillegaart *et al.* (1989) found decreased activity. In mice we found a hypoactivity, at least during the first 20 min, after treatment with 0.5 mg kg^{-1}. The hypothermic effect on 8-OH-DPAT in mice is consistent with other studies in mice and rats (Goodwin & Green, 1985; Hjorth, 1985; Goodwin *et al.*, 1987). In contrast to our results in mice, TFMPP, mCPP and MK 212 have been shown to induce hyperthermia in rats (Maj & Lewandowska, 1980; Yamawaki *et al.*, 1983; Pawlowski, 1984; Gudelski *et al.*, 1986). However, these authors gave a higher dose of the compounds and/or did the experiments in heat adapted rats. Both 8-OH-DPAT-induced hypoactivity and hypothermia in mice could be attenuated by DOI, TFMPP, mCPP and MK 212. These compounds bind preferentially to the $5\text{-}HT_{1C}$- and/or $5\text{-}HT_2$-receptors, suggesting that both activation of $5\text{-}HT_{1C}$- and/or $5\text{-}HT_2$-receptors can functionally inhibit these $5\text{-}HT_{1A}$-induced effects. The mixed $5\text{-}HT_{1A/1B}$ agonist CGS 12066B (Neale *et al.*, 1987; Schoeffter & Hoyer, 1989) had no effect on 8-OH-DPAT-induced hypolocomotion. However, hypothermia induced by 8-OH-DPAT was potentiated by a high dose of CGS 12066B. Previously, we demonstrated that lower lip retraction induced by 8-OH-DPAT is not influenced by CGS 12066B, whereas activation of $5\text{-}HT_{1C}$- and/or $5\text{-}HT_2$-receptors attenuated this effect (Berendsen *et al.*, 1989a). This might point to a lack of interaction between the $5\text{-}HT_{1A}$- and $5\text{-}HT_{1B}$-receptor-induced behaviours. It is also possible that the penetration of CGS 12066B into the brain is poor. A lack of interaction with the $5\text{-}HT_{1A}$- and $5\text{-}HT_{1B}$-receptors might also be inferred from the ability of the mixed $5\text{-}HT_{1A}/5\text{-}HT_{1B}$-agonist RU24969 to induce the $5\text{-}HT_{1A}$-related lower lip retraction (Berendsen *et al.*, 1989a). One might argue that the increased locomotor activity after DOI, TFMPP and mCPP contributes to the attenuation of 8-OH-DPAT-induced hypothermia. However, the effect of these compounds on hypothermia was strongest after 10 min, whereas the increase in locomotor activity occurred no earlier than 20–30 min after treatment. The forepaw treading induced by 8-OH-DPAT was influenced differently by the agonists: DOI dose-dependently potentiated the response, while TFMPP potentiated it at low doses but attenuated it at higher doses. mCPP and MK 212 both attenuated forepaw treading. These compounds bind to $5\text{-}HT_{1C}$ and $5\text{-}HT_2$ sites, but if the selectivity-ratio of these compounds for the $5\text{-}HT_{1C}$- over $5\text{-}HT_2$-receptors is calculated the sequence is MK 212 > mCPP > TFMPP > DOI (Table 1). It thus seems that as the preference for $5\text{-}HT_{1C}$-receptors over $5\text{-}HT_2$-receptors decreases the effect on forepaw treading changes from an inhibition to a potentiation. We have seen the same order of potency with these compounds for their ability to induce penile erections (Berendsen *et al.*, 1990). The potentiating effect of DOI and TFMPP on forepaw treading confirms previous findings (Berendsen *et al.*, 1989b; Arnt & Hyttel, 1989). Among the symptoms induced by 8-OH-DPAT, hypothermia and lower lip retraction are thought to be presynaptically mediated (Goodwin *et al.*, 1987; Wozniak *et al.*, 1988; Berendsen *et al.*, 1989a), whereas forepaw treading is thought to be a postsynaptically-mediated effect (Tricklebank *et al.*, 1985). The data thus suggest that presynaptically-induced $5\text{-}HT_{1A}$ effects are attenuated by both $5\text{-}HT_{1C}$- and $5\text{-}HT_2$-receptor activation, whereas postsynaptically-mediated $5\text{-}HT_{1A}$-effects are potentiated by concomitant activation of $5\text{-}HT_2$ and attenuated by concomitant activation of $5\text{-}HT_{1C}$-receptors. Injection of CGS 12066B did not change the 8-OH-DPAT-induced hypoactivity or forepaw treading response. Therefore, there is no evidence as yet to suggest an influence of $5\text{-}HT_{1B}$-receptor activation on $5\text{-}HT_{1A}$-receptor-mediated effects.

The functional effect of $5\text{-}HT_{1A}$-, $5\text{-}HT_{1B}$- and $5\text{-}HT_2$-receptor activation on a $5\text{-}HT_{1C}$-receptor-mediated effect has been discussed before (Berendsen *et al.*, 1990). It has been shown that activation of the $5\text{-}HT_{1A}$-receptor attenuated

the 5-HT_{1C}-mediated induction of penile erections (PE) and that induction of PE by mixed 5-HT_{1C}/5-HT_2 agonists depends on the selectivity of these compounds for 5-HT_{1C}- over 5-HT_2-receptors. This suggests that activation of the 5-HT_2-receptor inhibits or prevents expression of 5-HT_{1C}-receptor-mediated PE. DOI binds with similar affinity to 5-HT_2 and 5-HT_{1C} sites but induces 5-HT_2-mediated behaviours. This indicates that for a mixed 5-HT_{1C}/5-HT_2 agonist the behaviour related to activation of the 5-HT_2-receptor prevails over the behaviour induced by 5-HT_{1C}-receptor activation. The head shake response induced by DOI, could be attenuated by 8-OH-DPAT, TFMPP, mCPP and MK 212 suggesting that activation of both 5-HT_{1A}- and 5-HT_{1C}-receptors functionally inhibits this 5-HT_2-mediated response.

Functional interactions between the different 5-HT receptor subtype-mediated effects does not seem to be restricted to the behavioural expression of activation of these receptors. It has been shown that mCPP and TFMPP inhibit 5-HT_2-receptor-mediated vascular contractions (Cohen & Fuller, 1983) and that mCPP antagonizes cortical 5-HT_2-receptors that are linked to phosphoinositide turnover (Conn & Sanders-Bush, 1987). Moreover, it was shown in electrophysiological experiments that 5-HT_{1A} and 5-HT_{1B}/5-HT_{1C} agonists have different effects on neuronal cell firing in different brain regions (Sprouse & Aghajanian, 1988). Opposite effects of 5-HT_{1A}- and non-5-HT_{1A} (5-HT_{1B} or 5-HT_{1C})-receptor activation have been found in several experiments. For example, in the startle response in rats (Davis *et al.*, 1986), in aversive responding seen after electrical stimulation of the periaqueductal grey (Jenck *et al.*, 1989) and in several models of animal anxiety (Broekkamp & Jenck, 1989).

The observed functional interactions of 5-HT-receptor subtype-mediated effects may occur at different levels, such as a molecular allosteric mutual influence, opposing effects on second messengers or more broadly via presynaptic-postsynaptic locations or functional influences from different brain structures.

The data emphasize the difficulty of drawing conclusions from functional measurements when the compounds that are being investigated have mixed receptor affinities and efficacies. The availability of selective antagonists for the different 5-HT receptor subtypes would greatly facilitate the interpretation of such data.

We thank Mr R. Smets, J. van Schadewijk and A. Princen for their contribution and Prof. B. Bohus and Dr J. Hagan for their careful reading of the manuscript. The generous gifts of MK 212 and TFMPP by Merck, Sharpe and Dome and Duphar, respectively, are gratefully acknowledged.

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(Received September 21, 1989
Revised June 16, 1990
Accepted June 25, 1990)

Br. J. Pharmacol. (1990), **101**, 674–678

Evidence that the agonist action of dynorphin A(1–8) in the guinea-pig myenteric-plexus may be mediated partly through conversion to [Leu^5]enkephalin

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1 The agonist action of the opioid peptide dynorphin A(1–8) on the myenteric plexus-longitudinal muscle of the guinea-pig ileum has been characterized.

2 The endogenous opioid peptide dynorphin A(1–8) was rapidly degraded by slices of myenteric plexus-longitudinal muscle of the guinea-pig ileum.

3 A product of the degradation was the δ-receptor preferring [Leu^5]enkephalin. Levels of [Leu^5]enkephalin were markedly increased in the presence of the peptidase inhibitors bestatin, thiorphan and captopril.

4 In the myenteric plexus dynorphin A(1–8) acted as a κ-receptor agonist. In the presence of bestatin, thiorphan and captopril a μ-receptor agonist effect was observed. This μ-agonist action was lost in the presence of N-[1-(**RS**)-carboxy-2-phenylethyl]Ala-Ala-Phe-*p*-aminobenzoate, an inhibitor of the endopeptidase enzyme EC 3.4.24.15.

5 The results suggest that formation of [Leu^5]enkephalin from dynorphin A(1–8) may be an important conversion process. The enzyme responsible may be the Zn^{2+}-metalloendopeptidase, EC 3.4.24.15.

Introduction

The dynorphin family of opioid peptides are considered to be endogenous ligands for the κ-opioid receptor. The larger dynorphin peptides, namely dynorphin A (dynorphin A(1–17)) and dynorphin A(1–13) are potent selective κ-receptor agonists (Chavkin *et al.*, 1982; James *et al.*, 1984). However the shorter peptides, dynorphin A(1–8) and A(1–9), are less potent and less selective (Corbett *et al.*, 1982). In addition, these shorter sequences are highly susceptible to enzymic degradation in homogenates of the central nervous system (CNS) (Gillan *et al.*, 1985; Dixon & Traynor, 1987). Consequently, pharmacological studies of these smaller dynorphin fragments are performed in the presence of a cocktail of peptidase inhibitors to protect the C- and N-termini and the Gly^3-Phe^4 bond from hydrolysis (Corbett *et al.*, 1982; McKnight *et al.*, 1983). However, even in the presence of such a cocktail, [Leu^5]enkephalin may be produced (Miller *et al.*, 1985; Dixon & Traynor, 1987) and this pentapeptide may contribute to the observed pharmacological actions of the dynorphin fragments (Miller *et al.*, 1985). Indeed the peptidase inhibitors may stabilize [Leu^5]enkephalin and therefore favour formation of this pentapeptide (Dixon & Traynor, 1987). Cleavage of the Leu^5-Arg^6 bond of dynorphin A(1–8), which gives rise to [Leu^5]enkephalin, can be prevented by the EC 3.4.24.15 inhibitor N-[1-(**RS**)-carboxy-2-phenylethyl]-Ala-Ala-Phe-*p*AB (Chu & Orlowski, 1984; Dixon & Traynor, 1990).

The formation of [Leu^5]enkephalin from the smaller dynorphins would have important consequences if it occurred in physiological situations. As a preliminary to such investigations, we describe the formation of [Leu^5]enkephalin from exogenously added dynorphin A(1–8) in the myenteric plexus of the guinea-pig ileum, and its role in the observed agonist activity.

Methods

Male Dunkin-Hartley guinea-pigs (400–500 g) (David Hall, Burton-on-Trent) were killed by cervical dislocation. Segments of ileum were removed and placed in Krebs solution containing (mM): NaCl 118, KCl 4.7, $CaCl_2 \cdot 2H_2O$ 2.6, KH_2PO_4 1.2, $MgSO_4 \cdot 7H_2O$ 1.2, $NaHCO_3$ 25 and glucose 11.

Bioassays

The myenteric plexus-longitudinal muscle preparation of the guinea-pig small intestine was set up for field stimulation as previously described (Traynor *et al.*, 1987). The potencies of agonists were obtained from cumulative dose-response curves. The equilibrium dissociation constants (*Ke*, nM) of the antagonists were determined by the method of Arunlakshana & Schild (1959) or by the 'single-dose' method (Kosterlitz & Watt, 1968). Antagonists were allowed to equilibrate for 20 min, or 45 min for norbinaltorphimine, before repeating the addition of agonist.

Pre-incubation of myenteric plexus preparations with peptidase inhibitors

Myenteric plexus preparations were incubated in Krebs solution as above with the peptidase inhibitors bestatin (30 μM), captopril (10 μM), thiorphan (0.3 μM) (McKnight *et al.*, 1983) and, where stated, N-[1-(**RS**)-carboxy-2-phenylethyl]-Ala-Ala-Phe-*p*AB (30 μM) (Chu & Orlowski, 1984) for 30 min. Following this treatment potencies of agonists were determined without washout of the peptidase inhibitors.

Pre-incubation of myenteric plexus preparations with β-funaltrexamine

To block μ-opioid receptors irreversibly, preparations of myenteric plexus were incubated with β-funaltrexamine (100 nM) for 60 min and then washed with drug-free Krebs solution for 60 min. During washing the bath fluid was changed by overflow every 5 min. Agonist potencies were determined before and after β-funaltrexamine treatment.

Metabolism studies

Preparations of myenteric plexus-longitudinal muscle from the guinea-pig ileum were sliced (0.5 mm) on a McIlwain tissue chopper. The slices were washed three times in Krebs-solution

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containing HEPES buffer (pH 7.4, 25 mM) (Krebs-HEPES) and then suspended in Krebs-HEPES at a tissue concentration of 200 mg ml^{-1}. Aliquots of tissue slices (10 mg) were incubated in Krebs-HEPES solution (1 ml) in the presence or absence of peptidase inhibitors for 30 min at 37°C, prior to the addition of [^{3}H]-dynorphin A(1–8) at a final concentration of 12 nM. At timed intervals subsequent to this the reaction was terminated by the addition of phosphoric acid (50 mM final concentration). Aliquots (500 μl) were taken and stored at −20°C prior to analysis.

To aliquots of the incubation mixtures were added 20 μl of a solution containing the marker peptides Tyr, Tyr-Gly, Tyr-Gly-Gly, Tyr-Gly-Gly-Phe, [Leu^5]enkephalin, [Leu^5]enkephalyl-Arg^6, [Leu^5]-enkephalyl-Arg^6-Arg^7, and dynorphin A(1–8) each at a concentration of 100 μg ml^{-1}. The samples were applied to an Altex Ultrasphere ODS column (250 mm × 4.6 mm, particle size 5 μm) and eluted using a solvent gradient between solutions A (26 mM trifluoroacetic acid with triethylamine to pH 3.0) and B (49% acetonitrile in 13 mM trifluoroacetic acid with triethylamine to pH 3.0) as follows: 15–30%B in 15 min: 30–60%B in 30 min: 65–70%B in 5 min, at a flow rate of 1.0 ml min^{-1}. Peaks were detected at 280 nm and fractions (0.5 ml) collected from the column. The gradient system used did not readily separate the Tyr, Tyr-Gly, and Tyr-Gly-Gly fractions which were collected as a single 'N-terminal fraction'. Radioactivity in each fraction was determined after addition of 4 ml of scintillation cocktail (Ecoscint, National Diagnostics). Recovered radioactivity represented 94.1 ± 8.0% (n = 25) of the added activity.

Peptides and drugs

[^{3}H]-dynorphin A(1–8) (27.6 Ci mmol^{-1}) was obtained from New England Nuclear. Peptides were from Cambridge Research Biochemicals with the exception of Tyr, Tyr-Gly and Tyr-Gly-Gly which were from Sigma. Peptidase inhibitors were obtained from the following sources: bestatin (Cambridge Research Biochemicals), captopril (Squibb), thiorphan (Sandoz) and N-[1-(RS)-carboxy-2-phenylethyl]-Ala-Ala-Phe-*p*AB (where *p*AB = *p*-aminobenzoate, Professor M. Orlowski, Mount Sinai, New York, U.S.A.). The following drugs were used: [D-Ala^2, MePhe^4, Gly-ol^5]enkephalin (DAMGO, Sigma); *trans*, 3,4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)cyclohexyl]-benzeneacetamide (U-50488H) and 5α,7α,8β-(−)-N-methyl-N-(7-(1-pyrrolidinyl)-1-oxaspiro[4,5]-dec-8-yl)benzeneacetamide (U-69,593, Upjohn); β-funaltrexamine, (β-FNA, Research Biochemicals Inc.); naloxone hydrochloride (Endo Laboratories), 16-methylcyprenorphine (M8008, Reckitt and Colman); norbinaltorphimine (Glaxo). All other reagents were of analytical, or high performance liquid chromatography (h.p.l.c.) grade.

Results

Metabolism of [^{3}H]-dynorphin A(1–8) by the myenteric plexus-longitudinal muscle preparation of the guinea-pig ileum

Incubation of [^{3}H]-dynorphin A(1–8) (12 nM) with slices of myenteric plexus-longitudinal muscle at 37°C resulted in a rapid degradation of the tritiated octapeptide as determined by h.p.l.c. separation of all the possible tritiated products (Figure 1a). The half-life of [^{3}H]-dynorphin under these conditions was just 2.30 ± 0.46 min. The major products of the breakdown co-eluted with the Tyr, Tyr-Gly and Tyr-Gly-Gly markers. Radioactivity was also recovered in the fraction co-eluting with the [Leu^5]enkephalin marker. This product reached peak levels of 8.2 ± 1.2% of recovered activity after 10 min, but then declined. Other products of the metabolism eluting with the Tyr-Gly-Gly-Phe, [Leu^5]enkephalyl-Arg^6 and [Leu^5]enkephalyl-Arg^6-Arg^7 markers accounted for less than 5% of the recovered radioactivity.

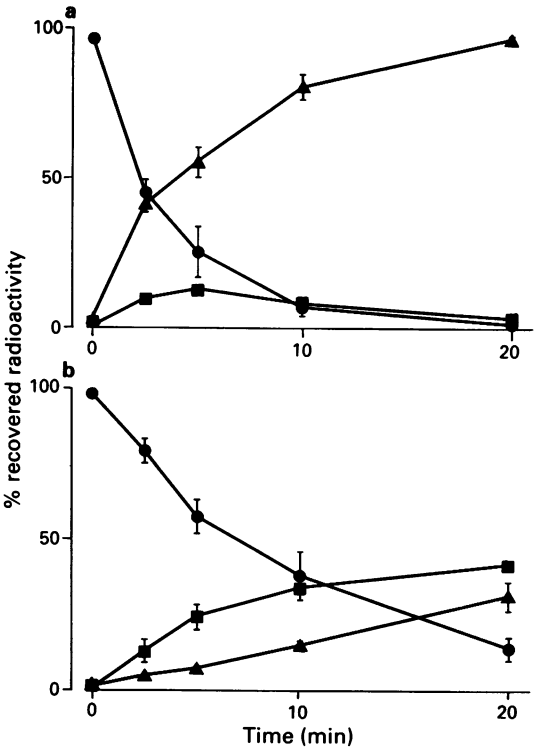

Figure 1 Metabolism of [^{3}H]-dynorphin A(1–8) (12 nM) by slices of myenteric plexus-longitudinal muscle of the guinea-pig ileum in the absence (a) or presence (b) of the peptidase inhibitors bestatin (10 μM), captopril (10 μM) and thiorphan (0.3 μM). [^{3}H]-dynorphin A(1–8) (●); [^{3}H]-[Leu^5]enkephalin (■); [^{3}H]-N-terminal fraction (▲). Points represent means of three determinations; vertical bars show s.e.means.

In the presence of the peptidase inhibitors bestatin (30 μM), captopril (10 μM) and thiorphan (0.3 μM) (McKnight *et al.*, 1983) the half-life of [^{3}H]-dynorphin A(1–8) in the presence of slices of myenteric plexus-longitudinal muscle increased to 7.0 ± 1.1 min. This was the result of a marked decline in the radioactivity attributed to the 'N-terminal' (Tyr, Tyr-Gly, Tyr-Gly-Gly) products. However, the level of radioactivity identified as [^{3}H]-[Leu^5]enkephalin was much increased and represented the major product of [^{3}H]-dynorphin A(1–8) metabolism accounting for 41.7 ± 1.0% of the recovered activity after 20 min (Figure 1b). There was no alteration in the levels of [^{3}H]-Tyr-Gly-Gly-Phe, [^{3}H]-[Leu^5]enkephalyl-Arg^6 and [^{3}H]-[Leu^5]enkephalyl-Arg^6-Arg^7 recovered.

Agonist action of dynorphin A(1–8) in the myenteric plexus longitudinal muscle preparation of the guinea-pig ileum

Dynorphin A(1–8) in the absence of peptidase inhibitors was able to depress the electrically-induced contractions of the myenteric plexus-longitudinal muscle preparation of the guinea-pig ileum, a tissue containing both μ- and κ-opioid receptors (Chavkin & Goldstein, 1981). The peptide acted as a full agonist in this tissue (Table 1). The action of dynorphin A(1–8) was antagonized by naloxone affording an equilibrium dissociation constant (Ke) for the antagonist of 14 nM. This value is in agreement with the Ke for naloxone against the selective κ-agonists U-50488H, U-69,593 and dynorphin A rather than against the μ-selective agonist DAMGO or [Leu^5]enkephalin (Table 1).

Table 1 The agonist actions of dynorphin A(1–8) and other opioids on the electrically induced contractions of the myenteric plexus-longitudinal muscle preparation of the guinea-pig ileum and their antagonism by naloxone

Opioid	*Peptidase inhibition**	*Agonist activity* (IC_{50}, nM)	*Antagonism by naloxone* (*Ke*, nM)	(Slope)
Dynorphin A(1–8)	No	27.4 ± 3.7	14.0 ± 2.4	0.92 ± 0.03 (19)
Dynorphin A(1–8)	Yes	1.80 ± 0.34	3.01 ± 0.79	0.86 ± 0.08 (9)
Dynorphin A(1–8) with E.C. 3.4.24.15 inhibitor†	Yes	1.89 ± 0.72	14.3 ± 2.1	single dose (3)
DAMGO	No	15.2 ± 0.14	2.09 ± 0.54	0.92 ± 0.04 (3)
DAMGO	Yes	21.3 ± 7.4	3.07 ± 0.82	1.00 ± 0.03 (3)
DAMGO with E.C. 3.4.24.15 inhibitor†	Yes	16.7 ± 0.90	3.00 ± 0.57	single dose (3)
[Leu^5]enkephalin	Yes	36.8 ± 7.0	1.63 ± 0.34	0.83 ± 0.03 (3)
U-50488H	No	1.52 ± 0.14	19.6 ± 1.4	single dose (6)
U-69,593	No	2.80 ± 0.47	31.4 ± 9.7	0.98 ± 0.08 (4)
Dynorphin A	No	0.32 ± 0.02	33.6 ± 1.02	1.07 ± 0.03 (4)

The values are the means ± s.e.mean; the number of observations is given in parentheses. * Bestatin (30 μM), captopril (10 μM) and thiorphan (0.3 μM).
† N-[1-(**RS**)-carboxy-2-phenylethyl]-Ala-Ala-Phe-pAB (30 μM). Individual preparations of myenteric plexus were pre-incubated with peptidase inhibitors for 30 min prior to determination of agonist potency and degree of antagonism by naloxone.
DAMGO: [D-Ala^2, MePhe^4, Gly-ol^5]enkephalin.

In the presence of the peptidase inhibitors bestatin (30 μM), captopril (10 μM) and thiorphan (0.3 μM) (McKnight *et al.*, 1983), the agonist potency of dynorphin A(1–8) was increased 15 fold. The *Ke* for naloxone was also shifted to give a value similar to that obtained against DAMGO in the presence and absence of peptidase inhibitors, and [Leu^5]enkephalin (Table 1). When the EC 3.4.24.15 inhibitor N-[1-(**RS**)-carboxy-2-phenylethyl]-Ala-Ala-Phe-*p*AB (30 μM) (Chu & Orlowski, 1984) was added to the bath fluid, in addition to the standard inhibitors, the potency of dynorphin A(1–8) as an agonist did not change. However the *Ke* for naloxone against dynorphin A(1–8) reverted back to a value seen in the absence of any peptidase inhibitors (Table 1). N-[1-(**RS**)-carboxy-2-phenylethyl]-Ala-Ala-Phe-*p*AB (30 μM) had no effect on the IC_{50} for DAMGO or on the naloxone *Ke* determined against DAMGO (Table 1).

Table 2 The effectiveness of 16-methylcyprenorphine (M8008) and norbinaltorphimine in antagonizing the agonist actions of opioids in the myenteric plexus-longitudinal muscle preparation of the guinea-pig ileum

Compound	*16-Methylcyprenorphine* Ke (nM)	*Slope*	*Norbinaltorphimine* Ke (nM)	*Slope*
DAMGO	3.50 ± 1.21	0.91 ± 0.05 (4)	13.0 ± 1.91	1.04 ± 0.08 (5)
U-69,593	85.8 ± 14.4	0.96 ± 0.11 (4)	0.07 ± 0.01	1.07 ± 0.03 (3)
Dynorphin A(1–8)	76.9 ± 11.1	0.91 ± 0.03 (4)	0.17 ± 0.04	0.98 ± 0.08 (3)
Dynorphin A(1–8) with peptidase inhibition*	31.4 ± 8.81	0.79 ± 0.01** (4)	0.62 ± 0.07	1.07 ± 0.03 (7)

The values are means ± s.e.mean; the number of observations is given in parentheses.
* Bestatin (30 μM), thiorphan (0.3 μM), captopril (10 μM).
** Significantly less than unity ($P < 0.05$) (Mann-Whitney).
DAMGO: [D-Ala^2, MePhe^4, Gly-ol^5]enkephalin.

Table 3 The effects of preincubation with β-funaltrexamine on the agonist potencies of opioids in the myenteric plexus-longitudinal muscle preparation of the guinea-pig ileum

Opioid	*Agonist potency* (IC_{50}, nM) *Before β-FNA*	*After β-FNA*	*Dose-ratio*
Dynorphin A(1–8)	18.6 ± 5.6	11.6 ± 2.5	0.6 ± 0.1 (3)
Dynorphin A(1–8) in presence of peptidase inhibition*	1.2 ± 0.35	1.57 ± 0.27	1.6 ± 0.4 (6)
DAMGO	12.2 ± 1.8	263 ± 48.8	26.1 ± 7.5 (12)
[Leu^5]enkephalin in presence of peptidase inhibition*	33.1	552	15.9 (2)
U-50488H	1.73 ± 0.56	2.1 ± 0.2	1.1 ± 0.5 (4)

The values are means ± s.e.mean; the number of observations is given in parentheses. Individual preparations of myenteric plexus were pre-incubated with 100 nM β-funaltrexamine (β-FNA) for 60 min, then washed for 60 min with drug-free Krebs solution. Dose-ratio is the ratio of IC_{50} after preincubation with β-FNA to IC_{50} before treatment.
*Bestatin (30 μM), thiorphan (0.3 μM), captopril (10 μM). DAMGO: [D-Ala^2, MePhe^4, Gly-ol^5]enkephalin.

The antagonist properties of the more selective antagonists 16-methylcyprenorphine (M8008), which is selective for μ- and δ- over κ-opioid receptors (Smith, 1987), and norbinaltorphimine which is selective for κ- over μ- and δ-opioid receptors (Portoghese *et al.*, 1987), are given in Table 2. The *Ke* value for M8008 against dynorphin A(1–8) agrees with the *Ke* determined against the selective κ-agonist U-69, 593 but differs from that obtained against the μ-receptor ligand DAMGO. In the presence of bestatin (30 μM), captopril (10 μM) and thiorphan (0.3 μM) the *Ke* shifted to a value midway between that determined against U-69,593 and the selective μ-agonist DAMGO (Table 2) and the slope of the Schild plot shifted to a value less than 1.0. The κ-selective antagonist norbinaltorphimine showed a similar pattern of activity (Table 2).

Effects of pre-incubation with β-funaltrexamine on the agonist activity of dynorphin A(1–8) in the guinea-pig myenteric plexus

Blockade of μ-receptors by pretreatment with β-FNA did not alter the agonist potency of dynorphin A(1–8) in the myenteric plexus, either in the absence or presence of the peptidase inhibitors bestatin (30 μM), captopril (10 μM) and thiorphan (0.3 μM). The agonist potency of U-69,593 was similarly unchanged. However, the dose-response curves for DAMGO and [Leu^5]enkephalin were markedly shifted to the right (Table 3).

Discussion

The results demonstrate that the endogenous opioid peptide dynorphin A(1–8), a κ-opioid receptor agonist (Corbett *et al.*, 1982), is rapidly metabolized by slices of myenteric plexus-longitudinal muscle preparation from the guinea-pig ileum. One product of this metabolism is the smaller δ-receptor preferring [Leu^5]enkephalin, which has little affinity for κ-opioid receptors (Gillan & Kosterlitz, 1982). This is similar to findings in CNS tissue (Griffiths *et al.*, 1983; Gillan *et al.*, 1985; Dixon & Traynor, 1987; 1990) and mouse vas deferens (Miller *et al.*, 1985). Since the process in myenteric plexus-longitudinal muscle occurs in well-washed tissue slices it is unlikely to be due to non-specific hydrolases such as might be released by damaged cells.

The rate of breakdown of dynorphin A(1–8) is retarded in the presence of the peptidase inhibitors captopril, bestatin and thiorphan (McKnight *et al.*, 1983) in accordance with the findings of Corbett *et al.* (1982). These inhibitors protect the C and N termini and the Gly^3-Phe^4 bond, respectively, of the larger peptide. However the inhibitors do not seem to prevent cleavage of dynorphin A(1–8) at the Leu^5-Arg^6 bond. Breakage of this bond leads to the production of [Leu^5]enkephalin, which in the presence of the peptidase inhibitors becomes the major tyrosine containing product. Since there is no evidence that significant amounts of [Leu^5]enkephalyl-Arg^6-Arg^7 or [Leu^5]enkephalyl-Arg^6 are produced under the assay conditions, the findings support our earlier suggestion (Dixon & Traynor, 1987) that a single cleavage step is involved, rather than sequential breakdown from the C-terminus. It also is clear that the peptidase inhibitors stabilize the [Leu^5]enkephalin formed from further metabolism thus leading to an accumulation of this pentapeptide.

The [Leu^5]enkephalin formed from exogenously added dynorphin A(1–8) appears to play a role in the observed agonist action of dynorphin A(1–8) in the myenteric plexus. The action of dynorphin A(1–8) in this tissue is antagonized by naloxone. The affinity of naloxone for the receptors at which dynorphin A(1–8) is acting is low and similar to the naloxone affinity (*Ke*) at κ-opioid receptors as measured against the selective synthetic κ-receptor agonists U-69,593 and U-50488H and the endogenous stable peptide dynorphin A. Thus, although dynorphin A(1–8) is a relatively non-selective ligand (Corbett *et al.*, 1982) in the myenteric plexus, it appears to have a preference for the κ-receptor. On the other hand this profile is changed markedly when the experiments are repeated in the presence of the peptidase inhibitors bestatin, thiorphan and captopril. Under such conditions the potency of dynorphin A(1–8) is greatly increased. Moreover the effectiveness of naloxone in antagonizing the effect is also increased, affording a *Ke* value similar to that obtained when using the ligands DAMGO, in the presence or absence of the inhibitors, and [Leu^5]enkephalin which act *via* the μ-opioid receptor. It may be hypothesized that in the presence of the peptidase inhibitors the increased levels and stability of [Leu^5]enkephalin formed from dynorphin A(1–8) becomes the major determinant of specificity. After addition of the EC 3.4.24.15 enzyme inhibitor N-[1-(RS)-carboxy-2-phenylethyl]-Ala-Ala-Phe-*p*AB (Chu & Orlowski, 1984) to the usual cocktail of inhibitors, the potency of dynorphin A(1–8) in the myenteric plexus is not further improved. However the effectiveness of naloxone in antagonizing dynorphin A(1–8) under these conditions is reduced, affording a value for *Ke* compatible with an interaction via κ-opioid receptors, while the observed parameters with the μ-receptor ligand DAMGO are not altered. This suggests that the EC 3.4.24.15 enzyme inhibitor is stabilizing the dynorphin A(1–8) and preventing formation of [Leu^5]enkephalin. This idea is supported by metabolism studies which demonstrate that N-[1-(RS)-carboxy-2-phenylethyl]-Ala-Ala-Phe-pAB is able to protect dynorphin A(1–8) from cleavage at the Leu^5–Arg^6 bond. Thus in slices of rat CNS tissue, in the presence of captopril, bestatin and thiorphan, N-[1-(RS)-carboxy-2-phenylethyl]-Ala-Ala-Phe-*p*AB (30 μM) stabilizes dynorphin A(1–8) such that after a 10 min incubation at 37°C, a 70% recovery of the peptide is obtained (Dixon & Traynor, 1990). In the absence of this latter inhibitor less than 10% of the intact peptide is recovered.

It seems clear from the above discussion that the apparent non-selectivity of dynorphin A(1–8) may be attributed, at least partly, to metabolism to the μ/δ-preferring [Leu^5]enkephalin. Further studies however suggest the action of exogenous dynorphin A(1–8) in the presence of the peptidase inhibitors bestatin, thiorphan and captopril cannot be wholly attributed to an action of formed [Leu^5]enkephalin at μ-receptors. Thus, results with the selective κ-antagonist norbinaltorphimine and the μ/δ-receptor antagonist 16-methylcyprenorphine suggest a mixed action of dynorphin A(1–8) at μ- and κ-opioid receptors. Indeed the slope of the Schild plot for M8008 was less than 1.0. This could have been due to the inherent non-selectivity of the dynorphin A(1–8) or to an action of unmetabolised dynorphin A(1–8) at κ-receptors and formed [Leu^5]enkephalin at μ-receptors. Thus, after alkylation of μ-receptors with the irreversible antagonist β-funaltrexamine (Corbett *et al.*, 1985), the agonist potency of exogenous dynorphin A(1–8) was not altered, as would have been expected if it had an action at κ-opioid receptors. This suggests that with a large reduction in the number of μ-opioid receptors as shown by a shift in the dose-response curves for DAMGO and [Leu^5]enkephalin, the observed agonist action of dynorphin A(1–8) was governed by the unmetabolised dynorphin A(1–8) activating κ-opioid receptors, rather than formed [Leu^5]enkephalin acting at μ-receptors.

A further problem with interpretation of the results is the time course of metabolism of dynorphin A(1–8) to [Leu^5]enkephalin as compared with the much more rapid onset of agonist action in the myenteric plexus. This may be explained if it is considered that the important [Leu^5]enkephalin concentration is that in the immediate vicinity of the opioid receptors, rather than the overall concentration in the tissue bath. This would indicate that the enzyme is close to the receptors and therefore affords a high local [Leu^5]enkephalin concentration.

Several studies have suggested that in certain areas of the CNS [Leu^5]enkephalin may be formed from prodynorphin, rather than from proenkephalin (Zamir *et al.*, 1983; 1984; Taquet *et al.*, 1985). The present study suggests an extra dimension in that the released dynorphin A (1–8) may be converted to [Leu^5]enkephalin outside the cell. Whether this is an important process in controlling the physiological role of dynorphin A(1–8) will have to await further study.

This work was supported by an MRC project grant. We thank Professor M. Orlowski for the supply of N-[1-(RS)-carboxy-2-phenylethyl]Ala-Ala-Phe-*p*AB.

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(Received October 11, 1989
Revised June 11, 1990
Accepted July 5, 1990)

Br. J. Pharmacol. (1990), **101**; 679–685

The effects of cocaine on intracellular Ca^{2+} handling and myofilament Ca^{2+} responsiveness of ferret ventricular myocardium

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1 When ferret right ventricular papillary muscles were stimulated with threshold punctate pulses (0.33 Hz; 30°C), cocaine, 10^{-5} M, increased peak tension development from 815 ± 120 to 1125 ± 180 mg ($P < 0.05$) and increased the rate of relaxation from peak tension (time to 80% decline from peak tension decreased from 155 ± 11 to 144 ± 11 ms; $P < 0.05$). These changes in the twitch were associated with comparable changes in the amplitude and time course of the calcium transient measured with aequorin (amplitude increased from 62 ± 4 to 90 ± 7% ($P < 0.05$) of maximal values; time to 80% decline from peak amplitude decreased from 84 ± 8 to 64 ± 3 ms; $P < 0.05$). These effects were markedly attenuated in the presence of the β-adrenoceptor-blocking agent, propranolol, 6×10^{-7} M, or by maximization of catecholamine release from the adrenergic nerve endings with field pulses of supratheshold strength, indicating that catecholamine release from the adrenergic nerve endings is responsible for the positive inotropic and lusitropic responses to low and moderate doses of cocaine (i.e., $\leqslant 10^{-5}$ M).

2 High doses of cocaine (i.e., $> 10^{-5}$ M) produced negative inotropic and lusitropic effects that were associated with a decreased amplitude and prolonged duration of the calcium transient.

3 In aequorin-loaded intact fibres, cocaine 10^{-5} M did not affect the force-calcium relationship unless catecholamines were present. Cocaine, 10^{-5} M, significantly shifted the force-calcium relationship of saponin-skinned muscles ($pCa_{50} = 6.14 \pm 0.05$ versus 5.92 ± 0.07; $P < 0.05$), indicating reduced responsiveness of the myofilaments to calcium. F_{max} (maximal Ca^{2+}-activated force) was reduced to 58% of control in the presence of 10^{-5} M cocaine, while the slope of the calcium-force curve remained unchanged. These data indicate that cocaine may also decrease myofilament calcium sensitivity and maximal calcium-activated force, via mechanisms independent of catecholamines, when cellular diffusion barriers are eliminated.

Introduction

The past several years have witnessed a dramatic increase in the non-medical use of cocaine and the advent of 'free-basing', which introduces large quantities of active drug into the bloodstream. Consequently, the incidence of drug-related cardiovascular complications is on the increase in Europe and the United States and now accounts for a significant fraction of cocaine-related disability and death. There are five clinically documented cardiac and vascular complications of cocaine use and abuse: (1) coronary and peripheral vascular spasm with regional ischaemia of the heart and mesenteric viscera, (2) myocardial infarction, (3) hypertension and stroke, (4) dysrhythmia and sudden cardiac death, (5) myocarditis, dilated cardiomyopathy and heart failure. Although the pathogenesis of the cardiovascular toxicity of cocaine remains to be defined, available evidence suggests that cocaine has medical consequences that are equal in importance to its well-documented psychosocial consequences (Nicholi, 1983; Isner *et al.*, 1986; Smith *et al.*, 1987).

Because calcium is an essential second messenger in excitation-contraction coupling of both the heart and blood vessels, the major cardiovascular toxicity of cocaine may be due to drug-induced alterations in intracellular Ca^{2+} handling and possibly the development of a Ca^{2+} overload state (Reiter, 1988; Morgan *et al.*, 1990). Depending upon the species studied and the precise experimental conditions, cocaine has been shown to have a positive inotropic, negative inotropic, or biphasic inotropic effect (Covino, 1986). In general, positive inotropic actions of this drug appear to correlate with increased catecholamine levels related to cocaine-induced blockade of the $Uptake_1$ system of the adrenergic nerve endings (Muscholl, 1961; Burgen & Iversen, 1965; Iversen, 1971). In contrast, negative inotropic actions appear to correlate with the local anaesthetic effects of higher concentrations of cocaine (Covino, 1986). In many experimental and clinical situations, the effects of cocaine are mixed and appear to involve a combination of these two mechanisms. In addition, direct actions on the sodium-calcium exchange mechanism and on the calcium pump of the sarcoplasmic reticulum have also been proposed for cocaine or related local anaesthetic agents (Blinks *et al.*, 1972; Josephson & Sperelakis, 1976).

The purpose of the present study was to test the hypothesis that the positive and negative inotropic actions of cocaine are related to changes in intracellular Ca^{2+} handling (Hague *et al.*, 1988; Perreault *et al.*, 1989). We also investigated, in skinned ferret ventricular muscles, the effects of cocaine on myofilament calcium responsiveness, which includes calcium sensitivity, maximal calcium-activated force and the slope of the calcium-force relationship (Perreault *et al.*, 1990a).

Although a wide range of blood levels has been obtained in conjunction with the cardiovascular actions or toxicity of cocaine in man, values usually range from between 10^{-6} to 5×10^{-5} M (Van Dyke *et al.*, 1976; Connor & Macleod, 1976; Wetli & Wright, 1979; Paly *et al.*, 1982; Isner *et al.*, 1986; Foltin *et al.*, 1988). On this basis, we selected a cocaine concentration of 10^{-5} M for use in the majority of our experiments.

Methods

Tissue acquisition and initial preparation

Papillary muscles of 1.0 mm or less in diameter were excised from the right ventricles of hearts removed from adult male ferrets, 12–14 weeks of age, under chloroform anaesthesia. The methods of preparation and instrumentation used in these

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studies have been described in detail (Gwathmey & Morgan, 1985; McKinnon et al., 1988; Kihara et al., 1989). After removal from the hearts, muscles were placed in baths containing bicarbonate-buffered physiological salt solution bubbled with a gas mixture of 95% O_2 and 5% CO_2 to pH 7.4. The experiments were performed at 30°C. Muscles were stimulated to contract at three second intervals with pulses of 5 ms duration. Unless otherwise specified, threshold voltage (i.e., $\leqslant$ 10% above threshold) delivered via punctate or field electrodes was used to stimulate the muscles; under some circumstances, maximal field stimulation was used in order to maximize catecholamine release from the adrenergic nerve endings (Blinks, 1966). Pulses were applied through either punctate or field electrodes located at the base of the muscle. An initial two hour equilibration period was allowed during which muscles were gradually stretched to the length where maximal isometric force developed. Since it was possible to obtain up to four papillary muscles per ferret, our n refers to the number of papillary muscles, not the number of ferrets.

Intact muscle studies

One group of muscles ($n = 9$) was used to characterize the actions of chlorobutanol, atropine and propranolol under conditions of threshold punctate or threshold field stimulation. In another group of punctate stimulated muscles ($n = 16$), cumulative concentration-response relationships for cocaine were determined. Tension, time to peak tension, and time from peak tension to 50% and 80% decline from peak tension were measured on chart strip paper which simultaneously recorded the tension response and the stimulus artifact at 100 mm s^{-1}. All measures were made under steady state conditions. In order to avoid precipitation of Ca^{2+} during the cumulative calcium concentration-response determinations, a phosphate-free solution was used and calcium was added in incremental concentrations up to 16 mM.

In a third group of muscles ($n = 7$ threshold punctate; $n = 7$ threshold field and maximal field), aequorin was loaded by macroinjection, as described in detail elsewhere (Kihara & Morgan, 1989). Light signals were recorded with a photomultiplier by means of a light collecting apparatus of a design described by Blinks (1982). In order to obtain a satisfactory signal-to-noise-ratio, it was usually necessary to average successive signals (from 16 to several hundred depending on the light intensity). Signal averaging was performed only after responses had reached the steady state. The light and tension responses and stimulus artifact were recorded simultaneously both on magnetic tape and on chart strip recording paper; light signals were passed through a filter with a 10 ms time constant. The light signal was measured in nanoamperes of anodal current. The amplitude and time course of the aequorin light signal were analysed in the same manner described above for tension. It is possible for drugs to interact directly with aequorin and modify the luminescent reaction or the sensitivity of aequorin to Ca^{2+} (Kamaya et al., 1977; Baker & Schapira, 1980; Endoh & Blinks, 1988). Therefore, the potential for interaction of cocaine and aequorin was tested *in vitro* by use of the basic method and calibration device described by Endoh & Blinks (1988). Briefly, aequorin was added to a solution containing free $[Ca^{2+}]$ between 10^{-7} and 10^{-6} M; under these conditions a low level of luminescence persists for several minutes until the aequorin is gradually consumed. After initiation of the luminescence reaction, cocaine dissolved in an aqueous diluent containing 0.5% chlorobutanol (see below), was added to the reaction pipette. We found that the drug had no effect on the luminescence reaction until concentrations in excess of 10^{-4} M were achieved. In these concentrations, the combination of cocaine plus aequorin appeared to decrease the light emission.

All data were compared by unpaired or paired (when appropriate) Student's t test or multiple sample comparison tests. Statistical significance was set at $P < 0.05$.

Skinned muscle studies

Muscles ($n = 18$) were skinned by a 30 min exposure to a solution containing: saponin 250 μg ml^{-1}, K_2ATP 5 mM, $MgCl_2$ 7 mM EGTA 5 mM KCl 60 mM imidazole 60 mM, creatine phosphate 12 mM and creatine phosphokinase 15 u ml^{-1}; pH = 7.1 at 21°C. Saponin, in this concentration, has been shown to remove functionally both the sarcolemma and sarcoplasmic reticulum (Endo & Iino, 1980). Total salt concentrations needed to obtain the desired pCa, pMg, pMgATP and pH at a constant ionic strength of 0.16 M were calculated using a program developed by Fabiato & Fabiato (1979). The absolute stability constants used were as described by Fabiato (1981). Muscles were subjected to activation-relaxation cycles which consisted of increasing the calcium concentration in a stepwise fashion until no further increase in force was produced, i.e., maximal calcium-activated force (F_{max}).

The isometric force versus pCa ($-\log[Ca^{2+}]$M) data were fitted to a modified Hill equation. Values of pCa_{50} (the pCa that produced 50% of maximal force development), F_{max} and n (the slope of the calcium-force curve) were compared by analysis of variance and Student's t tests. Statistical significance was set at $P < 0.05$.

Drugs

The aequorin used in these experiments was obtained from the laboratory of Dr J.R. Blinks of Rochester, Minnesota, U.S.A.; propranolol was purchased from Sigma Chemical Company, St. Louis, Missouri, U.S.A. and dissolved in aqueous solution. A stock solution of cocaine, 10^{-1} M, (purchased from Mallinckrodt, Inc., St. Louis, MO, U.S.A.) was obtained from the Beth Israel Hospital Pharmacy dissolved in a diluent containing 0.5% chlorobutanol. In the volumes added to the bath in these experiments, the diluent had no effect on the amplitude or time course of muscle contraction (see below).

Results

Effect of stimulus mode

Table 1 and Figure 1 show the relative effects of three different stimulus modes on the amplitude and time course of the intracellular calcium transient ($[Ca^{2+}]_i$), recorded with aequorin, and the isometric tension response. Note that threshold punctate and field stimulation produced similar peak-tension responses. In contrast, maximal field stimulation (the voltage that produced maximal activation) produced a significantly greater amount of tension development. Previous work has shown that the maximal response to field stimulation is equivalent to that produced by maximally effective concentrations of exogenously applied noradrenaline (Blinks, 1966). Table 1 also demonstrates the positive lusitropic (i.e., enhanced rate of relaxation) effect that occurred with threshold and maximal field stimulation compared to the punctate modality. Note that maximal field stimulation markedly abbreviated the duration of the twitch and enhanced relaxation, while threshold field stimulation produced a trend in that direction which did not reach statistical significance, except in the case of the time to 80% relaxation from peak tension (t8t).

Peak $[Ca^{2+}]_i$ was not measured in these experiments, and the peak aequorin light signals could not be accurately compared between different experiments due to variable degrees of aequorin loading and consequent muscle brightness; therefore, no value for peak $[Ca^{2+}]_i$ could be included in Table 1. However, Table 1 demonstrates that maximal field stimulation significantly abbreviated the time course of the Ca^{2+} transient, which correlates with the positive lusitropic response to this mode of stimulation.

Figure 1 shows the effect of the stimulation mode on the inotropic response to cocaine. Note that in both the punctate

Table 1 Effect of stimulus parameters on amplitude and time course of the aequorin light signal and tension

	Punctate (threshold V) ($n = 16$)	Field (threshold V) ($n = 7$)	Field (maximal V) ($n = 7$)
Pt (mg)	815 ± 120	760 ± 134	1951 ± 334*#
tPt (ms)	179 ± 10	162 ± 7	124 ± 4*#
t5t (ms)	101 ± 7	88 ± 8	59 ± 4*#
t8t (ms)	155 ± 11	72 ± 5#	87 ± 4*#
tPL (ms)	54 ± 3 ($n = 7$)	54 ± 2	41 ± 2*#
t5L (ms)	46 ± 2 ($n = 7$)	43 ± 2	30 ± 2*#
t8L (ms)	84 ± 8 ($n = 7$)	70 ± 5	49 ± 2*#

Data shown are means ± s.e.mean.
# $P < 0.05$ versus punctate; * $P < 0.05$ versus threshold field; Pt = peak tension; tPt = time to Pt, t5t and t8t = time to 50% and 80% decline from peak tension, respectively; tPL = time to peak light; t5L and t8L = time to 50% and 80% decline from peak light, respectively. Note that tPL and tPt were measured from stimulus artifact and t5L, t5T, t8L and t8T from peak response. Muscles were bathed in 1 mM calcium at 30°C and stimulated at 3 s intervals. V = voltage of 5 ms duration pulses.

and field modes, when threshold voltage was utilized, cocaine 10^{-5} M produced a significant positive inotropic effect. In contrast, when this concentration was administered to muscles field stimulated with a maximally effective voltage, no significant change was observed, although a negative inotropic trend was present in some experiments. In view of these responses, we used threshold stimulation to evaluate the sympathomimetic actions of cocaine.

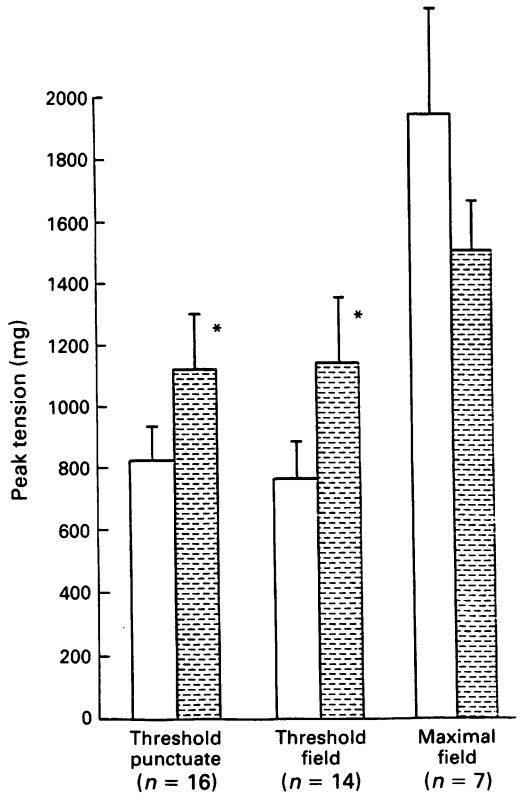

Figure 1 Dependence on stimulus parameters of peak tension development and inotropic responses to cocaine, 10^{-5} M, of ferret right ventricular papillary muscles. Open columns represent control responses in absence of cocaine and stippled columns, responses in presence of 10^{-5} M cocaine. Values shown are means and bars indicate s.e.mean. * $P < 0.05$ versus control.

Table 2 shows the effects of several bath additives on the amplitude of contraction in muscles stimulated with threshold voltage via field electrodes ($n = 9$). First, it should be noted that chlorobutanol in the amounts used in these experiments produced no significant effect on the contractile state. Under control conditions, cocaine 10^{-5} M, produced a statistically significant positive inotropic effect. Atropine, 2×10^{-6} M also increased the force of contraction of the preparations, an effect that was reversed by the subsequent addition of propranolol, 6×10^{-7} M. Table 2 demonstrates the dependence of the positive inotropic action of cocaine on the presence of functionally intact cardiac autonomic receptors. In the presence of atropine plus propranolol, the inotropic response to cocaine, 10^{-5} M, was markedly attenuated.

Effects of cocaine on the amplitude of the aequorin light signal and tension response

Figure 2 shows the effects of cocaine, 10^{-5} M, on the intracellular $[Ca^{2+}]_i$ transients recorded with aequorin and isometric contractions of a ferret papillary muscle. In this experiment the muscle was stimulated to contract with threshold field pulses. Note that this concentration of cocaine produced a marked increase in $[Ca^{2+}]_i$ that was associated with an increase in developed force. Table 3 illustrates these effects for 3 different concentrations of cocaine, applied to muscles being stimulated to contract with threshold voltage delivered via punctate electrodes. Note that 10^{-6} and 10^{-5} M cocaine produced similar increases in peak tension to levels that were significantly greater than the control. These increases in tension were associated with parallel increases in the amplitude of the intracellular Ca^{2+} transient. Higher concentrations (i.e., 10^{-4} M) of cocaine produced a negative inotropic effect that was associated with a decrease in the amplitude of the aequorin signal, reflecting decreased availability of $[Ca^{2+}]_i$ for excitation-contraction coupling.

Table 2 Effects of bath additives on amplitude of contraction of ferret papillary muscles stimulated with threshold field voltage

	Diluent#	*Cocaine* (10^{-5} M) *W*	*Atropine* (2×10^{-6} M) *W*	*Atropine* (2×10^{-6} M) + *propranolol* (6×10^{-7} M)	*Atropine* (2×10^{-6} M) + *propranolol* (6×10^{-7} M) + *cocaine* (10^{-5} M)
% change	−1.5 ± 1.9%	+21 ± 7.7%*	+16.6 ± 2.6*	−14 ± 6*	+6.8 ± 4.5*

Data shown are means ± s.e.mean, $n = 9$.
* $P < 0.05$ versus previous steady state value.
−, indicates decrease; +, increase from control values.
Cocaine vehicle containing 0.5% chlorobutanol. *W* indicates washout.

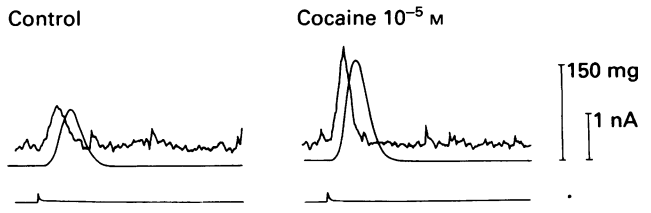

Figure 2 Effects of cocaine on a ferret right ventricular papillary muscle stimulated to contract at 3 s intervals with threshold field pulses of 3 V, 5 ms duration. Upper trace, aequorin light signal expressed in nanoamperes of anodal current from photomultiplier; middle trace, isometric force in g, lower trace, stimulus artifact. Forty successive signals were averaged under steady-state conditions to obtain these aequorin signals.

Effects of cocaine on the time course of the light signal and isometric contraction

Table 3 shows that low and moderate concentrations of cocaine (i.e., 10^{-6} and 10^{-5} M, respectively) significantly abbreviated the mechanical twitch, as reflected by the time to 80% relaxation from peak tension (t8t), and the corresponding Ca^{2+} transient, as reflected by the time to 80% decline from peak light (t8L). Cumulative additions of higher concentrations of cocaine (i.e., 10^{-4} M) tended to prolong both the twitch and calcium transient back towards control levels (although this change reached significance only for the time to peak light).

Effects of cocaine on myofilament Ca^{2+} responsiveness

In order to determine what effect, if any, cocaine has on myofilament Ca^{2+} responsiveness, we evaluated the force versus pCa relationship in aequorin-loaded fibres by determining the Ca^{2+} sensitivity index from the light versus force relationship in the absence and presence of the drug. This relationship can be expressed as the ratio of the amplitude of the light signals at equi-inotropic concentrations of drug and $[Ca^{2+}]_o$, i.e., peak light (cocaine) ÷ peak light ($[Ca^{2+}]_i$), expressed in nanoamperes. In muscles stimulated with threshold punctate pulses, low doses of cocaine had no significant effect on this ratio, producing light signals (i.e., intracellular Ca^{2+} transients) with equivalent amplitudes to those of equi-inotropic $[Ca^{2+}]_o$ (sensitivity index = 0.9 ± 0.1, n = 11). Concentrations of cocaine in excess of 10^{-5} M tended to increase this ratio in a direction suggesting decreased Ca^{2+} responsiveness, although this effect did not achieve statistical significance (data not shown). In contrast, when cocaine 10^{-5} M was added to the bathing medium of muscles being stimulated with maximal field pulses, the ratio increased to 1.7 ± 0.5, n = 5, ($P < 0.05$ versus punctate), suggesting that decreased Ca^{2+} responsiveness can occur at moderate concentrations of cocaine when catecholamine release is maximally facilitated. This effect of cocaine on maximal field stimulated muscles did not occur in the presence of propranolol, 6×10^{-7} M (sensitivity index = 0.9 ± 0.1, n = 5).

To evaluate more directly the effects of cocaine on myofilament Ca^{2+} responsiveness, which includes calcium sensitivity, maximal calcium activated force and the slope of the calcium-force relationship, we examined the effect of the drug on skinned muscle preparations. Cocaine, 10^{-5} M, decreased the responsiveness of the myofilaments to calcium in saponin-skinned ferret ventricular myocardium (n = 18). As shown in Figure 3, the sigmoidal force versus calcium curve was significantly shifted to the right in the presence of 10^{-5} M cocaine (6.14 ± 0.05 versus 5.92 ± 0.07, $P < 0.05$), thus indicating a decreased sensitivity of the contractile proteins to calcium (Figure 3). However, the Hill coefficient, which reflects the slope of the fit at the level of half maximal Ca^{2+} activation, was not altered by cocaine (1.28 ± 0.02 versus 1.25 ± 0.03 before and after cocaine 10^{-5} M, respectively). Maximal calcium-activated force, the maximal amount of force that a muscle can generate in the presence of calcium, was significantly decreased in the presence of 10^{-5} M cocaine to 58 ± 7.5% of the control level ($P < 0.05$).

Discussion

Interpretation of aequorin light signals in heart muscle

As shown in Figure 2 for ferret papillary muscles, the aequorin signals from mammalian working myocardium consist of a single component that temporally precedes the corresponding tension response. These findings are consistent with current models of excitation-contraction coupling, which predict that mechanical contractile events are preceded by changes in intracellular calcium concentrations (Rüegg, 1988). Interpreta-

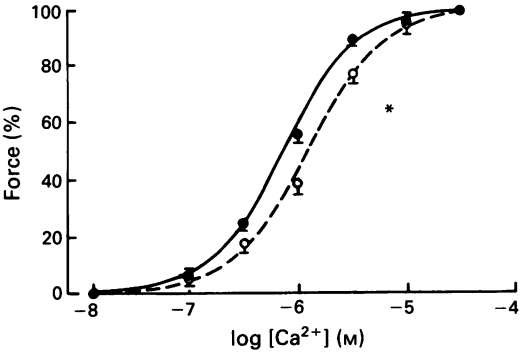

Figure 3 Effects of cocaine on saponin-skinned muscles. Maximal force was normalized and expressed as 100% for each muscle. (●—●) Control; (○- - -○) effect of cocaine 10^{-5} M. Values shown are means and vertical lines indicate s.e. * $P < 0.05$ versus control.

Table 3 Effects of cocaine on aequorin loaded right ventricular papillary muscles stimulated with threshold punctate voltage

	Control	*10^{-6} M Cocaine*	*10^{-5} M Cocaine*	*10^{-4} M Cocaine*
Peak tension (mg)	815 ± 120	1122 ± 174*	1125 ± 180*	674 ± 127*
Peak light (% max Ca^{2+}; n = 7)	62 ± 4	94 ± 6*	90 ± 7*	40 ± 11
tPL (ms; n = 7)	54 ± 3	50 ± 0	49 ± 1.5	61 ± 7*
tPt (ms)	179 ± 10	167 ± 8*	166 ± 7	164 ± 10
t5t (ms)	101 ± 7	96 ± 8	95 ± 8*	96 ± 8
t8t (ms)	155 ± 11	147 ± 12*	144 ± 11*	148.5 ± 11
t5L (ms; n = 7)	46 ± 2	36 ± 2*	40 ± 0*	41 ± 6
t8L (ms; n = 7)	84 ± 8	61 ± 3*	64 ± 3*	64 ± 6

Data shown are means ± s.e.mean, n = 16 unless indicated otherwise. % max Ca^{2+} = % of maximal response to Ca^{2+}. t5L = time to 50% decline from peak light; t8L = time to 80% decline from peak light; t5t = time to 50% decline from peak tension; t8t = time to 80% decline from peak tension; tPt = time to peak tension; tPL = time to peak light.
* $P < 0.05$ compared to control. All muscles were stimulated with threshold voltage via punctate electrodes at 0.33 Hz and bathed in 1 mM Ca^{2+} at 30°C.

tion of the aequorin light signals recorded from mammalian working myocardium have been detailed elsewhere (Morgan *et al.*, 1984; Morgan & Morgan, 1984; 1989). In brief, the aequorin signal in mammalian working myocardium appears to reflect predominantly the release and uptake of Ca^{2+} from the sarcoplasmic reticulum. Therefore, the peak of the aequorin light signal can be used as a measure of the amount of Ca^{2+} released by the sarcoplasmic reticulum. The time to peak of the aequorin light signal appears to reflect the relative balance between the phase of the Ca^{2+} transient during which Ca^{2+} release predominates versus the phase during which resequestration of Ca^{2+} predominates (descending phase). The half-time of decline from the peak of the aequorin light signal can be used as a measure of the speed of Ca^{2+} re-uptake by the sarcoplasmic reticulum. These interpretations are undoubtedly over-simplifications of the complex sequence of events that occur during excitation-contraction coupling in the heart, but are consistent with our current understanding of Ca^{2+} movement in myocardial cells. Determination of the relative effects of drugs on the amplitude of the aequorin light signal at similar levels of tension development provides an index for changes in the sensitivity of the contractile apparatus to Ca^{2+}; an index that generally correlates well with results from experiments in skinned cardiac muscle fibres (Gwathmey & Morgan, 1985; Morgan & Morgan, 1984; 1989).

Effects of autonomic agents and specific stimulus modalities on ferret myocardium

In this regard, it is worth noting that muscarinic agonists such as carbachol inhibit the adenosine 3':5'-cyclic monophosphate (cyclic AMP)-dependent positive inotropic effects of drugs that act by increasing intracellular cyclic AMP levels. This muscarinic action presumably occurs through blockade of the inhibitory effect of cholinoceptor agonists on adenylate cyclase activity, which is mediated through activation of an inhibitory guanine nucleotide regulatory protein. In dog and human myocardium, this muscarinic effect is not observed unless cyclic AMP levels are raised above those present with threshold stimulation alone (Endoh *et al.*, 1986; Warren *et al.*, 1989).

It is of interest that the effects of atropine and propranolol in our ferret muscle preparations, under conditions of threshold stimulation, were significantly greater than those obtained for similar concentrations of these autonomic receptor blocking agents in guinea-pig atria and atria and papillary muscles of kittens. In these species, concentrations of atropine or propranolol sufficient to prevent or greatly diminish the parasympathomimetic or sympathomimetic effects of maximal field stimulation had virtually no effect on the strength of contraction of preparations driven with threshold stimuli (Blinks, 1966). These results suggest a much greater dependence of ferret ventricular muscle on intracellular cyclic AMP levels for modulation of contractile function under basal conditions. These results also indicate that the data of studies in which ferret papillary muscles are driven electrically must be interpreted in light of the strong likelihood that liberation of autonomic transmitters by the stimuli is appreciable, even under conditions where threshold punctate stimulation is employed. Therefore, a combination of threshold stimulation plus β-adrenoceptor blockade must be employed to unmask the direct effect of cocaine on the sarcolemmal Na^+ channels; such experiments are complicated by the additive effect of local anaesthetic drugs. Consequently, in experiments where we wished to minimize catecholamine release to as large an extent as possible threshold stimulation without addition of other agents was employed alone. As shown in Table 1, threshold field stimulation of ferret papillary muscles produced effects intermediate between threshold punctate and maximal field stimulation, but which were more similar to the former than the latter. These effects of threshold stimulation are also presumably related to a greater quantity of catecholamine released from the larger number of sympathetic nerve endings which are affected by the field (versus punctate) electrodes.

Effects of cocaine on amplitudes of the aequorin light signal and tension responses

The amplitude of the aequorin light signal in ferret working myocardium is increased by most positive inotropic drugs, including isoprenaline, dibutyryl cyclic AMP, isobutylmethylxanthine, forskolin and increases in extracellular $[Ca^{2+}]$. Similar findings have been noted in other mammalian species (Morgan & Morgan, 1984; 1989; Perreault *et al.*, 1990a). These results are consistent with the expectation that an increased concentration of intracellular calcium would be reflected at the level of the myofilaments by an enhanced degree of activation. Agents like tyramine, amphetamine and bretylium produce a positive inotropic effect that is mediated by noradrenaline released from adrenergic nerve endings (Muscholl, 1961; Burgen & Iversen, 1965; Iversen, 1971). It is reasonable to hypothesize, therefore, that the positive inotropic effect of cocaine would be mediated by similar mechanisms.

As shown in Table 3, low concentrations of cocaine produce positive inotropic and lusitropic responses that are associated with an increase in amplitude and abbreviation of the duration of the Ca^{2+} transient. Moreover, when catecholamine release is facilitated, the ratio of the peak light responses at equi-inotropic concentrations of cocaine and $[Ca^{2+}]_i$ is increased in a direction suggesting desensitization of the contractile apparatus to calcium. This combination of effects is characteristic of agents that act by increasing intracellular cyclic AMP concentrations (Morgan & Morgan, 1989).

Phosphorylation of the Ca^{2+}-regulatory protein, phospholamban, results in a markedly increased rate of calcium resequestration by the calcium pump (Katz, 1979). This is reflected in the aequorin signal by an increased rate of decline (i.e., decreased t5L and t8L as shown in Table 3) and by a positive lusitropic effect as reflected by the decreased t5t and t8t (see Table 3). Moreover, phosphorylation of the myofilaments themselves, in particular, the troponin-C regulatory subunit, results in a decreased myofilament calcium sensitivity. This is reflected in aequorin-loaded fibres by the rightward shift which occurs in the ratio of the peak light responses at equi-inotropic concentrations of cocaine and $[Ca^{2+}]_0$. In the presence of low concentrations of cocaine, a much larger Ca^{2+} transient is required to produce an equi-inotropic response compared to control conditions. Therefore, the combination of effects noted at concentrations of cocaine $\leqslant 10^{-5}$ M are consistent with the actions of β-adrenoceptor stimulation, due to increased noradrenaline content in the synaptic cleft and biophase caused by cocaine-induced blockade of the $Uptake_1$ system of the adrenergic neurones. Additional substantiation of this interpretation is provided by attenuation of these responses when catecholamine effects are minimized via the use of threshold stimulation, and by the marked attenuation noted in the presence of propranolol (Table 2).

Biphasic action of cocaine

Higher concentrations of cocaine produce opposite effects to those described above. Concentrations of 10^{-4} M not only decreased the amplitude of the light and tension responses but also tended to prolong the time course towards control levels. These effects are probably related to the local anaesthetic actions of cocaine, since they were not affected by changes in the strength of field stimulation or altered by the addition of atropine. Local anaesthetics decrease the amount of Na^+ that enters the sarcoplasm via the voltage-dependent sarcolemmal Na^+ channel and could thereby decrease the amount of Ca^{2+} entering the cell via the Na^+–Ca^{2+} exchanger (Reiter, 1988). This in turn would be expected to decrease Ca^{2+} loading at

the sarcoplasmic reticulum and produce a lower-amplitude Ca^{2+} transient and twitch during subsequent cardiac cycles. Although we have found that concentrations of cocaine in the range of 10^{-4} M or higher may affect the interaction between aequorin and Ca^{2+} *in vitro*, such concentrations would not be expected to be achieved intracellularly in the present experiments. However, it must be noted that when applied in millimolar concentrations or higher, esteric local anaesthetics like cocaine may be expected to produce significant effects on subcellular Ca^{2+} handling (Blinks *et al.*, 1972). In the present study, effects of these large concentrations were not evaluated, not only because of a possible interaction with aequorin, but also because of the lack of clinical relevance of these very high concentrations.

Our data with skinned fibres also indicate that cocaine has the potential to alter the direct interaction of the cardiac contractile proteins with calcium, by decreasing both myofilament calcium sensitivity and maximal calcium-activated force (Figure 3). This effect must be due to a direct interaction of cocaine with the contractile proteins because these saponin-skinned fibres are devoid of functional autonomic nerve endings, the sarcolemma is removed as a diffusion barrier, and (in our preparations) the sarcoplasmic reticulum is destroyed. Free diffusion of cocaine into the sarcoplasm may explain why a 10^{-5} M concentration of cocaine produced a marked decrease in Ca^{2+} sensitivity in skinned cardiac muscle fibres, but not on intact papillary muscles stimulated with threshold voltage (see above). One implication of these results is that in a normally innervated heart, cocaine could decrease myofilament calcium responsiveness both by a drug-mediated rise in catecholamines and by its direct effects on the contractile apparatus. The mechanism of the direct myofilament action of cocaine remains to be determined, but could be related to an alteration in thin filament force regulation such as a change in the affinity of troponin-C for Ca^{2+}.

Toxic cardiovascular effects of cocaine

We and others have previously demonstrated that higher concentrations of drugs that increase intracellular cyclic AMP concentrations are often associated with the occurrence of Ca^{2+} oscillations and dysrhythmias (Orchard *et al.*, 1983; Wier *et al.*, 1983; Morgan & Morgan, 1984; Gwathmey & Morgan, 1985). Previous studies have shown that after-contractions and after-depolarizations occur with toxic doses of most positive inotropic agents that increase intracellular $[Ca^{2+}]$, particularly those that also increase intracellular cyclic AMP concentrations (Morgan & Morgan, 1984). These so-called 'triggerable' responses are an important cause of dysrhythmogenesis in addition to the mechanisms of automaticity and re-entry. Experimental evidence suggests that these after-responses are produced by an oscillatory release of calcium from intracellular stores. This oscillatory Ca^{2+} release may be caused by increased sarcoplasmic levels of calcium produced by high doses of inotropic drugs, that is, calcium-induced calcium release. Of interest, despite the marked positive inotropic response that we observed in some experiments, which we believe to be due to the effects of endogenous noradrenaline, cardiac dysrhythmias were not observed in any of our experiments. This suggests that the arrhythmogenic effects of catecholamines may be offset by the Na^{+} channel blocking properties of these concentrations of cocaine, at least in the ferret.

In summary, our findings suggest that an increase in intracellular calcium and increased isometric tension development are characteristic of low and moderate concentrations of cocaine in ferret working myocardium. However, they are not due to an increased myofilament Ca^{2+} responsiveness. Such an increase in contractility would be expected to translate into an increase in myocardial oxygen consumption, which would become significant in patients with coronary artery disease. Moreover, since these effects are mediated by increased cyclic AMP, we would also expect, in the intact animal or patient, a positive chronotropic effect to occur which would additionally increase myocardial oxygen demands. The negative inotropic effect which occurs at larger concentrations, appears to be due to Na^{+} channel blocking effects on the sarcolemma and, perhaps, direct interaction with the contractile proteins, would only be clinically relevant for patients consuming very large amounts of cocaine. In some reported pathological series, however, cocaine concentrations in excess of 5×10^{-5} M have been found in the blood (Van Dyke *et al.*, 1976; Paly *et al.*, 1982).

This work was supported in part by grant DA05171 from the National Institute of Drug Abuse, Bethesda, Maryland, U.S.A. and a Research Career Development Award (HL01611) to J.P.M. from the National Heart, Lung and Blood Institute, Bethesda, Maryland, U.S.A. Data analysis was performed on the PROPHET system, a computer resource sponsored by the Division of Research Resources, National Institutes of Health.

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(Received November 7, 1989
Revised June 5, 1990
Accepted June 21, 1990)

Br. J. Pharmacol. (1990), **101**, 686–690

Haemodynamic profile of an inhibitor of phosphodiesterase III, adibendan (BM 14.478): comparison with nitroprusside and dobutamine in conscious dogs

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1 This study was performed to investigate whether cardiac positive inotropic as well as peripheral vasodilator properties of adibendan contribute to its overall haemodynamic profile in conscious dogs.

2 Haemodynamic measurements were carried out in conscious chronically instrumented dogs after administration of adibendan, sodium nitroprusside or dobutamine.

3 The cardiovascular changes induced by adibendan (0.01 and 0.03 mg kg^{-1}) resembled those of dobutamine (1.0–4.0 μg kg^{-1} min^{-1}): left ventricular dP/dt_{60} (LV dP/dt_{60}), stroke volume (SV) and cardiac output (CO) increased to a similar extent, but mean arterial pressure (MAP) and heart rate (HR) remained unchanged.

4 In contrast to dobutamine, higher doses of adibendan (0.1–1.0 mg kg^{-1}) decreased MAP and LVEDP. These effects were of a similar magnitude to those observed following nitroprusside administration (0.5–12.5 μg kg^{-1} min^{-1}). In contrast to nitroprusside, adibendan still showed additional effects on LV dP/dt_{60} and CO.

5 From these results, it is concluded that both the peripheral vasodilator and the cardiac positive inotropic action of adibendan contribute to its overall haemodynamic profile.

Introduction

Adibendan (BM 14.478) is a benzimidazole derivative (Mertens *et al.*, 1987) with marked haemodynamic effects after acute i.v. and oral administration (Müller-Beckmann *et al.*, 1988a). Experiments *in vitro* have demonstrated positive inotropic and vasodilator properties (Müller-Beckmann *et al.*, 1988b) and have characterized the compound as a strong inhibitor of phosphodiesterase III (PDE-III = guanosine 3':5'-cyclic monophosphate (cyclic GMP)-inhibited PDE; Bethke *et al.*, 1988) and a calcium-sensitizing agent (Freund *et al.*, 1987; Gärtner *et al.*, 1987). Thus, the compound is related to amrinone (Farah & Alousi, 1978), enoximone (Dage *et al.*, 1982) and pimobendan (van Meel, 1985).

Because of their pronounced vasodilator properties these compounds have been classified as 'inodilators' (Opie, 1988). It is often claimed that their clinical efficacy is exclusively due to the peripheral action with a subsequent reflex increase in contractility (Firth *et al.*, 1984; Wilmshurst *et al.*, 1984; Franciosa, 1985; Miller *et al.*, 1987).

The aim of this study was to clarify whether the cardiac positive inotropic as well as the peripheral vasodilator properties of adibendan contribute to its overall haemodynamic profile. The cardiovascular effects of adibendan were compared with those of sodium nitroprusside, a pure vasodilator substance, and dobutamine, a strong positive inotropic drug, in an experimental model of conscious chronically instrumented dogs.

Methods

A left-side thoracotomy was carried out in mongrel dogs of either sex, weighing 25–34 kg, under aseptic conditions and general anaesthesia (halothane 0.5 vol%, 70% N_2O, 30% O_2). A Konigsberg manometer (model P5, Konigsberg Instruments, Pasadena, California, U.S.A.) was inserted through the apex of the heart into the left ventricle and an electromagnetic flowprobe (Speth, Dallenwil, Switzerland) was implanted around the aortic root. Finally, two polypropylene catheters (PP 120, e.d. 2 mm, i.d. 1 mm, Portex, Hythe, Kent) were placed in the abdominal aorta and in the inferior vena cava via the left femoral artery and vein, respectively. All implants were exteriorized between the shoulder blades and covered by a special suit.

Dogs were used in the experiments after a recovery period of at least 8 days. During the experiment they lay quietly in a cage. Arterial blood pressure (MAP) was recorded via a Statham P 23 db transducer, left ventricular pressure (LVP), left ventricular end-diastolic pressure (LVEDP) and cardiac output (CO; Hillers flowmeter, Hellige, Freiburg, F.R.G.) were measured. Heart rate (HR) was derived from the LVP signal by use of a pulse counter (MINO-Berlin, F.R.G.).

The contractility was measured as dP/dt_{60}, i.e., the derivative of LVP at a LV pressure of 60 mmHg (physiodifferentiator, Hugo Sachs, Hugstetten, F.R.G.). Peak negative dP/dt (dP/dt_{min}) was used as a measure of left ventricular relaxation. All parameters were recorded continuously on a universal amplifier 47/Varioscript 8008 (Schwarzer, Munich, F.R.G.).

Because the flow probes were not calibrated, the control values were taken as 100%. Total peripheral arterial resistance (TPR) was calculated according to Ohm's law. The control value of TPR was taken as 100% and the changes obtained after drug administration calculated relative to this value. Stroke volume (SV) was calculated as a ratio of CO and HR in percent, control value was expressed as 100%.

Three series of experiments were performed, with adibendan, nitroprusside, and dobutamine. Control values were taken after the baseline was constant for at least 30 min and calculated as the mean of two independent measures within 10 min before drug administration. The stability of the haemodynamic parameters during the experiments was achieved in previous investigations from our laboratories (Sponer *et al.*, 1987; Müller-Beckmann *et al.*, 1988a).

Drugs used

For intravenous administration 60.0 mg adibendan was dissolved in 1.0 ml dimethylformamide and 1.0 ml 1 N lactic acid.

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This solution was further diluted with distilled water. Adibendan was injected intravenously every 15 min in increasing doses. The cumulative doses were 0.01, 0.03, 0.1, 0.3, 1.0 mg kg^{-1} adibendan. Parameters were determined 14 to 15 min after each injection. Sodium nitroprusside (Nipruss) 60.0 mg was prepared as a solution with 27 mg sodium citrate and diluted with isotonic saline. Dobutamine (Dobutrex) 100 mg was dissolved and diluted with isotonic saline. Both drugs were administered as a continuous i.v. infusion over 10 min. Data were obtained at the end of each infusion period. Doses administered were 0.5, 1.0, 2.5, 5.0, 12.5 μg kg^{-1} min^{-1} sodium nitroprusside and 1.0, 2.0, 4.0, 8.0, 20.0 μg kg^{-1} min^{-1} dobutamine.

Statistics

Results are presented as arithmetic means and the standard error of the means (s.e.mean). Significant effects were calculated by Student's t test for paired observations. Significance was assumed at a level of $P < 0.05$.

Results

Haemodynamic effects of adibendan (Table 1)

Intravenous injections of adibendan at doses between 0.01 and 0.03 mg kg^{-1} caused no relevant change of MAP or HR (control values: 106 ± 3 mmHg and 66 ± 3 b.p.m.). At higher doses, MAP fell to 76 ± 2 mmHg and HR increased up to 125 ± 9 b.p.m. (1.0 mg kg^{-1}). However, LVEDP decreased continuously over the entire dose-range (7.1 ± 1.1 mmHg, control value; 3.7 ± 1.1 mmHg, 0.03 mg kg^{-1}; −1.0 ± 1.3 mmHg, 1.0 mg kg^{-1}).

Adibendan induced a dose-dependent rise of dP/dt_{60} (2200 ± 130 mmHg s^{-1}, control value; 3310 ± 170 mmHg s^{-1}, 0.03 mg kg^{-1}; 4130 ± 220 mmHg s^{-1}, 1.0 mg kg^{-1}). Changes in dP/dt_{min} were only significantly different from the control values at 0.03 mg kg^{-1}.

CO increased from 100% (control value) to 120 ± 5% at 0.03 mg kg^{-1} and finally to 157 ± 8% (1.0 mg kg^{-1}). In contrast, SV only increased at doses of 0.01 and 0.03 mg kg^{-1} from 100% (control value) to 115 ± 3%. At doses in excess of 0.3 mg kg^{-1} SV fell below the control value. TPR decreased continuously from 100% (control value) to 79 ± 4% at 0.03 mg kg^{-1} and finally to 47 ± 3% after administration of 1.0 mg kg^{-1}.

Haemodynamic effects of nitroprusside (Table 2)

Nitroprusside lowered MAP from 104 ± 3 mmHg (control value) to 65 ± 3 mmHg and increased HR from 75 ± 3 b.p.m. (control value) to 116 ± 4 b.p.m. when infused intravenously at doses between 0.5 and 12.5 μg kg^{-1} min^{-1}. LVEDP was reduced from 6.7 ± 1.2 mmHg (control value) to 0.9 ± 1.5 mmHg (12.5 μg kg^{-1} min^{-1}).

In the same dose range, dP/dt_{60} increased from 2100 ± 150 mmHg s^{-1} (control value) to 2750 ± 280 mmHg s^{-1} (12.5 μg kg^{-1} min^{-1}) and dP/dt_{min} decreased from 1850 ± 70 mmHg s^{-1} to 1250 ± 60 mmHg s^{-1}.

Nitroprusside caused an increase of CO from 100% (control value) up to 124 ± 6% (12.5 μg kg^{-1} min^{-1}). SV and TPR fell from 100% (control value) to 82 ± 4% and 51 ± 2% (12.5 μg kg^{-1} min^{-1}), respectively.

Haemodynamic effects of dobutamine (Table 3)

Intravenous infusion of dobutamine at doses between 1.0 and 4.0 μg kg^{-1} min^{-1} did not markedly affect MAP and HR (control values: 108 ± 4 mmHg; 69 ± 3 b.p.m.) while at higher doses MAP fell to 99 ± 4 mmHg and HR increased to 114 ± 2 b.p.m. (20.0 μg kg^{-1} min^{-1}). A significant change of LVEDP was observed only at the highest dose, from 8.9 ± 0.9 mmHg (control value) to 6.4 ± 0.8 mmHg (20.0 μg kg^{-1} min^{-1}).

Dobutamine produced a dose-dependent increase of dP/dt_{60} (2380 ± 140 mmHg s^{-1}, control value; 2970 ± 160 mmHg s^{-1}, 4.0 μg kg^{-1} min^{-1}; 4860 ± 100 mmHg s^{-1},

Table 1 Cardiovascular effects of intravenous adibendan in conscious dogs

		Adibendan (mg kg^{-1})					
		C	0.01	0.03	0.10	0.30	1.00
MAP	(mmHg)	106 ± 3	103 ± 3†	100 ± 4†	89 ± 4#	78 ± 1#	76 ± 2#
LVEDP	(mmHg)	7.1 ± 1.1	5.4 ± 1.1*	3.7 ± 1.1#	0.9 ± 1.2#	−0.4 ± 1.4#	−1.0 ± 1.3#
dP/dt_{60}	(mmHg s^{-1})	2200 ± 130	2710 ± 140#	3310 ± 170#	3940 ± 210#	4120 ± 240#	4130 ± 220#
dP/dt_{min}	(mmHg s^{-1})	1950 ± 90	2110 ± 110	2200 ± 150	2060 ± 150	2010 ± 170	1970 ± 220
HR	(b.p.m.)	66 ± 3	66 ± 2	69 ± 4	92 ± 7*	110 ± 7#	125 ± 9#
CO	(%)	100	108 ± 3†	120 ± 2*	143 ± 7#	147 ± 7#	157 ± 8#
SV	(%)	100	108 ± 2*	115 ± 3*	105 ± 6	90 ± 5	86 ± 6
TPR	(%)	100	90 ± 2*	79 ± 4#	60 ± 4#	51 ± 3#	47 ± 3#

Results are expressed as mean ± s.e.mean (n = 11). Different from control (C): † $P < 0.05$, * $P < 0.01$, # $P < 0.001$. MAP, arterial blood pressure, LVEDP, left ventricular end-diastolic pressure, dP/dt_{60}, derivative of LVP at a LV pressure of 60 mmHg; dP/dt_{min}, peak negative dP/dt; HR, heart rate; CO, cardiac output; SV, stroke volume; TPR, total peripheral arterial resistance.

Table 2 Cardiovascular effects of intravenous sodium nitroprusside in conscious dogs

		Nitroprusside (μg kg^{-1} min^{-1})					
		C	0.5	1.0	2.5	5.0	12.5
MAP	(mmHg)	104 ± 3	98 ± 3*	91 ± 3#	81 ± 3#	77 ± 3#	65 ± 3#
LVEDP	(mmHg)	6.7 ± 1.2	5.7 ± 1.2*	4.3 ± 1.2#	3.4 ± 0.9#	2.5 ± 0.9#	0.9 ± 1.5#
dP/dt_{60}	(mmHg s^{-1})	2100 ± 150	2210 ± 180*	2250 ± 170*	2350 ± 200*	2460 ± 250*	2750 ± 280†
dP/dt_{min}	(mmHg s^{-1})	1850 ± 70	1830 ± 70	1740 ± 50†	1620 ± 70*	1570 ± 90#	1250 ± 60#
HR	(b.p.m.)	75 ± 3	78 ± 4†	79 ± 3	94 ± 6*	108 ± 5#	116 ± 4#
CO	(%)	100	105 ± 3	109 ± 4†	120 ± 5*	128 ± 5#	124 ± 6*
SV	(%)	100	101 ± 3	102 ± 2	96 ± 5	88 ± 4*	82 ± 4*
TPR	(%)	100	91 ± 4†	82 ± 2#	66 ± 3#	58 ± 2#	51 ± 2#

Results are expressed as mean ± s.e.mean (n = 11). Different from control (C): † $P < 0.05$; * $P < 0.01$; # $P < 0.001$. For abbreviations see footnote to Table 1.

Table 3 Cardiovascular effects of dobutamine in conscious dogs

		Dobutamine ($\mu g\,kg^{-1}\,min^{-1}$)					
		C	1.0	2.0	4.0	8.0	20.0
MAP	(mmHg)	108 ± 4	107 ± 3	104 ± 5	110 ± 5	106 ± 4	99 ± 4*
LVEDP	(mmHg)	8.9 ± 0.9	9.3 ± 1.0	9.7 ± 1.4	10.4 ± 0.8	8.2 ± 1.0	6.4 ± 0.8†
dP/dt_{60}	($mmHg\,s^{-1}$)	2380 ± 140	2490 ± 160†	2640 ± 180#	2970 ± 160#	3660 ± 160#	4860 ± 100#
dP/dt_{min}	($mmHg\,s^{-1}$)	2160 ± 100	2150 ± 120	2130 ± 80	2320 ± 110*	2510 ± 190†	2940 ± 320†
HR	(b.p.m.)	69 ± 3	67 ± 4	67 ± 3	69 ± 3	82 ± 2*	114 ± 2#
CO	(%)	100	103 ± 3	110 ± 6	122 ± 7†	162 ± 9#	226 ± 12#
SV	(%)	100	107 ± 2†	112 ± 2*	118 ± 3#	134 ± 4#	134 ± 6#
TPR	(%)	100	96 ± 3	90 ± 4	85 ± 6†	62 ± 4#	42 ± 3#

Results are expressed as mean ± s.e.mean ($n = 10$). Different from control (C): † $P < 0.05$, * $P < 0.01$, # $P < 0.001$. For abbreviations see footnote to Table 1.

$20.0\,\mu g\,kg^{-1}\,min^{-1}$) and a slight increase of dP/dt_{min} ($2160 \pm 100\,mmHg\,s^{-1}$, control value; $2320 \pm 110\,mmHg\,s^{-1}$, $4.0\,\mu g\,kg^{-1}\,min^{-1}$; $2940 \pm 320\,mmHg\,s^{-1}$, $20\,\mu g\,kg^{-1}\,min^{-1}$).

CO and SV (control values: 100%) rose to 122 ± 7% and 118 ± 3% at $4.0\,\mu g\,kg^{-1}\,min^{-1}$, respectively, and finally increased to 226 ± 12% and 135 ± 6%, respectively, after administration of $20.0\,\mu g\,kg^{-1}\,min^{-1}$. TPR was dose-dependently diminished from 100% (control value) to 42 ± 3% ($20\,\mu g\,kg^{-1}\,min^{-1}$).

Discussion

In accordance with the literature, the direct dilator effects of nitroprusside, on arterial and venous vessels, induced pronounced decreases in TPR, MAP and LVEDP (Rowe & Henderson, 1974; Brodie *et al.*, 1977; Pagani *et al.*, 1978; Dinerman *et al.*, 1988). Because no direct positive inotropic or chronotropic properties of nitroprusside have been found in several experimental models, the increase in HR and contractility under intact haemodynamic conditions is in general attributed to sympathetic reflex responses and parasympatholysis (Chatterjee *et al.*, 1973; Pagani *et al.*, 1978; Pennington *et al.*, 1979; Macho & Vatner, 1981; Dumont *et al.*, 1983). The reduced rate of left ventricular relaxation, observed in the present study, is probably due to its correlation with the magnitude of peak aortic systolic pressure (Weisfeldt *et al.*, 1974; Brodie *et al.*, 1977).

During dobutamine infusion, the predominant effects observed can be explained by direct stimulation of cardiac β_1-adrenoceptors and the subsequent intracellular increase of adenosine 3′:5′ cyclic monophosphate (cyclic AMP) (Katz, 1983; Sonnenblick *et al.*, 1979). There was a marked increase in contractility, ventricular relaxation and increases in SV and CO.

The chronotropic efficacy of dobutamine was more pronounced at higher doses. As previously described (Vatner *et al.*, 1974; Hinds & Hawthorne, 1975; Tuttle & Mills, 1975) the effects of dobutamine on pre- and afterload were small. The marked fall in TPR can be explained by reflex vasodilatation in skeletal muscle (Liang & Hood, 1979) and by an additional direct β_2-adrenoceptor-mediated dilator effect at high concentrations (Robie *et al.*, 1974; Vatner *et al.*, 1974).

In these experiments, adibendan and nitroprusside showed similar arterio- and venodilating properties, as illustrated by the decrease of MAP, TPR and LVEDP (Figure 1). The reduction in LVEDP may also have been caused by enhanced myocardial relaxation or improved pump function (Ludmer *et al.*, 1986). However, in the present study, neither adibendan nor nitroprusside improved ventricular relaxation, as measured by dP/dt_{min}. In addition, given the marked rise of CO with dobutamine and the concurrent minimal change of LVEDP, improved pump function also seems unlikely to explain the marked fall of LVEDP associated with adibendan and nitroprusside administration. Adibendan, like nitro-

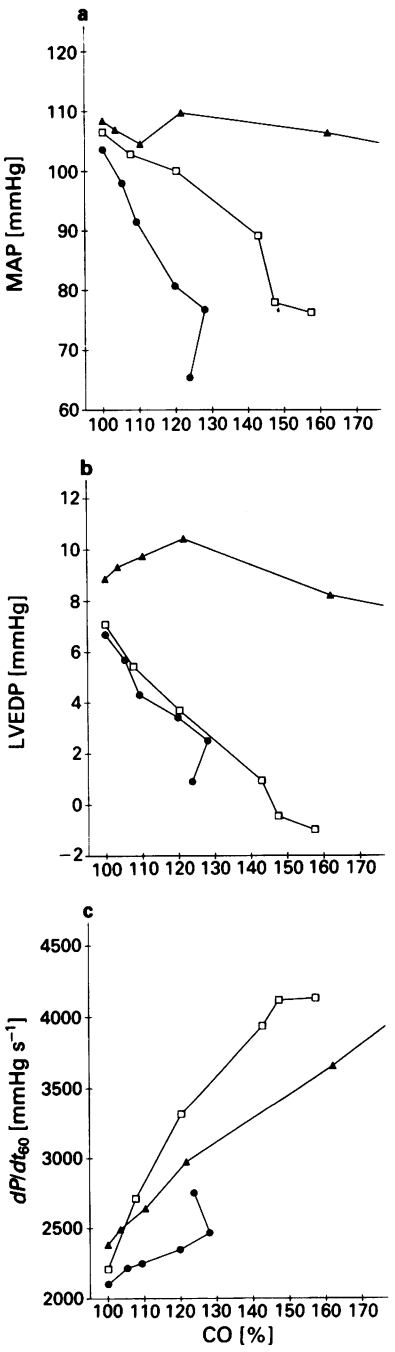

Figure 1 Effects of adibendan (□) ($n = 11$), nitroprusside (●) ($n = 11$) and dobutamine (▲) ($n = 10$) on mean arterial blood pressure (MAP), left ventricular end-diastolic pressure (LVEDP) and dP/dt_{60} in relation to the cardiac output (CO).

prusside might stimulate reflex mechanisms to increase cardiac contractility. However, since adibendan produced a greater increase in contractility and CO than nitroprusside and increased SV, at low doses, an additional direct positive inotropic effect seems likely.

In fact, the haemodynamic profile of adibendan at low doses (0.01 and 0.03 mg kg^{-1}) resembles the effects of dobutamine at a dose-range between 1.0 and 4.0 μg kg^{-1} min^{-1} (Figure 2). LV dP/dt_{60}, SV and CO increased to a similar extent, TPR fell slightly, but MAP and HR remained unchanged. The main difference was the marked decrease of LVEDP observed after adibendan administration, this is probably due to a direct venodilator effect as discussed above.

Higher doses of adibendan produced different haemodynamic changes. Instead of a further increase, SV decreased. This reduction might be caused by a lowered preload (Veit *et al.*, 1985; Müller-Beckmann *et al.*, 1988a). Contractility continued to increase, but was no longer accompanied by a corresponding increase of CO, as was the case with dobutamine (Figure 1). The reduction in MAP certainly contributed to a reflex increase of HR and to a further decrease of SV.

All three drugs increased the HR to a similar extent. This can be explained for dobutamine by its direct chronotropic effects and for nitroprusside by reflex chronotropy. Both mechanisms could account for the chronotropic activity of a PDE-III inhibitor. There was a similar ratio of increase of HR to decrease of MAP for adibendan and nitroprusside, indicating that the positive chronotropy observed after adibendan administration is mediated via reflex mechanisms (Figure 3). This would be in agreement with results obtained in atrial preparations *in vitro* which demonstrated only small positive chronotropic responses to adibendan (Bethke *et al.*, 1988; Müller-Beckmann *et al.*, 1988b).

No evidence for an improvement in ventricular relaxation, as may be expected following PDE inhibition, was observed after adibendan administration. As discussed for sodium nitroprusside the dependency of dP/dt_{min} on peak systolic aortic pressure may have disguised any change. In addition, the known Ca^{2+}-sensitizing action of adibendan (Freund *et al.*, 1987; Gärtner *et al.*, 1987) might have contributed to a slowing of ventricular relaxation.

In conclusion, both the cardiac inotropic and the peripheral vasodilator effects of adibendan contribute clearly to its overall haemodynamic profile.

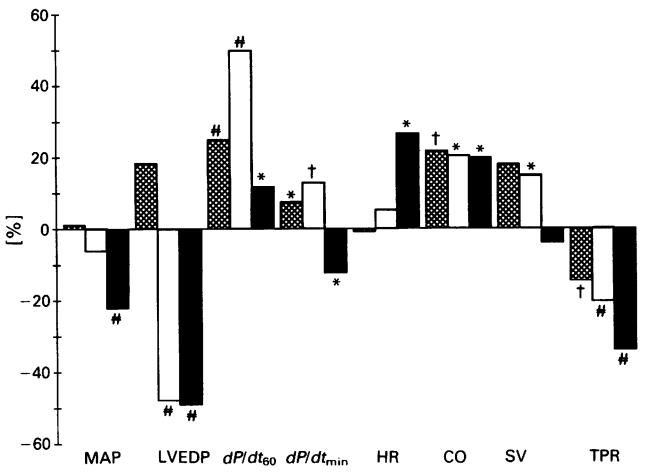

Figure 2 Cardiovascular effects of adibendan (open columns, 0.03 mg kg^{-1}), nitroprusside (solid columns, 2.5 μg kg^{-1} min^{-1}) and dobutamine (cross hatched columns, 4.0 μg kg^{-1} min^{-1}) are compared at doses which increase cardiac output by approximately 20%. Data presented as percentage change; † $P < 0.05$, * $P < 0.01$, # $P < 0.001$. For abbreviations, see footnote to Table 1.

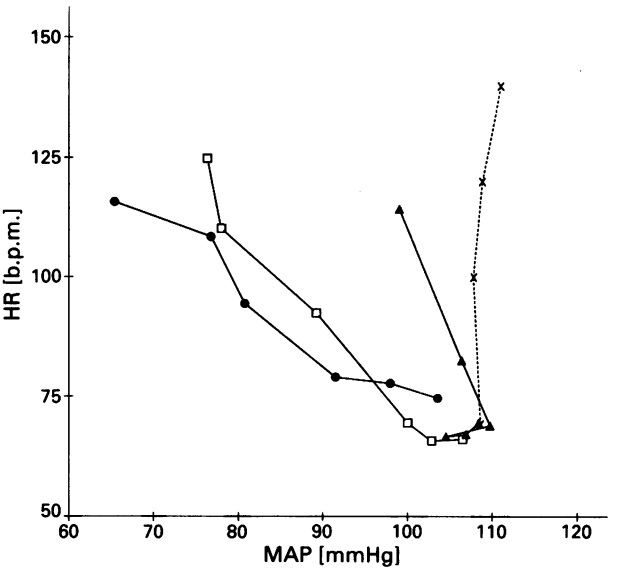

Figure 3 The relationship between heart rate (HR) and mean arterial blood pressure (MAP) is shown for adibendan (□) ($n = 11$), nitroprusside (●) ($n = 11$) and dobutamine (▲) ($n = 10$) as well as external pacing (×) ($n = 10$) in the same dogs.

The authors wish to express their thanks to Mrs B. Brömmert, Mrs H. Kroschel and Mr G. Muth for the skilful assistance with the experiments.

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(Received March 15, 1990
Revised June 11, 1990
Accepted June 28, 1990)

Br. J. Pharmacol. (1990), 101, 691–697

Nitrendipine decreases benzodiazepine withdrawal seizures but not the development of benzodiazepine tolerance or withdrawal signs

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1 The effects of the calcium channel blocking agent, nitrendipine, were studied on seizures in mice produced during withdrawal from chronic benzodiazepine treatment and on the development of tolerance to benzodiazepines.

2 Nitrendipine produced a dose-dependent decrease in seizure incidence, when seizures were produced by the partial inverse agonist FG7142 during withdrawal from seven days treatment with flurazepam.

3 Nitrendipine did not raise the seizure thresholds in naïve mice to the full inverse agonist methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate (DMCM), or to the γ-aminobutyric acid (GABA) antagonist, bicuculline.

4 When given concurrently with flurazepam for seven days, nitrendipine did not affect the incidence of seizures during flurazepam withdrawal.

5 When given concurrently with the benzodiazepines, nitrendipine did not prevent the development of tolerance to midazolam general anaesthesia or tolerance to the ataxic actions of flurazepam or midazolam.

6 Chronic treatment with flurazepam for seven days did not affect the K_d or B_{max} of [^{3}H]-nimodipine binding in mouse whole brain or cerebral cortex.

7 These results with benzodiazepines are partially in contrast with those for ethanol, where nitrendipine not only decreased ethanol withdrawal seizures when given acutely, but also prevented the development of tolerance and withdrawal signs when given concurrently with ethanol. However, they do confrm the selectivity of nitrendipine for withdrawal-induced seizures.

Introduction

The dihydropyridine binding site is thought to be an integral part of the voltage-operated calcium channel. Dihydropyridines include both calcium channel antagonists, such as nitrendipine, and calcium channel activators, such as Bay K 8644. Until recently these compounds have not been thought to have many actions on central neurones (e.g. Miller & Freedman, 1984) but effects on neuronal calcium channels have now been demonstrated (e.g. Spedding & Middlemiss, 1985). The compounds have been shown to affect physical dependence on sedative/hypnotic drugs.

Dihydropyridine calcium channel antagonists decreased seizure incidence during withdrawal from ethanol (Little *et al.*, 1986; Littleton *et al.*, 1990), barbiturates (Brown *et al.*, 1988) or nitrous oxide (Dolin & Little, 1989b). They also decreased the severity of the opiate withdrawal syndrome (Bongianni *et al.*, 1986). They were considerably less effective against other types of seizures (Dolin *et al.*, 1988) and appear to be relatively selective for drug withdrawal. Animals chronically treated with ethanol had increased [^{3}H]-nimodipine binding sites in cerebral cortex and in whole brain. The dihydropyridine calcium channel antagonist nitrendipine, given concurrently with ethanol, prevented the development of ethanol tolerance (Little & Dolin, 1987; Dolin & Little, 1989a), and the ethanol withdrawal syndrome (Whittington & Little, 1988). These data support the hypothesis that changes in dihydropyridine-sensitive calcium channels are important in the mechanism of ethanol tolerance and withdrawal phenomena.

In this paper we have extended our investigations from ethanol to the benzodiazepine group of compounds, and examined the effects of the dihydropyridine calcium channel antagonist, nitrendipine, on benzodiazepine tolerance and withdrawal, and the effects of chronic treatment with benzodiazepines on central dihydropyridine receptors, in order to see whether there was any common pattern in the effects of chronic treatment with alcohol and with benzodiazepines.

There is some evidence for interactions between benzodiazepines and calcium channels. Calcium channel antagonists have been shown to increase the hypothermic (Draski *et al.*, 1985) and anaesthetic (Dolin & Little, 1988) effects of benzodiazepines, but were found not to alter other central effects of benzodiazepines (Mendelson *et al.*, 1984b; Draski *et al.*, 1985). Nifedipine was shown to prevent sleep induction by flurazepam (Mendelson *et al.*, 1984b). When given alone, calcium channel antagonists did not produce general anaesthesia, even at very high doses (Dolin & Little, 1988). Micromolar concentrations of benzodiazepines inhibited [^{3}H]-nitrendipine binding (Bender & Hertz, 1985), and micromolar concentrations of calcium channel antagonists displaced binding at the peripheral benzodiazepine receptor (Cantor *et al.*, 1984). As the concentrations required to produce these effects were well in excess of the K_d values for other receptor ligands, a non-specific effect is the most likely explanation. However, micromolar concentrations of benzodiazepines may be found after doses used in behavioural experiments and in clinical practice (Garattini *et al.*, 1973; Klotz, 1979).

Benzodiazepines, in micromolar concentrations, have been shown to affect calcium flux (Leslie *et al.*, 1980; Skerritt *et al.*, 1984). The concentrations of a range of benzodiazepines required to decrease voltage-dependent calcium uptake into synaptosomes, were shown to correlate with their production of loss of righting reflex rather than with their anticonvulsant effects (Leslie *et al.*, 1986). Chronic treatment with benzodiazepines has been shown to result in tolerance to the inhibitory effects on voltage-dependent calcium uptake (Leslie *et al.*, 1980). However, diazepam increased depolarization-dependent calcium uptake into synaptosomes (Mendelson *et al.*, 1984*a*), an effect which was reversed by dihydropyridine calcium

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channel antagonists. Midazolam, at nanomolar concentrations, increased calcium spikes in neurones (Carlen et al., 1983). The reasons for these discrepancies is not immediately clear, although the doses of benzodiazepines in the studies by Mendelson et al. and Carlen et al. were lower than those used by other authors.

The chronic benzodiazepine treatment schedules used in the present experiments were sufficient to cause tolerance to the ataxic, anticonvulsant, sedative and anaesthetic properties of the benzodiazepines (Little et al., 1987; 1988). Spontaneous withdrawal seizures were not produced by these treatments, but we have shown previously that the partial inverse agonist, FG7142 (N-methyl-β-carboline-3-carboxamide), causes full seizures when given during the withdrawal period (Little et al., 1987, 1988; Little, 1988). This effect was used to study the actions of the calcium channel antagonist during withdrawal. For comparison, the effects of nitrendipine were examined on seizures in naïve mice produced by the full inverse agonist, DMCM (methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate), and the γ-aminobutyric acid (GABA) antagonist, bicuculline.

The 'peripheral' benzodiazepine site (Bender & Hertz, 1985) has a different pharmacological sensitivity from the central benzodiazepine site and is thought to operate a calcium conductance (Mestre et al., 1985; Holck & Osterreider, 1985). We therefore also investigated the actions of the 'peripheral' benzodiazepine antagonist, PK11195, on benzodiazepine tolerance and withdrawal.

Methods

Animals

Albino male mice (T.O. strain), 25–30 g were used in all experiments. They were allowed to acclimatize to the departmental animal house for several days before experimentation. They were housed in standard mouse cages with free access to food and water, with a 12 h day-night cycle. During chronic benzodiazepine treatment animals were weighed regularly to ensure that there was no loss of weight.

Benzodiazepine withdrawal seizures

Flurazepam, 40 mg kg^{-1}, or saline, were given once daily for seven days. Seizures were produced by the partial inverse agonist FG714, 24 h after the last of the chronic flurazepam treatment injections. Nitrendipine, 10, 50 or 100 mg kg^{-1}, was given i.p. 30 min, or 2 h later, FG7142, 40 mg kg^{-1}, was injected and the seizure incidence over the following 40 min noted by an observer who was unaware of the prior chronic treatments. A seizure was defined as tonic or clonic whole body movements associated with loss of posture. The seizure incidences in each group were compared by Fisher's exact probability test.

Effects of nitrendipine given during chronic flurazepam treatment

Once-daily injections were given for seven days. Each group of mice was divided into four subgroups: (1) Flurazepam, 40 mg kg^{-1}, plus Tween vehicle. (2) Flurazepam, 40 mg kg^{-1} plus nitrendipine 10 or 50 mg kg^{-1}, either 5 or 30 min before the flurazepam. (3) Nitrendipine 10 or 50 mg kg^{-1} plus saline. (4) Tween vehicle plus saline.

Twenty-four hours after the last chronic treatment injections, withdrawal seizures were precipitated by FG7142, 40 mg kg^{-1} (see above). Owing to the low incidence of seizures in the first group tested following treatment with flurazepam alone, this experiment was repeated. The results have been given separately, but the experimental conditions were the same in both cases. The incidence of seizures was also tested forty-eight hours after the last chronic injections, in case any changes were seen at that time.

Seizure incidence to bicuculline and DMCM

Seizure incidence with the full inverse agonist DMCM was measured by observation of the numbers of mice having full convulsions (defined above) after intraperitoneal doses of DMCM. Groups of naïve mice were given nitrendipine injections, 50 mg kg^{-1}, 30 min before i.p. injections of DMCM. The mice were observed for 40 min after the DMCM injections, and seizure incidence noted.

Bicuculline (60 mg kg^{-1}) was given by the intraperitoneal route (i.p.); this has been demonstrated previously to produce seizures in about 95% of control mice. Naïve mice were given nitrendipine injections, 50 or 100 mg kg^{-1}, 30 min before the i.p. injections of bicuculline, and the seizure incidence noted for 40 min following the bicuculline injections.

In all cases the observer was blind to the prior drug treatment. Seizure incidences were compared by Fisher's exact probability test.

Tolerance to rotating rod performance

The chronic treatments used to induce tolerance to the ataxic action of the benzodiazepines were as follows: flurazepam, 20 mg kg^{-1}, once daily for seven days; midazolam, 10 mg kg^{-1}, once daily for fourteen days, or clonazepam, 10 mg kg^{-1}, once daily for ten days. The dihydropyridine calcium channel antagonists, nitrendipine and PN 200–110 were given at 50 mg kg^{-1} and 4 mg kg^{-1}, respectively. Concurrently treated groups ($n = 8$ per group) received one of the benzodiazepines plus a dihydropyridine, the dihydropyridine alone, or vehicle treatment.

The ataxic effects of the benzodiazepines were measured by a rotating rod (rotorod), speed of rotation 4.5 r.p.m. Tests of the ataxic actions of the compounds were made at the beginning and end of the chronic treatment. There were no intermediate learning opportunities in the presence of drugs. The times on the rotorod for the groups of mice were compared by the Mann-Whitney 'U' test.

An initial learning period was found to be necessary in the rotorod studies. Before the experiments were started, each mouse was placed on a rotating rod (4.5 r.p.m.) three times, in a drug-free state, until it was able to stay on the rod for up to three minutes. Rarely, a mouse did not attain this and was omitted from the experiment. I.p. injections were given, then the mice were placed on the rotating rod every 15 min. The time spent on the rod was measured; an upper limit of 180 s was allowed.

Results from the initial rotorod tests, carried out at the beginning of the chronic treatments, have not been presented, as they were designed primarily to ensure that all mice in the treatment schedules would stay on the rotorod in the naïve state and to check the effects of the dose used in the chronic treatments. The tests made following chronic treatment, after administration of a single dose of the appropriate benzodiazepine to all groups of mice, were considered the most useful measure of tolerance. Studies on the acute effects of dihydropyridine calcium channel antagonists and benzodiazepines have shown that synergism occurs, that was not due to a pharmacokinetic interaction (Dolin & Little, 1988). Administration of each benzodiazepine alone in the tests of tolerance allowed the measurement of its action without interference from the dihydropyridine. The doses used for this tolerance testing were midazolam, 2.5 mg kg^{-1}, flurazepam, 20 mg kg^{-1}, or clonazepam, 10 mg kg^{-1}. These doses were chosen to produce effects in our rotorod test that were almost maximal, without causing total inability to stay on the rotating rod.

Tolerance to midazolam anaesthesia

Groups of ten mice were injected once daily with midazolam, 135 mg kg^{-1}, for four days. This dose has previously been shown to be the ED_{50} for loss of righting reflex in the TO strain of mice (Dolin et al., 1987a). Controls were injected with

vehicle only. Twenty-four hours after the last dose of midazolam or vehicle all mice were injected with midazolam 150 mg kg^{-1}, a dose chosen to cause loss of righting reflex in nearly all control mice. Tolerance was assessed by comparing numbers with loss of righting reflex in the midazolam-treated groups with the control group.

Loss of righting reflex was defined as the inability of a mouse to regain the upright posture (all four feet on the bench) within 60 s of being placed on its back. The numbers of mice that lost their righting reflex in each group were compared by Fisher's exact probability test.

The effects of nitrendipine on midazolam tolerance were assessed by giving nitrendipine, 10, 25 or 50 mg kg^{-1}, either 5 min or 2 h before midazolam, once daily for four days. The effects of the peripheral benzodiazepine antagonist PK11195 on midazolam tolerance were assessed by giving PK11195, 30 or 90 mg kg^{-1}, 5 min before midazolam once daily for four days.

Radioligand binding studies

Dihydropyridine radioligand binding assays (Glossmann *et al.*, 1982) were performed on whole brains and on cerebral cortices taken from mice 24 h after the last of seven daily injection of flurazepam, 40 mg kg^{-1}, with or without nitrendipine, 50 mg kg^{-1}. This chronic treatment schedule was the same as that used in the flurazepam withdrawal studies. Tissues were homogenised in a glass hand-held homogeniser and suspended in 50 mM Tris HCl (pH 7.4, 25°C). The membrane preparation was washed and centrifuged three times and finally suspended at 8 mg ml^{-1}. Tissue was incubated in the presence of [^{3}H]-nimodipine (135 μCi mol^{-1}) 0.125–8 nM, at a final volume of 0.5 ml. Non-specific binding was defined in the presence of an excess of unlabelled nitrendipine, 1 μM. Incubation was performed in the dark, at 25°C for 45 min. Incubation was stopped by rapid filtration with ice-cold 50 mM Tris HCl across Whatman GF/C filters set in a filtration manifold under suction. Filters were placed in a plastic vials and 5 ml of scintillant (Beckmann Hi-Safe) were added. Vials were counted on a Beckmann LS2000 counter. Scatchard analysis of binding data was performed to determine the affinity constant (K_d) and the numbers of binding sites (B_{max}). Comparisons were made by Student's t test.

Drugs

The dihydropyridine calcium channel antagonists, nitrendipine (Bayer) and PN 200-110 (isopropyl-4-(2,1,3-benzoxadiazol-4-yl-1,4-dihydro-2,6-dimethyl-5-methoxy-carbonyl-pyridine-3-carboxylate), Sandoz), were weighed under dimmed red lighting as they are light sensitive. They were kept in light-proof containers, and before use were suspended at 1–10 mg ml^{-1} in distilled water and Tween 80 (polyoxyethylene sorbitan monoleate) 0.5%, and sonicated.

FG7142 (Schering), PK11195 (1-(2-chlorophenyl)-N-methyl-N-(1-methylpropyl)-isoquinoline-3-carboxamide), Rhone-Poulenc), and clonazepam were suspended in water/Tween 80 0.5% and sonicated. Flurazepam, and midazolam were dissolved in saline. All the above drugs were injected i.p. at a volume of 0.01 ml g^{-1}.

DMCM was dissolved in 0.1 N HCl, then diluted 1 in 10 with distilled water. Bicuculline (Sigma) was dissolved in concentrated acetic acid, titrated to pH 3 with NaOH, and diluted with NaCl 0.9% brought to pH 3 with HCl.

Results

Actions of nitrendipine on seizures produced by FG7142 during withdrawal from flurazepam treatment

Nitrendipine 10, 50 and 100 mg kg^{-1}, produced a dose-dependent reduction in FG7142-induced seizures during flurazepam withdrawal (Table 1). This effect was significant when the seizure incidence after the two higher doses of nitrendipine were each compared with that after vehicle treatment ($P < 0.05$). FG7142 did not cause seizures in mice that were chronically treated with the vehicle only (Tween 80, 0.5%, in distilled water). Mice treated with flurazepam, 40 mg kg^{-1}, once daily for seven days did not show any spontaneous seizures after the last dose of flurazepam.

When nitrendipine, 10 and 50 mg kg^{-1}, was given concurrently with flurazepam throughout the seven day chronic

Table 1 Seizure incidence when nitrendipine or Tween vehicle was given with the inverse agonist, FG1742, 40 mg kg^{-1}, during flurazepam withdrawal, 24 h after the last chronic treatment injections

A Nitrendipine given 30 min before FG7142

Chronic treatment	*Drug given during withdrawal*	*Seizure incidence*
Flurazepam	Vehicle	12/20
Flurazepam	Nitrendipine 10 mg kg^{-1}	12/20
Flurazepam	Nitrendipine 50 mg kg^{-1}	5/20*
Flurazepam	Nitrendipine 100 mg kg^{-1}	4/20*

B Nitrendipine given 2 h before FG7142

Chronic treatment	*Drug given during withdrawal*	*Seizure*
Flurazepam	Vehicle	11/20
Flurazepam	Nitrendipine 10 mg kg^{-1}	8/19
Flurazepam	Nitrendipine 50 mg kg^{-1}	3/20*
Flurazepam	Nitrendipine 100 mg kg^{-1}	4/19*
Flurazepam	Vehicle	7/10
Flurazepam	Nitrendipine 50 mg kg^{-1}	2/10*
Vehicle	Nitrendipine 50 mg kg^{-1}	0/10*
Vehicle	Vehicle	0/10*

* $P < 0.05$ compared with chronic flurazepam-treated mice, given Tween vehicle before FG7142.

Table 2 Seizure incidence to FG7142, 40 mg kg^{-1}, when nitrendipine was given concurrently with flurazepam over 7 days

A FG7142 given 24 h after the last dose of flurazepam

Chronic treatment	*Seizure incidence*
(i) Nitrendipine given 5 min before flurazepam during chronic treatment	
Flurazepam plus Tween vehicle	4/10
Flurazepam plus nitrendipine 10 mg kg^{-1}	7/10
Flurazepam plus nitrendipine 50 mg kg^{-1}	4/10
Vehicle plus saline	0/10
Flurazepam plus Tween vehicle	4/10
Flurazepam plus nitrendipine 50 mg kg^{-1}	4/10
Nitrendipine 50 mg kg^{-1}	0/10
Vehicle plus saline	0/10
(ii) Nitrendipine given 30 min before flurazepam during chronic treatment	
Flurazepam plus Tween vehicle	5/10
Flurazepam plus nitrendipine 10 mg kg^{-1}	3/10
Flurazepam plus nitrendipine 50 mg kg^{-1}	1/10
Vehicle plus saline	0/10
Flurazepam plus Tween vehicle	7/20
Flurazepam plus nitrendipine 50 mg kg^{-1}	11/19

B FG7142 given 48 h after the last dose of flurazepam

Chronic treatment	*Seizure incidence*
Flurazepam plus saline	14/19
Flurazepam plus nitrendipine 50 mg kg^{-1}	16/20

No significant differences ($P > 0.05$) were found in any of the experiments, between the groups treated chronically with flurazepam plus Tween vehicle and those receiving flurazepam plus nitrendipine.

Table 3 Lack of effect of nitrendipine on seizures in naïve mice produced by methyl-6,7-dimethoxy-4-ethyl-B-carboline-3-carboxylate (DMCM) or bicuculline

A Intraperitoneal injection of DMCM

Drug pretreatment	*DMCM dose* ($mg\,kg^{-1}$)	*Seizure incidence*
Vehicle	3	3/10
Nitrendipine $50\,mg\,kg^{-1}$	3	5/10
Vehicle	3.5	4/10
Nitrendipine $50\,mg\,kg^{-1}$	3.5	5/10

B Intraperitoneal injection of bicuculline, $6\,mg\,kg^{-1}$

Drug pretreatment	*Seizure incidence*
Vehicle	7/8
Nitrendipine $50\,mg\,kg^{-1}$	7/8
Nitrendipine $100\,mg\,kg^{-1}$	7/8

No significant changes, $P > 0.1$ for comparisons, between nitrendipine-treatment and vehicle controls.

treatment period there was no reduction in seizure incidence, compared with the group given flurazepam alone during the chronic treatment (Table 2). This result was obtained whether nitrendipine was given 2 h or 30 min before flurazepam. Seizure incidence was unaltered by concurrent nitrendipine whether FG7142 was given at either 24 or 48 h after the last dose of flurazepam. Mice given chronic nitrendipine alone for 7 days did not have FG7142-induced seizures.

Lack of effect of nitrendipine on seizures induced by bicuculline or DMCM in naïve mice

Nitrendipine, 50 or $100\,mg\,kg^{-1}$, did not affect the seizures due to bicuculline, $P > 0.1$ (Table 3). Nitrendipine, $50\,mg\,kg^{-1}$, did not affect the seizures due to the full inverse agonist, DMCM, $P > 0.1$ (Table 3).

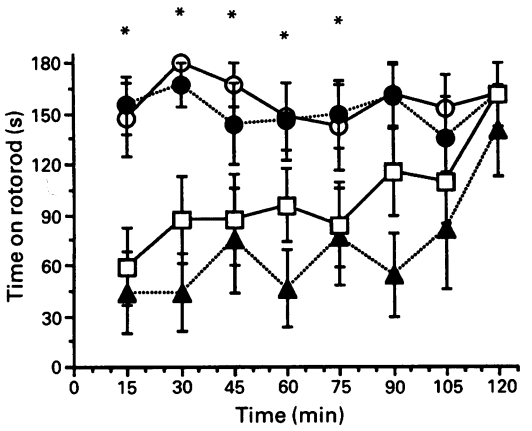

Figure 1 Tolerance to the ataxic actions of flurazepam, after seven days chronic treatment. On the eighth day, 24 h after the last of the chronic treatment injections, all mice were given i.p. injections of flurazepam, $20\,mg\,kg^{-1}$, then tested on the rotorod. Results are presented as mean and vertical lines indicate s.e.mean. Chronic treatments were as follows: (○) flurazepam, $20\,mg\,kg^{-1}$ plus Tween vehicle; (▲) nitrendipine $50\,mg\,kg^{-1}$ plus saline; (●) flurazepam, $20\,mg\,kg^{-1}$ plus nitrendipine $50\,mg\,kg^{-1}$; (□) controls = saline plus Tween vehicle. The asterisks mark the times at which the results for the groups given either flurazepam alone or flurazepam plus nitrendipine were significantly different ($P < 0.05$) from those of the vehicle-treated controls. At no times were the results for the two flurazepam treatment groups significantly different from each other, or the controls and the group given nitrendipine alone ($P > 0.1$).

Lack of effect of nitrendipine on tolerance to benzodiazepine ataxia

Nitrendipine did not affect the development of tolerance to the ataxic actions of flurazepam (Figure 1). After seven days of chronic treatment, all mice were given flurazepam $20\,mg\,kg^{-1}$. Both flurazepam alone and flurazepam plus nitrendipine groups were able to stay on the rotating rod for near the maximum allowed times. The rotorod times for both these groups were significantly greater than those for the control mice or those given nitrendipine alone during the chronic treatment. Tolerance therefore developed to the ataxic effects of flurazepam, whether given with or without nitrendipine.

The same result was found when another dihydropyridine calcium channel antagonist, PN 200-110, was given with flurazepam in the chronic treatment schedule. The rotorod times following a challenge dose of $20\,mg\,kg^{-1}$ flurazepam, 24 h after the end of chronic dosing with flurazepam (same doses as above) with and without PN 200-110, $4\,mg\,kg^{-1}$, were 116 ± 22 for chronic treatment with flurazepam alone and 98 ± 23 for chronic treatment with flurazepam plus PN 200-110, when measured 30 min after injection.

In each experiment, the group that received chronic nitrendipine alone showed a degree of impairment of rotorod performance that was not significantly different from that in controls, when given the acute challenge dose of flurazepam on day 8.

There were no significant differences between the tolerance developed by groups of mice chronically treated with midazolam only or midazolam plus nitrendipine ($P > 0.05$, Figure 2). Both groups were significantly different from controls after two weeks chronic treatment. Concurrent administration of nitrendipine did not therefore affect the development of tolerance to the ataxic effects of midazolam. Similar results were found whether nitrendipine was given immediately before (Figure 2) or two hours before the midazolam (results not shown).

The same pattern of results was seen when tolerance to the ataxic action of clonazepam was studied (results not shown). When clonazepam was tested after the chronic treatments, the groups of mice that had received control injections or treatment with nitrendipine alone during the chronic schedules had near maximal scores on the rotorod, while those given clonazepam alone or clonazepam plus nitrendipine during the chronic treatment had significantly lower scores. In neither case did the nitrendipine treatment have any effect.

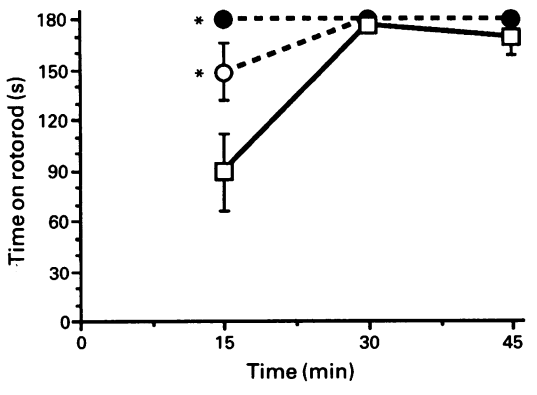

Figure 2 Lack of effect of chronic treatment with nitrendipine on tolerance to the ataxic actions of midazolam. The chronic treatments were given once daily for fourteen days. On the fifteenth day, 24 h after the last chronic treatment injections, all mice were given i.p. injections of midazolam, $2.5\,mg\,kg^{-1}$, then tested on the rotorod at 15 min intervals. The results are presented as mean and vertical lines indicate s.e.mean. Chronic treatments were as follows: (○) midazolam, $10\,mg\,kg^{-1}$ plus Tween vehicle; (●) midazolam, $10\,mg\,kg^{-1}$ plus nitrendipine $50\,mg\,kg^{-1}$; (□) saline plus Tween vehicle. * $P < 0.05$, compared with vehicle-treated controls.

Table 4 Tolerance to midazolam anaesthesia, after chronic treatment with midazolam alone or midazolam plus the peripheral benzodiazepine antagonist PK 11195

Chronic treatment	*Number of mice with loss of righting reflex after an accurate dose of midazolam 150 mg kg^{-1}*						
	5	*10*	*15*	*20*	*25*	*30*	*35 min*
*Midazolam	1/10	2/10	1/10	1/10	1/10	1/10	0/10
*Midazolam plus PK11195, 30 mg kg^{-1}	2/10	2/10	2/10	2/10	2/10	1/1	0.10
*Midazolam plus PK11195, 90 mg kg^{-1}	0/8	3/8	2/8	2/8	1/8	2/8	1/8
Vehicle	6/10	8/10	10/10	10/10	9/10	10/10	10/10

* $P < 0.05$, compared with vehicle-treated controls, at all times tested.

Lack of effect of nitrendipine on tolerance to midazolam anaesthesia

Nitrendipine, 10–50 mg kg^{-1}, did not affect the development of tolerance to midazolam anaesthesia. In the vehicle-treated control group midazolam, 150 mg kg^{-1}, produced loss of righting reflex in nearly all mice. The groups that had received four days chronic treatment with midazolam only and midazolam plus nitrendipine, 50 mg kg^{-1}, showed significantly less ($P < 0.05$) loss of righting reflex at all times tested, compared with controls (Figure 3). The nitrendipine-treated groups did not differ from vehicle-treated controls. The same pattern was seen when nitrendipine was used at 10 or 25 mg kg^{-1} (data not shown).

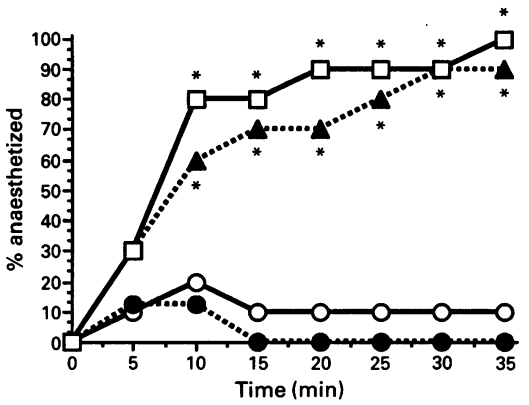

Figure 3 Lack of effect of nitrendipine, 50 mg kg^{-1}, on tolerance to midazolam anaesthesia, as measured by loss of righting reflex. All animals were given an acute dose of midazolam, 150 mg kg^{-1}, 24 h after the end of four days chronic treatment. Chronic treatments were as follows: (○) midazolam, 135 mg kg^{-1} plus Tween vehicle; (●) midazolam, 135 mg kg^{-1} plus nitrendipine 50 mg kg^{-1}; (▲) nitrendipine 50 mg kg^{-1} plus saline; (□) Tween vehicle plus saline (controls). * $P < 0.05$, compared with vehicle-treated controls. No significant differences were seen between results from groups that received midazolam alone or midazolam plus nitrendipine, or those given nitrendipine alone or vehicles, during the chronic treatments.

Table 5 Dihydropyridine binding, measured 24 h after the last of seven daily doses of flurazepam, with and without nitrendipine

A Whole brain			
Chronic treatment	K_d (nM)	B_{max} (fmol mg^{-1} protein)	
Flurazepam 40 mg kg^{-1}	2.86 ± 0.65	241 ± 23	(5)
Flurazepam 40 mg kg^{-1} plus nitrendipine 50 mg kg^{-1}	2.01 ± 0.17	244 ± 15	(5)
Nitrendipine 50 mg kg^{-1}	2.35 ± 0.51	239 ± 33	(5)
Vehicle	2.07 ± 0.24	234 ± 18	(5)
B Cerebral cortex			
Chronic treatment	K_d (nM)	B_{max} (fmol mg^{-1} protein)	
Flurazepam 40 mg kg^{-1}	1.33 ± 0.69	301 ± 56	(8)
Vehicle	1.79 ± 1.29	346 ± 95	(8)

No significant differences between any of the groups, $P > 0.05$.

The lack of effect of the peripheral benzodiazepine antagonist PK11195, at 30 or 90 mg kg^{-1}, on the tolerance to the anaesthetic action of midazolam is shown in Table 4. Tolerance to midazolam anaesthesia was clearly evident after four days treatment with midazolam alone. Concurrent treatment with either dose of PK11195 did not affect the development of midazolam tolerance.

Lack of effect of chronic benzodiazepine treatment on dihydropyridine binding

No significant differences were seen between the K_d or B_{max} values for any of the groups after the chronic treatments, $P > 0.05$ (Table 5). The same results were obtained when either whole brains or cerebral cortices were used for the binding studies.

Discussion

The doses of dihydropyridines used in the present study were the same as those that have been found to be effective in our studies on ethanol tolerance and dependence (Dolin & Little, 1987, 1989a; Dolin *et al.*, 1987b; Whittington & Little, 1988; Littleton *et al.*, 1990) and on nitrous oxide tolerance and withdrawal (Dolin & Little, 1989b). They were in the same range as those found by other authors to be necessary for other central nervous system actions (e.g. Hoffmeister *et al.*, 1982) and for displacement of radiolabelled dihydropyridines from brain *in vivo* (Supervilai & Karobath, 1984). The central concentrations of nitrendipine achieved after the doses used in the present study were in the low micromolar range (Dolin *et al.*, 1986; Dolin, 1988). It has been suggested that dihydropyridines do not enter the CNS well, but this is untrue, as they are highly lipid soluble compounds that pass the blood brain barrier easily. It is well established that the concentrations required to affect CNS tissues *in vitro* are within this order of magnitude.

The effects of FG7142 in the present study reflect changes caused or unmasked by withdrawal from chronic benzodiazepine treatment. Our chronic treatment schedules produced tolerance and withdrawal changes, but we have not studied self-administration of benzodiazepines. FG7142 was given at a time when there was residual benzodiazepine activity still present in the brain. At 24 h after the last flurazepam injection, we found no evidence of residual flurazepam or metabolites, but there was some occupation of benzodiazepine receptors (Little *et al.*, 1987). Some of the increased action of FG7142 was therefore likely to be due to precipitated withdrawal, with competition between FG7142 and metabolites of flurazepam.

However, the increased action of FG7142 can also be demonstrated at 48 h after the last of our chronic flurazepam injections, when we found no evidence of residual benzodiazepine activity, by either of the above methods (Little *et al.*, 1987). At this time, therefore, the seizures induced by FG7142 reflect alterations, caused by the chronic treatment, unmasked by the withdrawal from such treatment.

When nitrendipine was given chronically, with the benzodiazepine treatment, it did not affect the seizures produced by the partial inverse agonist, FG7142, during withdrawal or tolerance to the benzodiazepines. After seven days treatment with flurazepam there was no increase in the number of central dihydropyridine binding sites, as was seen after chronic ethanol or chronic morphine treatment (Dolin *et al.*, 1987b; Ramkumar & El-Fakahany, 1984). The results suggest that neuronal calcium channels do not play a large part in the development of changes during long-term administration of benzodiazepines, in contrast to their roles in the adaptations to chronic ethanol and opiate treatment.

However, nitrendipine, given acutely, produced a dose-dependent decrease in seizure incidence, when the seizures were precipitated by FG7142, during withdrawal from chronic flurazepam treatment. Nitrendipine did not reduce the seizure thresholds or seizure incidence following administration of the full inverse agonist, DMCM, or the $GABA_A$-antagonist, bicuculline, indicating that it does not have a general anticonvulsant action against seizures due to decreases in GABA transmission. These findings confirm our earlier observations that the anticonvulsant effects of nitrendipine are selective for drug withdrawal seizures (Dolin *et al.*, 1988).

We suggest that the effect of nitrendipine on the FG7142 induced seizures may have been due to an action on the mechanism(s) responsible for the increased effect of FG7142 and that this mechanism might involve neuronal calcium channels. Recent, *in vitro* evidence showed that the increased effect of FG7142 was a neuronal change, as the action of FG7142 on extracellular field potentials in the hippocampus was increased by chronic flurazepam treatment (Little *et al.*, 1989). These alterations have been suggested to occur at the GABA-benzodiazepine receptor ionophore complex (Lewin *et al.*, 1989). However, the protective effect of nitrendipine, seen in the present study, suggests that this is not the whole story, and that alterations in calcium fluxes or in the state of the calcium channels may be involved in the neuronal adaptation to chronic benzodiazepine treatment. One possibility is that the benzodiazepine might increase the inactivation of voltage-sensitive calcium channels, as the dihydropyridines have higher affinity for the inactivated form (Bean, 1984). However, in view of the variability in the effects of benzodiazepines on calcium currents (see Introduction), we need to know the changes in calcium fluxes after the treatments used in the present study before such ideas can advance beyond the stage of speculation.

The development of tolerance to midazolam anaesthesia was unaffected by PK11195. In addition, tolerance developed to the ataxic effects of clonazepam, which is known to act only at the benzodiazepine-GABA-chloride channel complex (Rampe & Triggle, 1986). It would seem that the peripheral benzodiazepine site is not involved in the development of tolerance to benzodiazepines.

It would appear that the calcium channel antagonists may be useful drugs for reducing the incidence of seizures during benzodiazepine withdrawal. However, the development of tolerance to and physical withdrawal from benzodiazepines does not appear to involve dihydropyridine-sensitive calcium channels in brain, in contrast to tolerance and withdrawal with ethanol and opiates.

We thank the Wellcome Trust, the Mental Health Foundation and the East Anglian Health Authority for financial assistance with this work and Bayer AG for gifts of nitrendipine. H.J.L. is a Wellcome Trust Senior Lecturer.

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(Received November 29, 1989
Revised June 29, 1990
Accepted July 11, 1990)

Br. J. Pharmacol. (1990), **101**, 698–702

Inhibitory effect of 5-hydroxytryptamine on penile erectile function in the rat

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1 An increase in corporal pressure was elicited in pithed rats by stimulation of the sacral part of the spinal cord. This response was inhibited by intravenous injection of 5-hydroxytryptamine (5-HT) ($ED_{50} = 28.5 \pm 2.2\,\mu g\,kg^{-1}$).

2 The inhibitory effect of 5-HT was blocked by methysergide and methiothepin (each $1\,mg\,kg^{-1}$), but not by ketanserin ($0.02\,mg\,kg^{-1}$), MDL 72222 ($1\,mg\,kg^{-1}$) or prazosin ($0.1\,mg\,kg^{-1}$).

3 An inhibitory effect on the corporal pressure response to spinal stimulation was also produced by 5-carboxyamidotryptamine ($ED_{50} = 5.6 \pm 2.8\,\mu g\,kg^{-1}$), but not by m-chlorophenylpiperazine (mCPP), RU 24969, 8-hydroxy-2-[di-n-propyl-amino]-tetralin (8-OH-DPAT) or fenfluramine (doses up to 1–2 $mg\,kg^{-1}$).

4 Neither methiothepin ($1\,mg\,kg^{-1}$) nor clomipramine ($1\,mg\,kg^{-1}$) had any effect on the frequency-response curve for increase in corporal pressure by spinal stimulation.

5 The results indicate that 5-HT exerts an inhibitory action on penile erection by a peripheral mechanism. This effect may be mediated by vasoconstriction in cavernosal vessels, or inhibition of release of a vasodilator neurotransmitter. From the spectrum of agonist and antagonist responses, the receptor involved may be of the $5\text{-}HT_{1D}$ subtype.

Introduction

Penile erection is mediated by coordinated function of the peripheral autonomic nerves following appropriate CNS stimuli. Activation of the pelvic parasympathetic nerve, together with reduction in sympathetic tone, results in relaxation of the helicine arterioles and of trabecular smooth muscle tissue in the corpora cavernosa, permitting inflow of blood, and filling of the corporal lacunae. Subsequently, increased venous outflow resistance leads to turgor of the corpora and penile rigidity (Lue & Tanagho, 1987; Krane *et al.*, 1989). Drugs and autacoids may therefore cause inhibition of erection by vasoconstriction of the helicine arterioles supplying the lacunar spaces or contraction of the trabecular smooth muscle surrounding the lacunae, as well as by inhibition of neurotransmitter release.

5-Hydroxytryptamine (5-HT) could potentially affect the erectile process by both central and peripheral actions. In rats, 5-HT receptor agonists induce penile erections, with a spectrum of agonist and antagonist effects indicative of a $5\text{-}HT_{1B}$-receptor mediation (Berendsen & Broekkamp, 1987). The 5-HT receptor agonist m-chlorophenylpiperazine (mCPP) was found to stimulate electrophysiological activity along the cavernous nerve, by an action within the spinal cord (Steers & de Groat, 1989).

Other observations indicate an inhibitory effect of 5-HT on erection. Administration of 5-methoxydimethyltryptamine inhibits the spinal reflex erectile response to tactile stimulation (Mas *et al.*, 1985). Direct injection of 5-HT markedly increases mount and intromission latency in male rats (Gonzales *et al.*, 1982).

Research on the mechanisms whereby drugs can affect the erectile process has been hampered by the lack of a suitable small animal model. Recently, we have described the development of such a model system in the pithed rat, for studying the action of drugs on the peripheral arc of the erectile process (Vardi *et al.*, 1989). In this model, we found that 5-HT produces a profound inhibition of the erectile stimulation, and present here the characteristics of this effect and its possible receptor mediation. A preliminary account of these observations has been communicated to the British Pharmacological Society (Finberg & Vardi, 1989b).

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Methods

Pithed rat preparation

Corporal pressure response to spinal nerve root stimulation was determined essentially as described previously (Vardi *et al.*, 1989). Rats were pithed under halothane (3% in oxygen) anaesthesia, by use of a hollow trochar. Systemic blood pressure was measured from a carotid artery, and drugs were administered via a jugular vein cannula. (+)-Tubocurarine ($1\,mg\,kg^{-1}$) was injected i.v. to prevent skeletal muscle contraction. An insulated stimulating electrode was passed down the spinal column to the level L5-S2, and a second electrode inserted under the skin of the back. The skin covering the penis was removed, a fine (26 gauge) needle was inserted into one corpus cavernosum, and connected to a pressure transducer (Statham p23Db) via a saline-filled polyethylene tube. Square wave pulses (50 V, 1 ms duration) were applied to the spinal electrode with a Grass 44B stimulator, and the position of the electrode adjusted for optimal corporal pressure response.

Determination of the effect of 5-HT, and of 5-HT receptor agonists and antagonists

For determination of the effect of 5-HT, electrical stimulation was applied at 15 Hz for 30 s, with 2–3 min between successive stimulation periods. Following 3 or 4 control stimuli, 5-HT or the agonist drug was administered i.v. and stimulation given 15 s later. Following return of blood pressure to control levels, increasing doses of 5-HT were administered, with stimulation repeated at 2–3 min intervals. The effect of other 5-HT receptor agonists was examined similarly, except that stimulation was given 30 s to 2 min after drug injection. Antagonists were administered i.v. in the doses indicated, immediately after initial determination of control response to stimulation, and further control stimuli given 10 min later. A dose-response curve to 5-HT was then determined as described above. In

separate experiments, the effect of methiothepin and clomipramine was studied on the frequency-response curve for spinal stimulation. Stimulation was applied for 1 min at 1, 2, 5, 10 and 20 Hz, with 3 min between each stimulus. Methiothepin or clomipramine (each 1 mg kg^{-1} i.v.) was administered, and responses to each frequency of stimulation redetermined.

Calculation of response

Mean arterial blood pressure (MAP) was calculated by use of the formula: MAP = diastolic pressure + 1/3 pulse pressure. Blood pressure was measured 45 s after injection of 5-HT, at the time of peak corporal response to stimulation, so the pressor responses to 5-HT described here are less than the peak pressure response. Since the value of corporal pressure achieved during spinal nerve stimulation (CP) varies with the level of systemic arterial pressure, corporal pressure response to spinal stimulation (CPR) was calculated as a fraction of simultaneous MAP by the formula: CPR = CP/MAP. Following administration of 5-HT receptor agonists, CPR was expressed as a percentage of the control response, obtained before commencement of agonist treatment.

Statistical analysis

Data are presented as arithmetic means ± s.e.mean. Significance of difference between means was calculated by Student's *t* test (probability level for significance <0.05).

Drugs

We are indebted to the following companies for samples of various compounds: methiothepin maleate (Roche); methysergide maleate (Sandoz); 1αH, 3αH, 5αH-tropan-3-yl 3,5-dichlorobenzoate methane sulphonate (MDL 72222; Merrell-Dow); ketanserin tartrate (Janssen); 5-carboxyamidotryptamine maleate (5-CT; Glaxo); 5-methoxy, 3(1,2,3,6-tetrahydro,4-pyridinyl) indole succinate (RU 24969; Roussel). 5-Hydroxytryptamine creatinine sulphate (doses refer to the base), clomipramine and fenfluramine were purchased from Sigma, and mCPP from Research Biochemicals Inc. (+)-Tubocurarine was purchased from Taro, Tel Aviv, Israel.

Results

The effects of 5-HT and 5-HT receptor agonists were studied at maximal frequency of stimulation (15 Hz). At this frequency, control CPR averaged 0.65 ± 0.032 ($n = 20$). Control MAP averaged 57.3 ± 1.8 mmHg at the start of the experiment, and did not change significantly as a result of the spinal stimulation. Resting corporal pressure averaged 6.4 ± 0.8 mmHg. Repeated application of spinal stimuli (15 Hz) in control experiments without injection of drugs produces consistent corporal pressure responses over the course of 1 h (Vardi *et al.*, 1989).

Intravenous injection of 5-HT produced an initial pressor followed by a depressor response, as described by Fozard & Leach (1968). The resting level of corporal pressure was not changed by 5-HT, except for an occasional small transient increase coincident with the pressor response to the higher doses (Figure 1). The CPR, however, was strongly inhibited, as shown in the example in Figure 1. Following the pressor response to 5-HT, blood pressure returned to control levels before full recovery of corporal response. After doses of 5-HT higher than 64 μg kg^{-1}, corporal response occasionally remained suppressed for more than 30 min, but normally recovered by 2–5 min after injection. The dose-response relationship of the 5-HT inhibitory effect is shown in Figure 2. The ED_{50} for inhibition of corporal response was

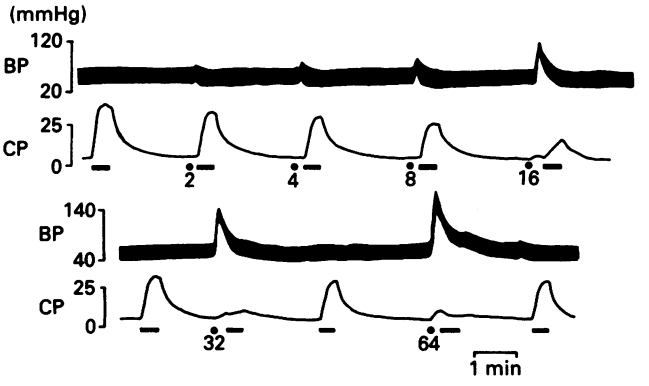

Figure 1 Suppression of corporal response to spinal stimulation by 5-hydroxytryptamine (5-HT) in a pithed rat. Responses of carotid arterial blood pressure (BP) and corporal pressure (CP) to i.v. injection of 5-HT (●) at doses shown (μg kg^{-1}). Spinal stimulation (15 Hz) applied at periods shown by horizontal bars.

28.5 ± 2.2 μg kg^{-1}, and a dose of 96 μg kg^{-1} produced complete inhibition of the CPR.

The modification of the 5-HT response by various 5-HT receptor antagonists is shown in Figure 2. Methysergide

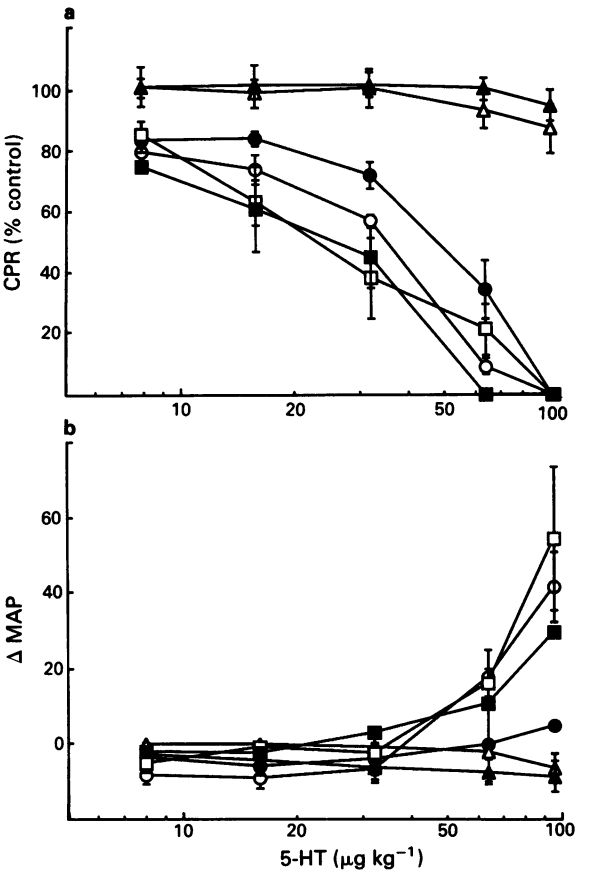

Figure 2 Effects of 5-hydroxytryptamine (5-HT) receptor antagonists and prazosin on 5-HT-induced inhibition of corporal response to spinal stimulation in the pithed rat. (a) Corporal pressure response to spinal stimulation at 15 Hz (CPR), and (b) increment in mean arterial blood pressure (MAP), following i.v. injection of 5-HT (doses shown on abscissa scale) in pithed rats. Corporal pressure response during spinal stimulation (CPR) was calculated as described in Methods, and expressed as a percentage of the control value at the start of the experiment. Blood pressure increment following 5-HT injection was measured at the time of spinal stimulation, and does not represent the peak response to 5-HT. Groups of animals were treated with methysergide (△) 1 mg kg^{-1} ($n = 5$), methiothepin (▲) 1 mg kg^{-1} ($n = 5$), ketanserin (●) 0.02 mg kg^{-1} ($n = 5$), MDL 72222 (□) 1 mg kg^{-1} ($n = 4$), prazosin (■) 0.1 mg kg^{-1} ($n = 4$) or saline (○; $n = 5$) before determination of response to 5-HT. Vertical bars show s.e.mean.

(1 mg kg^{-1}) and methiothepin (1 mg kg^{-1}) blocked both the pressor and the corporal inhibitory response to 5-HT. Ketanserin (0.02 mg kg^{-1}) blocked the pressor response but had no significant effect on the corporal inhibitory response, while MDL 72222 (1 mg kg^{-1}) had no effect on either pressor or corporal inhibitory responses to 5-HT. None of the above antagonists themselves had any significant effect on the corporal response to spinal stimulation, at 15 Hz. Control values of CPR in rats treated with ketanserin, methysergide and MDL 72222 were respectively: 0.72 ± 0.01, 0.64 ± 0.08 and 0.61 ± 0.08 ($n = 5$–6). In addition, the effects of methiothepin and clomipramine (both 1 mg kg^{-1}) were studied on the frequency-response curve to spinal stimulation (Figure 3). Neither substance had an effect on CPR at stimulation rates between 1 and 20 Hz. Prazosin (0.1 mg kg^{-1}) had no significant effect on the pressor or corporal inhibitory response to 5-HT (Figure 2). Control CPR following prazosin was 0.73 ± 0.05 ($n = 4$; not significant).

An inhibition of the corporal response to spinal stimulation was also produced by the 5-HT$_1$ receptor agonist 5-carboxyamido-tryptamine (5-CT; see Figures 4 and 5), which caused a pure depressor response, as described by Docherty (1988). Doses of 5-CT above 0.05 μg kg^{-1} produced a detectable inhibition of CPR, while the ED_{50} for inhibition of corporal response was 5.6 ± 2.8 μg kg^{-1}. 5-CT was, therefore, 5.1 times more potent than 5-HT in its effect on the corpora cavernosa, but did not consistently produce complete inhibition of the corporal response at higher doses, in contrast to the observation with 5-HT. The time course for suppression of CPR by 5-CT wsa similar to that for reduction in arterial pressure. After the higher doses of 5-CT, blood pressure and CPR were reduced for more than 60 min (see Figure 5). Administration of methiothepin restored both blood pressure and CPR to control levels, when these were depressed subsequent to 5-CT administration (see Figure 5).

The effects of other 5-HT receptor agonists, or releasers, are shown in Table 1. Neither 8-hydroxy-2-[di-n-propyl-amino] tetralin (8-OH-DPAT), mCPP, RU 24969 nor fenfluramine had any effect on the corporal response to stimulation (15 Hz) at doses of up to 1–2 mg kg^{-1}.

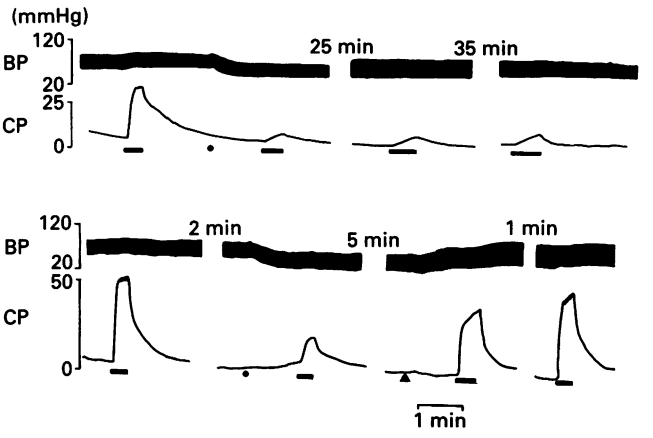

Figure 5 Carotid arterial pressure (BP) and corporal pressure (CP) records from two separate pithed rat experiments. Suppression of corporal pressure response to spinal stimulation (15 Hz, applied at period shown by horizontal bars) and reduction of BP following i.v. injection of 5-carboxyamidotryptamine (5-CT, ●, 50 μg kg^{-1}). In the lower record, methiothepin (3 mg kg^{-1}) was injected at point indicated (▲). Traces broken for periods shown (in min).

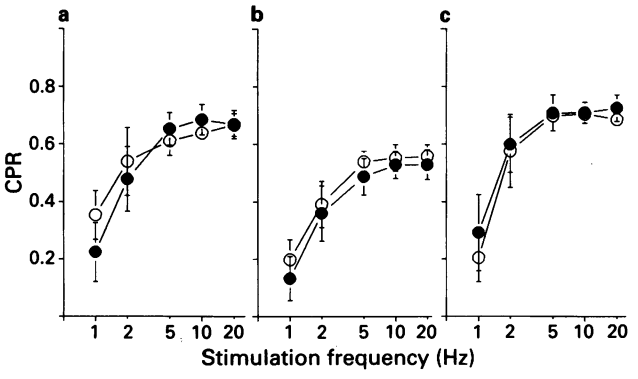

Figure 3 Corporal pressure response (CPR) at different frequencies of spinal stimulation in pithed rats, before (○) and after (●) injection of saline (a; $n = 6$), clomipramine 1 mg kg^{-1} (b; $n = 7$) and methiothepin 1 mg kg^{-1} (c; $n = 6$). Vertical bars show s.e.mean.

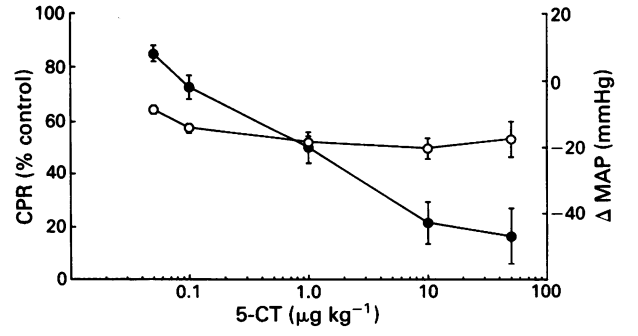

Figure 4 Inhibition of corporal pressure response to spinal stimulation at 15 Hz (CPR; ●), and reduction in mean arterial blood pressure (△ MAP; ○) by 5-carboxyamidotryptamine (5-CT) in pithed rats. CPR expressed as percentage of control value before administration of 5-CT. Blood pressure and corporal pressure responses were measured 1–2 min after injection of 5-CT. Vertical bars show s.e.mean of 10 experiments.

Discussion

The increased corporal pressure following spinal stimulation in the pithed rat preparation is presumed to be the result of vasodilatation in corporal arterioles. The value of CPR attained during spinal stimulation indicates the relative degree of vasoconstriction in corporal blood vessels in relation to the general peripheral resistance. If a vasoconstrictor agent produces an equal degree of vasoconstriction in corporal vessels as in the general circulation, then blood pressure and corporal

Table 1 Effect of 5-hydroxytryptamine (5-HT) agonists and antagonists on blood pressure and corporal response in pithed rat

Drug	n	*Dose*	*ΔMAP* (mmHg)	*CPR* (% control)
8-OH-DPAT	4	1	+9 ± 5	103 ± 7
		2	+24 ± 5	100 ± 10
mCPP	4	0.1	+13 ± 1	97 ± 6
		0.5	+22 ± 7	92 ± 7
		1	+35 ± 12	96 ± 10
RU-24969	3	1	+20 ± 1	98 ± 9
		2	+25 ± 6	91 ± 12
Fenfluramine	3	0.5	−7 ± 2	109 ± 4
		1	−5 ± 9	100 ± 10

Doses of agonists (i.v.) shown in mg kg^{-1}. Data presented refer to change in mean arterial blood pressure (ΔMAP) and corporal response to spinal stimulation (CPR) ± s.e.mean (n = number of experiments). 8-OH-DPAT = 8-hydroxy-2-[di-n-propyl-amino]tetralin and mCPP = m-chlorophenyl-piperazine.

pressure will increase to a similar degree, and the CPR will remain constant. On the other hand, if corporal vessels are constricted to a greater degree than the remaining vasculature, the CPR will decrease, while a selective vasodilatation in corporal vessels will lead to an increase in the ratio. The fall in CPR produced by 5-HT is, therefore, indicative of a selective vasoconstrictor effect of 5-HT in the corporal vasculature. The profound inhibitory action of 5-HT on the CPR may be effected by either a direct vasoconstrictor action on corporal arterioles, counteracting the neuronally mediated vasodilatation, or by a presynaptic action to inhibit the release of the vasodilatator transmitter. Further experiments will be required to elucidate which mechanism is involved in the response. The 5-HT-induced suppression of CPR is unlikely to be mediated by release of noradrenaline acting at postsynaptic α_1-adrenoceptors, since the 5-HT response was not modified by prazosin. Consistent with this, we have observed that α_1-adrenoceptor agonists are much less effective than 5-HT in inhibiting the corporal response to spinal stimulation, and are incapable of producing complete inhibition when given i.v. (Finberg & Vardi, 1989a).

The fact that the 5-HT inhibitory response on the corporal pressure was blocked by methysergide and methiothepin, but not by ketanserin or MDL 72222, indicates that the receptor involved belongs to the 5-HT_1-like category (Bradley *et al.*, 1986). From radioactive ligand binding site analysis, 8-OH-DPAT has been classed as selective for 5-HT_{1A} sites, RU 24969 for 5HT_{1A} and 5-HT_{1B} sites, mCPP for 5-HT_{1B}, 5-HT_{1C} and 5-HT_2 sites and 5-CT for 5-HT_{1A}, 5-HT_{1B}, and 5-HT_{1D} sites (Peroutka, 1988; Hoyer *et al.*, 1985; Engel *et al.*, 1986; Bagdy *et al.*, 1989). The lack of effect of 8-OH-DPAT, RU 24969 and mCPP, together with the effectiveness of 5-CT in inhibiting CPR, is therefore indicative of an action at a 5-HT_{1D} receptor type.

Since mCPP did not produce an increase in cavernosal pressure in the pithed rat as it did in intact animals (Steers & de Groat, 1989), it appears that the neuronal elements mediating this stimulating action are destroyed by the pithing process. The lack of modification of the CPR by methiothepin, the 5-HT releasing agent fenfluramine, or the 5-HT uptake blocker clomipramine, is indicative of an absence of 5-HT-containing nerves in the corpora cavernosa. Alternatively, if such nerves do exist, they may not be activated by the spinal stimulation.

The importance of the present finding for human physiology, is the possibility that 5-HT released from platelets or nerves could activate inhibitory receptors and suppress erection. Recently, evidence has been produced that 5-HT exists in and/or can be released from coronary, cerebral and mesenteric arteries of rats and rabbits (Griffith *et al.*, 1982; Cohen, 1985; Gale & Cowen, 1988; Kawasaki *et al.*, 1989). Evidence for 5-HT receptor-mediated vasoconstriction in human skin has recently been published by Coffman & Cohen (1988). If 5-HT exerts an appreciable corporal vasoconstriction in man, then enhanced release of 5-HT as a result of vascular or neuronal pathology, could play a role in some cases of human erectile disorders. In this regard, it is interesting that in a number of cases of carcinoid syndrome, complete erectile impotence has also been reported (Feldman *et al.*, 1974). Plasma levels of 5-HT are elevated in diabetic patients (Barradas *et al.*, 1988), so that 5-HT may be a contributing factor in some cases of impotence in these patients. During preparation of this article, a study was published by Kirkeby *et al.* (1989), describing the potent ($pD_2 = 6.9$) contractile property of 5-HT in isolated smooth muscle strips prepared from human corpora cavernosa, as we have also observed in preliminary experiments. 5-HT may, therefore, also inhibit erectile function by an action on the corporal smooth muscle.

The present work indicates a suppressant effect of 5-HT on erection mediated by a peripheral action. The effect of 5-HT receptor agonists and antagonists in the whole animal, therefore will be complex, and the outcome will depend on which effect predominates, i.e. inhibition at the peripheral site, or activation and inhibition at sites in the spinal cord and brain. The final effect produced will also depend on the receptor type involved in each action.

We wish to thank Ilana Spanir and Shulamit Levy for skilled technical assistance. This work was supported by grants from the Office of the Chief Scientist, Israel Ministry of Health, and the A & E Blum Medical Research Fund (Office of the Vice-President for Research, Technion).

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(Received March 19, 1990
Revised June 18, 1990
Accepted June 25, 1990)

Br. J. Pharmacol. (1990), **101**, 703–709

Interleukin-1 potentiates histamine-induced release of prostacyclin from human endothelial cells

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1 In human cultured umbilical vein endothelial cells, interleukin-1 potentiated histamine-induced release of prostacyclin in a time- and concentration-dependent manner.

2 In cells incubated with interleukin-1 for 24 h, maximal potentiation was observed when cells were pre-incubated with 0.5 u ml^{-1} interleukin-1 before stimulation with histamine (1 μM–1 mM).

3 In cells incubated with 0.5 u ml^{-1} interleukin-1, 20 min pre-incubation was sufficient to induce a statistically significant potentiation of prostacyclin release induced by 1 μM histamine ($P < 0.05$).

4 Nifedipine but not cycloheximide, significantly ($P < 0.05$) inhibited histamine-induced release of prostacyclin and interleukin-1 potentiation of histamine-induced release of prostacyclin ($P < 0.05$).

5 Incubation with 1 u ml^{-1} interleukin-1 induced a two fold increase in cellular prostaglandin synthetase activity within 30 min. The enzyme activity increased up to 6 h and was maintained up to 24 h. In cells co-incubated with cycloheximide and 1 u ml^{-1} interleukin 1, prostaglandin synthetase activity at 24 h was the same as that in unstimulated cells. Prostacyclin release was not significantly inhibited in cells co-incubated with cycloheximide and interleukin 1.

6 These results suggest that interleukin-1 potentiates histamine-induced release of prostacyclin by rapid up-regulation of prostaglandin synthetase activity as well as by inducing synthesis of enzyme protein. These mechanisms may act to potentiate/regulate vascular endothelial responses in inflammatory reactions.

Introduction

Interleukin-1 (IL-1) and histamine are both able to induce release of prostaglandin from vascular endothelial cells; however, their mechanisms of activation are quite different. Histamine induces an immediate release of prostacyclin (PGI_2) from human umbilical vein endothelial cells (HUVEC), which is maximal within 30 min and inhibited by the H_1 receptor antagonist, pyrilamine (Baenziger *et al.*, 1981; Bull *et al.*, 1989). In contrast, release of PGI_2 from HUVEC incubated with human recombinant interleukin 1 α (hrIL-1α) is characterized by a delayed onset of 4–6 h but is then continuous up to at least 48 h, so long as the agonist is present (Rustin *et al.*, 1989).

Mechanisms regulating receptor-mediated prostaglandin synthesis by endothelial cells are poorly understood, however, the actions of IL-1 and histamine indicate that these events are mediated by at least two pathways, one involving rapid stimulation of pre-existing enzymes and release of PGI_2 and the other characterized by slow activation and sustained PGI_2 release.

Two key reactions mediating cellular synthesis of prostaglandins are mobilization of arachidonic acid from membrane phospholipids, catalysed by phospholipases A_2 and C and conversion of arachidonic acid into the prostaglandin endoperoxides catalysed by the cyclo-oxygenase/peroxidase enzyme system.

Experiments on human dermal fibroblasts have indicated that IL-1-induced release of prostaglandin E_2 (PGE_2) is mediated primarily, if not solely, via induction of cyclo-oxygenase synthesis, in a manner that is concentration- and time-dependent (Raz *et al.*, 1988).

Fibroblasts do not release PGI_2 and do not respond well to agonists known to induce immediate release of PGI_2 from HUVEC, such as histamine or thrombin; however, prior exposure of fibroblasts to the supernatant from stimulated monocytes, which contains IL-1 activity, potentiates release of PGE_2 induced by the pro-inflammatory agonist bradykinin. This potentiation was, at least partially, inhibited by cycloheximide and actinomycin D, suggesting that IL-1 had increased the availability of cyclo-oxygenase enzyme by inducing its synthesis (Whiteley & Needleman, 1984). Similar results have recently been reported in human keratinocytes, which do not release prostaglandins in response to histamine alone but following incubation with IL-1, release PGE_2 in response to histamine (Pentland & Mahoney, 1988).

In the above reports of IL-1 potentiation of agonist-induced release of PGE_2, the cells were pre-treated with IL-1 for at least 12 h before stimulation and although the results indicate that the effect of IL-1 may be mediated by increased synthesis of cyclo-oxygenase enzyme they do not address the question of whether or not IL-1 is able to increase the immediate availability of cyclo-oxygenase or modulate release of prostaglandins by alternate mechanisms.

In addition to release of PGI_2, histamine and IL-1 also induce specific responses in endothelial cells. IL-1 potentiates neutrophil and monocyte adherence (Bevilacqua *et al.*, 1985), and expression of surface antigens (Pober *et al.*, 1986), while histamine potentiates plasma extravasation and leukocyte margination (Beaven, 1976). These individual functions, as well as the different time-courses of PGI_2 release, suggest that IL-1 and histamine may act interdependently to promote or regulate vascular inflammatory responses.

We have therefore sought to determine whether histamine and IL-1 interact to regulate release of PGI_2 from human endothelial cells.

Methods

Endothelial cell culture

Human endothelial cells were harvested from umbilical cord veins and cultured as originally described by Jaffe *et al.* (1973). Umbilical cords were collected immediately following delivery and placed in Hanks balanced salt solution (HBSS). Only untraumatized sections were used. The vein was cannulated and flushed with sterile HBSS until the effluent ran clear

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of red blood cells. At this stage, collagenase (type 1 CLS *Clostridium histolyticum*) (0.1% w/v) was prepared in HBSS. The vein was filled with collagenase solution, both ends sealed and the cord was incubated for 20 min at 37°C. At the end of the incubation period, the contents of the vein were flushed out with culture medium 199 (M199) containing 100 u ml^{-1} penicillin, 100 $\mu g\,mg^{-1}$ streptomycin, 50 $\mu g\,ml^{-1}$ amphotericin B and 2 mM glutamine. The cells were centrifuged at 200*g* for 5 min, resuspended in complete culture medium consisting of M199 supplemented with 20% v/v pooled human serum and plated out in 24 well culture dishes. The endothelial cell nature of the cultured cells was confirmed by positive staining for von Willebrand Factor protein (Wagner *et al.*, 1982).

In these experiments primary cultures were used and each experiment was performed with cells cultured from a different umbilical cord.

Agonist-induced release of prostacyclin from endothelial cells

Confluent monolayers of endothelial cells were washed once with M199 alone. Each well of endothelial cells was then overlayed with 500 μl of M199 containing hrIL-1α at concentrations of 0.05 u ml^{-1} to 5.0 u ml^{-1}. The cells were incubated at 37°C in 5% CO_2/95% humidified air for 24 h and then histamine was added to give a final concentration (f/c) of 0.1 μM to 1 mM. The incubations were continued for a further 30 min and then the supernatants were removed from individual wells and frozen at −20°C for subsequent radioimmunoassay of 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$). At the end of the experiment, the endothelial cells were trypsinized (0.25% w/v trypsin in calcium- and magnesium-free HBSS) and counted manually in an improved Neubauer Chamber (Weber, Lancing). Concentrations of 6-keto-$PGF_{1\alpha}$ were expressed as ng per 10^4 cells.

Separate experiments were undertaken to determine whether prolonged pre-incubation with hrIL-1α was required to potentiate histamine-induced release of PGI_2. Endothelial cells were incubated concomitantly with hrIL-1α and histamine for 30 min or pre-incubated for varying lengths of time with hrIl-1α before being stimulated with histamine for 30 min.

The effect of specific antagonists of prostacyclin release from endothelial cells

The concentrations of antagonists used in these experiments were not cytotoxic as they did not induce morphological change, as assessed by phase contrast microscopy and trypan blue exclusion. Furthermore, they did not stimulate PGI_2 release following 24 h incubation with cells alone.

Confluent monolayers of endothelial cells were washed with M199 and pre-incubated for 30 min at 37°C with 500 μl of M199 containing the different antagonists. Histamine was then added and the incubations continued for another 30 min or hrIL-1α was added and the incubations continued for up to 24 h. In separate experiments, histamine (1 μM f/c) was added for 30 min to cells pre-incubated with the antagonists and hrIL-1α. At the end of the experiments, the supernatant was removed and frozen at −20°C for subsequent radioimmunoassay for 6-keto-$PGF_{1\alpha}$.

Assay of prostaglandin synthetase enzyme activity

Confluent monolayers of endothelial cells, grown in 75 cm^2 culture flasks, were either incubated with hrIl-1α (1 u ml^{-1}) alone for up to 24 h or pre-incubated with cycloheximide (0.2 μM) for 30 min prior to the addition of hrIL-1α (1 u ml^{-1} f/c) for 24 h. At the end of the incubation, the supernatant was removed, the cells washed with HBSS and overlayed with 1 ml HBSS containing 10 mM diethyldithiocarbamic acid. The cells were lifted from the bottom of the culture flasks with a rubber policeman, collected into microfuge tubes and centrifuged (10,000*g*, 10 min). The supernatant was aspirated and the cell pellets frozen at −20°C.

Endothelial cell prostaglandin synthetase enzyme activity was assayed by measuring 6-keto-$PGF_{1\alpha}$ formation by the method described by Raz *et al.* (1988) with modifications.

The endothelial cell pellets were resuspended in solubilizing buffer (50 mM Tris, 1 mM diethyldithiocarbamic acid, 10 mM EDTA, 1% v/v Tween-20 (pH 8.0) containing 0.2 mg ml^{-1} α_2-macroglobulin), vortexed and sonicated and then centrifuged (10,000*g*, 10 min) to precipitate insoluble material. Prostaglandin synthetase activity was determined by adding an aliquot of solubilized cell supernatant to assay buffer (0.1 M NaCl, 0.02 M sodium borate, 0.015 M EDTA, 0.3 mM phenylethylsulphonyl fluoride, 0.5% w/v bovine serum albumin and 0.5% v/v Triton X100, pH 9.0) containing 1 mM arachidonic acid. The reaction was allowed to proceed for 60 min at 37°C and the incubation mixture assayed for 6-keto-$PGF_{1\alpha}$ by radioimmunoassay.

The protein content of the solubilized cell suspensions was determined by the Bradford (1976) protein assay. Bovine serum albumin prepared in the assay buffer was used as a standard. The amount of 6-keto-$PGF_{1\alpha}$ formed was expressed as pg 6-keto-$PGF_{1\alpha}$ per μg protein.

Prostacyclin assay

PGI_2 was measured using a radioimmunoassay for 6-keto-$PGF_{1\alpha}$ (the stable hydrolysis product of PGI_2) as previously described (Bull *et al.*, 1988). Briefly, each assay tube contained 50 μl of endothelial cell supernatant, 3,000 c.p.m. [^{3}H]-6-keto-$PGF_{1\alpha}$ in 50 μl assay buffer (0.07 M NaCl, 0.07 M phosphate, pH 7.4) and 50 μl of antiserum. Standard curves covered the range 5 pg up to 1 ng of 6-keto-$PGF_{1\alpha}$. The assay tubes were incubated overnight at 4°C and the bound tritiated 6-keto-$PGF_{1\alpha}$ was separated from unbound by dextran charcoal precipitation; 200 μl of supernatant was counted in 2 ml of Ecoscint scintillation fluid (National Diagnostics). The 6-keto-$PGF_{1\alpha}$ antiserum exhibited crossreactivity of less than 1% with other major prostaglandins of the 1 and 2 series and the limit of sensitivity of the assay was 5 pg. The inter-assay and intra-assay coefficients of variation for the 6-keto-$PGF_{1\alpha}$ radioimmunoassay were 7.56 ($n = 10$) and 3.88 ($n = 10$) respectively.

The cell supernatants were assayed without prior extraction and measurements were made at two dilutions.

Analysis of data

Statistical analysis of endothelial cell responses to hrIL-1α and histamine was by the Mann Whitney 'U' test and results considered to be significant if $P < 0.05$. Data from experiments in which specific antagonist effects were examined, was analysed by the Student's t test for paired data. $P < 0.05$ was considered to be significant.

Materials

Culture medium and antibiotics were obtained from Gibco, Paisley. Histamine, cycloheximide and arachidonic acid were obtained from Sigma Chemical Co., Poole. Collagenase was obtained from Worthington Biochemicals, Cambridge.

The 6-keto-prostaglandin $F_{1\alpha}$ antiserum was a gift from Dr M. Greaves, Royal Hallamshire Hospital, Sheffield. The standard was obtained from Cayman Chemicals, Ann Arbor, Michigan, U.S.A. and the tritiated compound was obtained from Amersham PLC.

Human recombinant interleukin 1α was a gift from Dr P. Lomedico, Hoffman La Roche Inc., Nutley, NJ, U.S.A. This preparation consisted of the carboxy terminal 154 amino acids of the 271 amino acid human IL-1 α precursor and was essentially pure protein with specific activity of 1.3×10^6 half maximal u ml^{-1}, 5×10^6 half maximal u mg^{-1} protein and

contained less than 3×10^{-5} u ml^{-1} endotoxin, as measured by the *Limulus* assay.

Results

Potentiation of histamine-induced release of prostacyclin by interleukin 1

Histamine-induced release of PGI_2 from confluent monolayers of HUVEC after 30 min incubation was concentration-dependent with threshold stimulation of less than 1 μM and maximal stimulation with 100 μM (Figure 1). PGI_2 release from HUVEC incubated with hrIL-1α for 24 h was also concentration-dependent with threshold stimulation of less than 0.1 u ml^{-1} and maximal stimulation with 5.0 u ml^{-1}. When HUVEC were pre-incubated with hrIL-1α for 24 h before stimulation with histamine for 30 min, there was a concentration-dependent potentiation of histamine-induced PGI_2 release. Significant potentiation occurred in HUVEC pre-incubated with 1 u ml^{-1} hrIL-1α prior to stimulation with 0.1 μM histamine ($P < 0.05$) (Table 1); however, 0.05 u ml^{-1} hrIL-1α was sufficient to induce significant potentiation of PGI_2 release by 1 μM histamine ($P < 0.05$). Maximal potentiation of histamine-induced PGI_2 release was observed in HUVEC pre-incubated with 0.5 u ml^{-1} hrIL-1α. In HUVEC pre-incubated with concentrations of hrIL-1α greater than 0.5 u ml^{-1} before being stimulated with histamine concentrations greater than 1 μM, release of PGI_2 was sub-maximal.

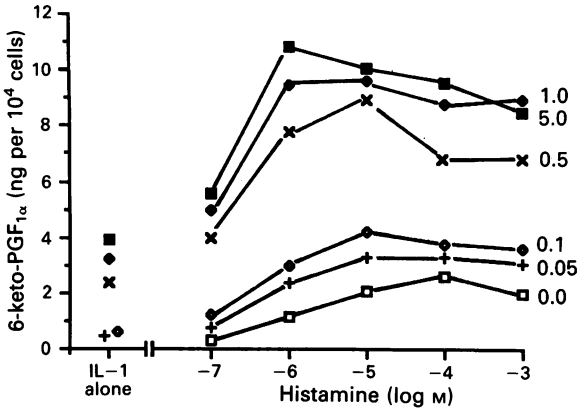

Figure 1 Human recombinant interleukin-1α (hrIL-1α) potentiation of histamine-induced release of prostacyclin (PGI_2) from human umbilical vein endothelial cells (HUVEC). HUVEC were incubated with hrIL-1α (0.05 to 5.0 u ml^{-1}) for 24 h and then stimulated with histamine (0.1 μM to 1 mM) for 30 min. In control cultures, HUVEC were incubated alone with hrIL-1α for 24 h (left hand column) or histamine for 30 min (□). This is a representative experiment which was repeated 4 times with similar results.

Mechanisms facilitating interleukin-1 potentiation of histamine induced release of prostacyclin

Effect of the antagonists Nifedipine (0.1 mM), significantly reduced PGI_2 release from HUVEC incubated with histamine (1 μM–100 μM) for 30 min ($P < 0.05$) (Figure 2a). In HUVEC incubated with hrIL-1α (0.1–1.0 u ml^{-1}) in the presence of nifedipine (0.1 mM) for 24 h, PGI_2 release was reduced but this did not reach significance (Figure 2b). hrIL-1α (0.1–1.0 u ml^{-1}) significantly potentiated release of PGI_2 induced by 1 μM histamine ($P < 0.05$) and this potentiation was completely abolished by 0.1 mM nifedipine (Figure 2c).

Cycloheximide (0.2 μM) did not inhibit release of PGI_2 from HUVEC incubated with histamine (1–100 μM) for 30 min (Figure 3a). In HUVEC co-incubated with the highest concentration of hrIL-1α (1.0 u ml^{-1}) and cycloheximide for 24 h, PGI_2 release was reduced but not significantly so (Figure 3b). hrIL-1α (0.1–1.0 u ml^{-1}) significantly potentiated release of PGI_2 induced by histamine (1 μM) ($P < 0.05$), and in HUVEC pre-incubated with cycloheximide (0.2 μM) and hrIL-1α (1.0 u ml^{-1}), release of PGI_2 induced by 1 μM histamine was significantly reduced ($P < 0.05$) (Figure 3c).

Effect of interleukin-1 on prostaglandin synthetase activity hrIL-1α-induced release of PGI_2 from HUVEC was associated with a time-dependent increase in prostaglandin synthetase activity (Figure 4). A two fold increase in enzyme activity was observed within 30 min exposure to 1 u ml^{-1} hrIL-1α. By 6 h, the enzyme activity was more than three fold greater than that in unstimulated HUVEC and although there was a slight reduction after 24 h incubation with hrIL-1α, the enzyme activity was still nearly three fold greater than that in unstimulated cells. The increase in prostaglandin synthetase activity was inhibited in HUVEC co-incubated with 1 u ml^{-1} hrIL-1α and 0.2 μM cycloheximide for 24 h, indicating that the hrIL-1α induced increase in prostaglandin synthetase activity was, at least in part, due to protein synthesis. PGI_2 release from HUVEC co-incubated with hrIL-1α and cycloheximide was not significantly reduced, indicating firstly, that cycloheximide was not acting as a prostaglandin synthetase inhibitor and secondly, that new enzyme protein was not an absolute requirement for the release of PGI_2 by hrIL-1α during the 24 h incubation.

Effect of short exposure to interleukin-1 on histamine-induced release of prostacyclin The inhibitory effect of nifedipine and

Table 1 Relative potentiation of histamine-induced release of prostacyclin (PGI_2) by human recombinant interleukin-1α (hrIL-1α)

hrIL-1α (u ml^{-1})	*Histamine* (log M) −7	−6	−5	−4	−3
0.05	1.10 ± 0.11	1.63 ± 0.19	1.39 ± 0.02	1.36 ± 0.30	1.43 ± 0.23
	NS	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$
0.1	1.17 ± 0.15	1.59 ± 0.23	1.59 ± 0.04	1.32 ± 0.12	1.78 ± 0.33
	NS	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$
0.5	1.26 ± 0.15	1.96 ± 0.36	1.92 ± 0.24	2.28 ± 0.54	2.41 ± 0.38
	NS	$P < 0.02$	$P < 0.02$	$P < 0.05$	$P < 0.05$
1.0	1.32 ± 0.10	1.72 ± 0.46	1.70 ± 0.14	1.73 ± 0.22	1.78 ± 0.05
	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$
5.0	1.28 ± 0.07	1.99 ± 0.30	1.68 ± 0.08	1.54 ± 0.09	1.51 ± 0.22
	$P < 0.05$	$P < 0.05$	$P < 0.02$	$P < 0.05$	$P < 0.05$

Human umbilical vein endothelial cells (HUVEC) were pre-incubated with hrIL-1α (0.05 u ml^{-1}–5.0 u ml^{-1}) for 24 h prior to stimulation with histamine (0.1 μM–1 mM) for 30 min. The relative potentiation of histamine-induced release of PGI_2 by HUVEC pre-incubated with hrIL-1α is expressed as a fraction of that induced by histamine alone + hrIL-1α alone. ([Histamine + hrIL-1α] ÷ [Histamine] + [hrIL-1α])
Results represent the mean ± s.e.mean of 4 experiments.
NS = not statistically significant.

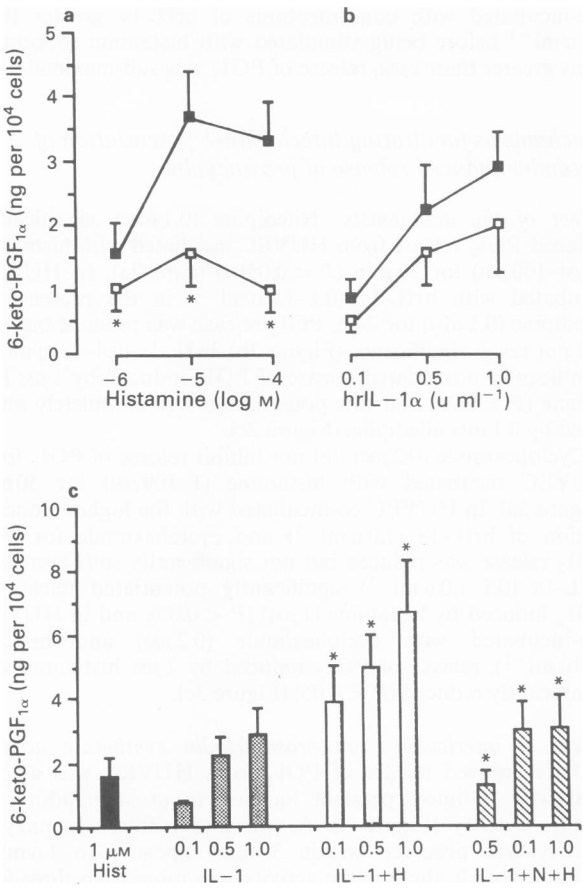

Figure 2 Effect of nifedipine on prostacyclin (PGI_2) release from human umbilical vein endothelial cells (HUVEC) incubated with: (a) histamine for 30 min in the absence (■) or presence (□) of 0.1 mM nifedipine or (b) human recombinant interleukin-1α (hrIL-1α) for 24 h in the absence (■) or presence (□) of 0.1 mM nifedipine or (c) pre-incubated with hrIL-1α (0.1–1.0 u ml^{-1}) for 24 h in the absence (open columns) or presence (hatched columns) of 0.1 mM nifedipine (N) prior to stimulation with 1 μM histamine (H) for 30 min. In control cultures, HUVEC were incubated with 1 μM histamine for 30 min (solid column) or hrIL-1α (IL, 0.1–1.0 u ml^{-1}) for 24 h (stippled columns) Results are expressed as mean for $n = 6$ (a), $n = 3$ (b) and (c) with s.e.mean shown by vertical bars. *$P < 0.05$.

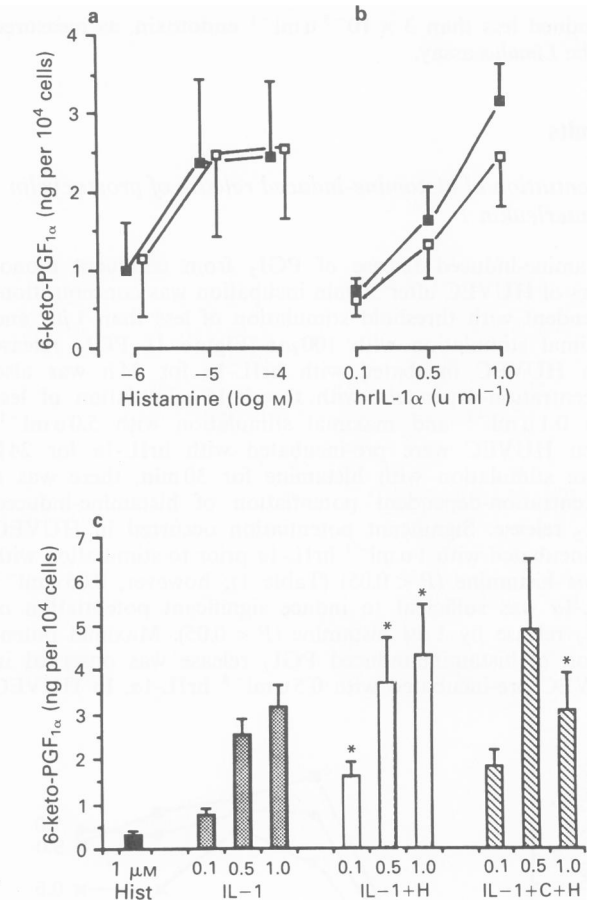

Figure 3 Effect of cycloheximide on prostacyclin (PGI_2) release from human umbilical vein endothelial cells (HUVEC) incubated with: (a) histamine or 30 min in the absence (■) or presence (□) of 0.2 μM cycloheximide or (b) human recombinant interleukin-1α (hrIL-1α) for 24 h in the absence (■) or presence (□) of 0.2 μM cycloheximide or (c) pre-incubated with hrIL-1α (0.1–1.0 u ml^{-1}) for 24 h in the absence (open columns) or presence (hatched columns) of 0.2 μM cycloheximide (C) prior to stimulation with 1 μM histamine (H) for 30 min. In control cultures HUVEC were incubated with 1 μM histamine for 30 min (solid column) or hrIL-1α (0.1–1.0 u ml^{-1}) for 24 h (stippled columns). Results are expressed as mean for $n = 5$ in (a) and (b), $n = 3$ in (c) with s.e.mean shown by vertical bars. * $P < 0.05$.

the rapid increase in prostaglandin synthetase activity indicated that histamine-induced release of PGI_2 from HUVEC may be potentiated by shorter exposure to hrIL-1α. Concomitant exposure of HUVEC to hrIL-1α (0.1–1.0 u ml^{-1}) and histamine (1 μM) did not increase PGI_2 release above that induced by histamine alone (Figure 5), however, potentiation of histamine-induced release of PGI_2 was concentration- and time-dependent. Pre-incubation for 10 min with 1.0 u ml^{-1} hrIL-1α was sufficient to induce a statistically significant increase in histamine-induced release of PGI_2 ($P < 0.05$); however, in HUVEC pre-incubated with 0.1 u ml^{-1} hrIL-1α, more than 30 min was required. The relative potentiation of histamine release of PGI_2 from HUVEC pre-incubated with hrIL-1α (0.5–1.0 u ml^{-1}) for 30 min was greater than that from HUVEC pre-incubated with hrIL-1α for 2, 6 or 24 h (Figure 6).

Potentiation of histamine-induced release of PGI_2 from HUVEC pre-incubated with hrIL-1α (0.1–1.0 u ml^{-1}) for 30 min was not significantly inhibited by either nifedipine or cycloheximide (Figure 7).

Discussion

Histamine and interleukin-1 are vasoactive and induce release of PGI_2 from HUVEC. We show here that hrIL-1α is able to

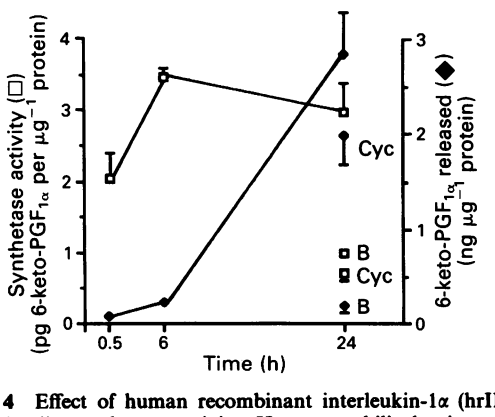

Figure 4 Effect of human recombinant interleukin-1α (hrIL-1α) on prostaglandin synthetase activity. Human umbilical vein endothelial cells (HUVEC) were incubated with 1 u ml^{-1} hrIL-1α for different lengths of time and prostaglandin synthetase activity in solubilized cells assayed as 6-keto-$PGF_{1\alpha}$ (□). Concomitant release of PGI_2 into cell supernatant during the incubation period was also measured (◆). Prostaglandin synthetase activity in and PGI_2 release from unstimulated HUVEC (B) and HUVEC co-incubated with hrIL-1α and 0.2 μM cycloheximide (Cyc) were determined following 24 h incubation. The results are expressed as the mean of 3 experiments with s.e.mean shown by vertical bars.

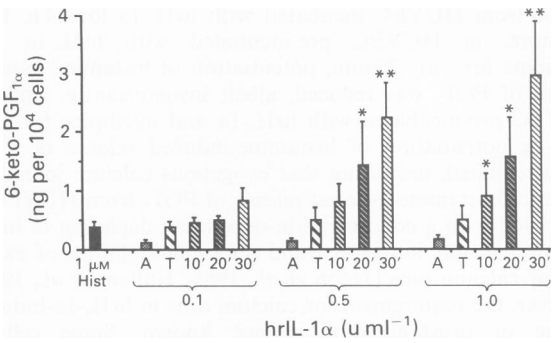

Figure 5 Effect of concomitant or short pre-exposure to human recombinant interleukin-1α (hrIL-1α) on histamine-induced release of prostacyclin (PGI_2). Human umbilical vein endothelial cells (HUVEC) were either incubated with hrIL-1α (0.1–1.0 u ml^{-1}) and 1 μM histamine together (T) for 30 min or pre-incubated with hrIL-1α for 10, 20 or 30 min prior to stimulation with 1 μM histamine for 30 min. In control cultures, HUVEC were incubated alone with 1 μM histamine (solid column) for 30 min or hrIL-1α (0.1–1.0 u ml^{-1}) for 1 h (A). Results are expressed as mean for 4 experiments with vertical bars showing s.e.mean. * $P < 0.05$; ** $P < 0.005$.

potentiate histamine-induced release of PGI_2 from HUVEC. This effect appears to be mediated by two mechanisms, IL-1-induced synthesis of prostaglandin synthetase enzymes and up-regulation of existing enzyme activity.

In HUVEC incubated with hrIL-1α for 30 min, there was a two fold increase in prostaglandin synthetase enzyme activity, as assessed by the ability of a solubilized cell sonicate to produce 6-keto-$PGF_{1\alpha}$ in the presence of exogenous arachidonic acid. This increased enzyme activity was associated with increasing potentiation of histamine-induced release of PGI_2 from intact HUVEC by hrIL-1α. The sustained release of PGI_2 from HUVEC incubated with hrIL-1α for 24 h was associated with enzyme protein synthesis as prostaglandin synthetase activity of HUVEC incubated with hrIL-1α in the presence of cycloheximide for 24 h was not raised above that of unstimulated cells. However, release of PGI_2 from those same HUVEC was not dependent on synthesis of new enzyme protein, as there was minimal reduction in release of PGI_2 from HUVEC co-incubated with hrIL-1α and cycloheximide. The amount of PGI_2 released from HUVEC incubated with hrIL-1α for 24 h is comparable to that released within 30 min of exposure to histamine, indicating that sufficient enzyme is already available to catalyse the hrIL-1α-mediated release of PGI_2 from HUVEC. The lack of effect of 0.2 μM cyclo-

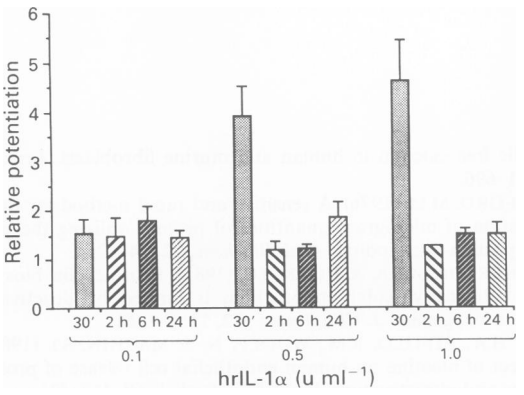

Figure 6 Human recombinant interleukin-1α (hrIL-1α) potentiation of histamine-induced release of prostacyclin (PGI_2). Human umbilical vein endothelial cells (HUVEC) were pre-incubated with hrIL-1α (0.1–1.0 u ml^{-1}) for 30 min, 2, 6 or 24 h prior to stimulation with 1 μM histamine for 30 min. The relative potentiation of histamine-induced release of PGI_2 by hrIL-1α is expressed as a fraction of that induced by histamine alone + hrIL-1α alone. ([Histamine + hrIL-1α] ÷ [Histamine] + [hrIL-1α]). Results represent the mean of 4 experiments with vertical bars showing s.e.mean.

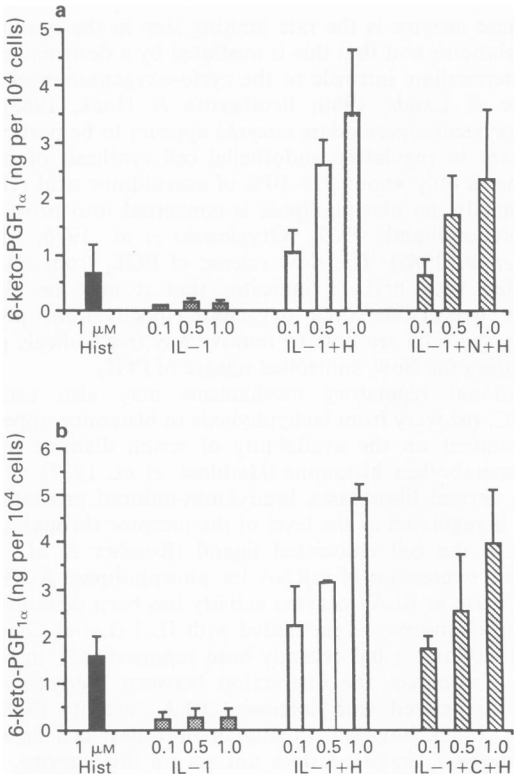

Figure 7 Inhibition of human recombinant interleukin-1α (hrIL-1α) potentiation of histamine-induced release of prostacyclin (PGI_2). Human umbilical vein endothelial cells (HUVEC) were pre-incubated with hrIL-1α (0.1–1.0 u ml^{-1}) for 30 min: (a) in the absence (open columns) or presence (hatched columns) of 0.1 mM nifedipine (N) or (b) in the absence (open columns) or presence (hatched columns) of 0.2 μM cycloheximide (C) prior to stimulation with 1 μM histamine (H) for 30 min. In control cultures HUVEC were incubated with either 1 μM histamine for 30 min (solid column) or hrIL-1α (0.1–1.0 u ml^{-1}) for 1 h (stippled columns). Results are expressed as mean of $n = 2$ experiments in (a) and (b) with vertical bars showing range.

heximide on hrIL-1α-induced release of PGI_2 from HUVEC is in agreement with our previous results (Rustin *et al.*, 1989) but in contrast to results obtained from experiments on human dermal microvascular endothelial cells (Bull *et al.*, 1990). Human dermal microvascular endothelial cells do not release PGI_2 when incubated with either hrIL-1α or histamine, however, they do release PGE_2 in a time- and concentration-dependent manner and hrIL-1α-induced release of PGE_2 is inhibited by 0.2 μM cycloheximide. Inhibition of IL-1-induced release of PGE_2 from other cell types by cycloheximide has also been reported (Newton & Covington, 1987; Raz *et al.*, 1988), however, in our experience, IL-1-induced release of PGI_2 from HUVEC cannot be inhibited by concentrations of cycloheximide that are not at the same time cytotoxic. The lack of contrary data from other laboratories would appear to support our observations. It has recently been reported that interleukin 2-induced release of PGI_2 from both HUVEC and bovine aortic endothelial cells was associated with a time-dependent synthesis of prostaglandin H synthetase (Frazier-Scott *et al.*, 1988), however the time-course of the relationship between synthesis of prostaglandin H synthetase and release of PGI_2 was not examined. Furthermore, although these authors demonstrated inhibition of interleukin 2-induced release of PGI_2 from bovine aortic endothelial cells with cycloheximide, they did not give any data regarding the effects of cycloheximide on release of PGI_2 from HUVEC.

In addition to inducing protein synthesis, hrIL-1α appears to be able to up-regulate prostaglandin synthetase enzyme activity; however, the means by which this occurs is unclear. Reports have indicated that irreversible inactivation of the cyclo-oxygenase activity of the prostaglandin endoperoxide

synthetase enzyme is the rate limiting step in the synthesis of prostaglandins and that this is mediated by a destructive reaction intermediate intrinsic to the cyclo-oxygenase mechanism (Hemler & Lands, 1980; Brotherton & Hoak, 1983). The cyclo-oxygenase/peroxidase enzyme appears to be particularly important in regulating endothelial cell synthesis of prostaglandins as only about 5%–10% of arachidonic acid released from membrane phospholipids is converted into prostaglandins, predominantly PGI_2 (Gryglewski *et al.*, 1976; Alhenc-Gelas *et al.*, 1982). The slow release of PGI_2 from HUVEC incubated with hrIL-1α indicates that it may be able to prevent irreversible cyclo-oxygenase inactivation, possibly because the cells are able to remove any free radicals generated during the slow, controlled release of PGI_2.

Additional regulatory mechanisms may also exist. In HUVEC, recovery from tachyphylaxis to histamine appears to be dependent on the availability of serum diamine oxidase which metabolizes histamine (Haddock *et al.*, 1987), while in human dermal fibroblasts, bradykinin-induced prostaglandin release is regulated at the level of the receptor through degradation of the cell associated ligand (Roscher *et al.*, 1984). Increased expression of mRNA for phospholipase A_2 (PLA_2) and increase in PLA_2 enzyme activity has been demonstrated in rabbit chondrocytes incubated with IL-1 (Lyons-Giordano *et al.*, 1989) and it has recently been reported that, in human dermal fibroblasts, the interaction between bradykinin and IL-1 is associated with increased PLA_2 activity (Solito & Parente, 1989), however, in human fibroblast cell lines, evidence suggests that IL-1 does not act on this enzyme, as no increased release of free arachidonic acid can be demonstrated in cells incubated with IL-1 (Newton & Covington, 1987). Release of arachidonic acid from membrane phospholipids may be a rate limiting step in hrIL-1α-induced release of PGI_2 from intact HUVEC, as in the presence of excess cellular prostaglandin synthetase enzyme activity, there is minimal release of PGI_2. Histamine has also been demonstrated to increase PLA_2 in guinea-pig isolated perfused lung (Blackwell *et al.*, 1978) and in HUVEC, histamine-induced release of PGI_2 is blocked by the PLA_2 inhibitor, mepacrine (Alhenc-Gelas *et al.*, 1982); however, as only a fraction of the available arachidonic acid is converted to PGI_2 by HUVEC stimulated with histamine (Alhenc-Gelas *et al.*, 1982), it would appear that the cyclo-oxygenase/peroxidase enzyme is the rate limiting enzyme in HUVEC stimulated with histamine. From these observations one may hypothesize that in HUVEC incubated with IL-1 and then histamine, both prostaglandin synthetase activity and PLA_2 activity are up-regulated, the result being increased synthesis and release of PGI_2.

Nifedipine significantly inhibited release of PGI_2 from HUVEC incubated with histamine for 30 min and reduced release from HUVEC incubated with hrIL-1α for 24 h. Furthermore, in HUVEC pre-incubated with hrIL-1α and nifedipine for only 30 min, potentiation of histamine-induced release of PGI_2 was reduced, albeit insignificantly, while in HUVEC pre-incubated with hrIL-1α and nifedipine for 24 h, hrIL-1α potentiation of histamine-induced release of PGI_2 was attenuated, indicating that exogenous calcium ions were required. Histamine-induced release of PGI_2 from HUVEC is associated with a concentration-dependent depletion of intracellular calcium ion stores and subsequent influx of extracellular calcium ions (Jacob *et al.*, 1988; Hallam *et al.*, 1989), however, the requirement for calcium ions in hrIL-1α-induced release of prostaglandins is not known. Some cellular responses to IL-1 appear to be calcium-dependent; however, the requirements appear to differ between cell types. IL-1 increases cytosolic free calcium ($[Ca^{2+}]_i$) in lymphocytes (Pincus *et al.*, 1988) but not in neutrophils (Georgilis *et al.*, 1987), demonstrating that even within 'excitable' cells such a lymphocytes and neutrophils, IL-1 has a differential effect on $[Ca^{2+}]_i$. Furthermore, in pancreatic β-islet cells, IL-1 does not have an acute effect on $[Ca^{2+}]_i$, however, verapamil (10 μM) blocked the inhibitory action of IL-1 on insulin release over a 24 h incubation period (Helqvist *et al.*, 1989). In contrast, IL-1 has been shown to induce a gradual increase in $[Ca^{2+}]_i$ in human dermal fibroblasts, which are a 'non-excitable' cell and this effect can be blocked by EGTA but not by verapamil or nifedipine (Bouchelouche *et al.*, 1988). The vascular endothelial cell is also a 'non-excitable' cell and the inhibitory effect of nifedipine in our experiments demonstrates a requirement for extracellular calcium ions in both the rapid release of PGI_2 induced by histamine and the protracted release of PGI_2 induced by hrIL-1α and indicates that depletion of intracellular calcium ion stores may be a rate-limiting step in prostaglandin synthesis. We have previously reported that HUVEC do not become tachyphylactic to hrIL-1α and that removal of the agonist results in a decline in PGI_2 release (Rustin *et al.*, 1989). Controlled influx and utilization of calcium ions might explain these observations.

The rapid up-regulation of prostaglandin synthetase activity induced by hrIL-1α without concomitant release of PGI_2 highlights the complexities of the mechanisms regulating PGI_2 synthesis in endothelial cells. Furthermore the effects of hrIL-1α and histamine on PGI_2 release support the role of prostaglandins as mediators of inflammatory reactions and underlines the importance of interactions between cytokines and other vasoactive agents in the regulation of inflammatory processes.

H.A.B. is supported by a grant from The Sir Jules Thorn Charitable Trust.

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(Received December 9, 1989
Revised May 1, 1990
Accepted July 4, 1990)

Br. J. Pharmacol. (1990), **101**, 710–714

Relative potencies for barbiturate binding to the *Torpedo* acetylcholine receptor

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1 The structural requirements of an allosteric barbiturate binding site on acetylcholine receptor-rich membranes isolated from *Torpedo* electroplaques have been characterized by the ability of fourteen barbiturates to displace [^{14}C]-amobarbitone binding.

2 The barbiturates could be grouped into two classes with ten barbiturates producing a strong inhibition of [^{14}C]-amobarbitone binding (class one) and with four exerting minimal effects (class two).

3 Eight of the ten class one barbiturates displaced essentially all of the [^{14}C]-amobarbitone from its binding site, while, at their respective aqueous solubility limits, two of these barbiturates (thiopentone and dimethylbutylbarbitone (DMBB)) inhibited [^{14}C]-amobarbitone binding by nearly 80%. The apparent inhibition constants (K_I) for the class one barbiturates ranged from 13 μM for amobarbitone to 2.8 mM for barbitone with the other eight agents lying in the range 100–600 μM, and having the rank order pentobarbitone $\approx$ secobarbitone > thiopentone > DMBB > butabarbitone $\approx$ phenobarbitone > aprobarbitone > allylbarbitone.

4 By contrast, the class two barbiturates had minimal effects even at close to saturating concentrations. [^{14}C]-amobarbitone binding was reduced slightly (<30%) by hexobarbitone, mephobarbitone and methohexitone and was enhanced slightly (<20%) by metharbitone.

5 All of the class two, but none of the class one barbiturates, were N-methylated.

Introduction

The molecular mechanisms underlying the various clinical actions of barbiturates remains unclear. Several studies suggest that barbiturates may exert some of their anticonvulsive, anxiolytic and anaesthetic actions by allosterically enhancing and/or inhibiting postsynaptic responses (Barker *et al.*, 1980; Ho & Harris, 1981; Willow & Johnston, 1983; MacDonald *et al.*, 1986). The most extensively studied of these possible sites has been in pathways using the inhibitory neurotransmitter, γ-aminobutyric acid (GABA) (Simmonds & Turner, 1987; Schwartz, 1988; Olsen, 1982). Studies have suggested the existence of a barbiturate binding site on the GABA receptor that appears to be capable of allosterically enhancing GABA binding. Less is known about the effect of barbiturates on excitatory postsynaptic responses. Several studies have focused on barbiturate-acetylcholine receptor interactions (reviewed in Richter & Holtman, 1982). Because nicotinic receptors in the central nervous system are not well characterized, electrophysiological studies have focused on the neuromuscular junction. Here, pharmacological studies show the ability of barbiturates to inhibit synaptic transmission parallels their anaesthetic potency, implying a nonspecific mechanism (Lee-Son *et al.*, 1975). However, mechanistic studies found barbiturates to inhibit transmission at the neuromuscular junction by selectively blocking open ion channels (Adams, 1976) in a manner most consistent with a mechanism involving an allosteric barbiturate site (Gage & McKinnon, 1985).

Because of the low density of nicotinic receptors in mammalian tissues, the question of the existence of specific barbiturate sites is best approached in other nicotinic systems, such as the *Torpedo* electroplaque. In acetylcholine receptor-rich membranes from this tissue, there is direct evidence for a stereoselective functional barbiturate binding site that is allosterically coupled to the acetylcholine binding site (Dodson *et al.*, 1987; Roth *et al.*, 1989). In this work we have extended our studies to a wider range of barbiturates, obtaining a self-consistent and accurate set of data for fourteen barbiturates in order to explore the molecular pharmacology of the barbiturate site.

Methods

Preparation of membranes

Acetylcholine receptor-rich membranes were prepared from freshly dissected *T. nobiliana* electroplaque by differential and sucrose density gradient centrifugation, as previously described (Dodson *et al.*, 1987). The resultant membranes, which contained 1–2 nmol [^{3}H]-acetylcholine binding sites mg^{-1} protein, were divided into 1 ml aliquots, frozen in liquid nitrogen and stored at −85°C. These aliquots were thawed as needed, stored under nitrogen at 4°C and used within three weeks. Membrane specific activity and results were unaffected by storage conditions.

Measurement of [^{14}C]-amobarbitone binding to membranes

[^{14}C]-amobarbitone binding to membranes was determined by a centrifugation assay, as previously described (Dodson *et al.*, 1987). Briefly, membranes (1 μM acetylcholine binding sites) were incubated with [^{14}C]-amobarbitone (5 μM) and varying concentrations of unlabelled barbiturates in *Torpedo* Ringer solution (composition, mM: NaCl 250, KCl 5, $CaCl_2$ 3, $MgCl_2$ 2, Na_2PO_4 5, pH 7.0) at 25°C for 30 min. Aliquots (100 μl) of the membrane suspension were then transfered in triplicate to microcentrifuge tubes and centrifuged (Beckman Airfuge, Ultracentrifuge 30° rotor, model A-100, 133,000 *g*, 30 min). After careful aspiration of the resultant supernatant, the pellet was quickly washed three times with 100 μl of ice-cold buffer. To solubilize the pellet, 10% sodium dodecyl sulphate (100 μl) was added to the centrifuge tubes, and the contents transferred to 7 ml glass scintillation vials containing 6 ml of scintillation cocktail. The vials were then kept at 37°C for at least 1 h. After vigorous vortexing, the samples were counted on a

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Table 1. Relative potencies and aqueous solubilities for fourteen barbiturates

Agent	$IC_{50}^{a,b}$ (±s.e.) (μM)	$K_I^{a,b}$ (±s.e.) (μM)	C_{sat}^{b} (mM)
Amobarbitone	18 (1.2)	13 (0.9)	5.0
Aprobarbitone	640 (31)	452 (22)	14.0
Barbitone	3,970 (240)	2,802 (169)	35.0
Butabarbitone	482 (51)	340 (35)	10.0
Allylbarbitone (Butalbarbitone)	827 (58)	584 (41)	6.0
DMBB	365 (30)	258 (21)	1.0
Hexobarbitone			2.0
Mephobarbitone			1.0
Metharbitone			8.0
Methohexitone			1.3
Pentobarbitone	154 (7)	109 (5)	10.0
Phenobarbitone	550 (24)	388 (17)	5.0
Secobarbitone	173 (11)	122 (8)	10.0
Thiopentone	261 (20)	184 (14)	0.7

[a] Individual binding parameters were obtained from fitting the combined data of two or more experiments to Equation 1 with $n_H = 1$ and to Equation 2 with $K_d = 12\,\mu M$ (see Results).
[b] Concentrations include both the unionized and ionized form of the barbiturate in *Torpedo* Ringer buffer, pH 7.0, at 25°C. DMBB = dimethylbutylbarbituric acid.

Packard Tri-Carb liquid scintillation spectrometer having 98% efficiency. Nondisplaceable binding was defined as binding in the presence of excess amobarbitone (3 mM) and represented approximately 30% of total binding. Maximum displaceable binding was defined as the difference between total and nondisplaceable [^{14}C]-amobarbitone binding under control conditions in a given experiment.

Data analysis

Values for 50% inhibition of displaceable [^{14}C]-amobarbitone binding (IC_{50}) were calculated by fitting the data to a logistic function of the form

$$I = (I_{max} - I_{min})\left(\frac{[barb]^N}{[barb]^N + IC_{50}^N}\right) - I_{min} \quad \text{Eq. (1)}$$

where I is the percentage inhibition for a barbiturate at concentration [barb], I_{max} and I_{min} are the maximum and minimum percentage inhibitions, respectively, and N is the slope parameter corresponding to the Hill coefficient. Apparent inhibition constants (K_I) were calculated from the equation

$$K_I = \frac{IC_{50} \times K_d}{K_d + \{[^{14}C]\text{-amobarbitone}\}} \quad \text{Eq. (2)}$$

where K_d is the previously determined equilibrium dissociation constant for displaceable [^{14}C]-amobarbitone binding under these conditions ($K_d = 12\,\mu M$, Dodson *et al.*, 1987). Binding data were analysed by an iterative, nonlinear least squares programme on a Macintosh SE computer as previously described (Dodson *et al.*, 1987). Results are expressed as mean ± standard deviation of the combined data of two or more experiments.

Materials

Torpedo nobiliana were purchased from Biofish Associates (Georgetown, MA, U.S.A.). [^{14}C]-amobarbitone (52 mCi mmol^{-1}) was purchased from American Radiolabeled Chemicals (St. Louis, MO, U.S.A.). [^{3}H]-acetylcholine (0.7–2.0 Ci mmol^{-1}) was obtained from Amersham-Searle (Arlington Heights, IL, U.S.A.). α-Bungarotoxin was purchased from Miami Serpentarium (Miami, FL, U.S.A.). All other materials and drugs were purchased from commercial sources.

Results

Effect of barbiturates on displaceable [^{14}C]-amobarbitone binding to acetylcholine receptor-rich membranes

Fourteen barbiturates were examined in a self-consistent set of experiments with at least two independent determinations being made for each agent. Ten barbiturates (Table 1) inhibited displaceable [^{14}C]-amobarbitone binding to acetylcholine receptor-rich membranes, while four had minimal effect. The ten inhibiting barbiturates all appeared to act similarly with typical results shown in Figure 1. Eight of these barbiturates (amobarbitone, aprobarbitone, barbitone, butabarbitone, allylbarbitone, pentobarbitone, phenobarbitone and

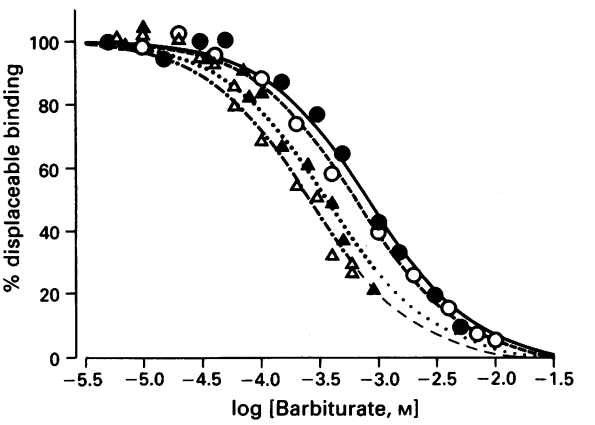

Figure 1 Barbiturate inhibition of displaceable [^{14}C]-amobarbitone binding: the effect of various concentrations of aprobarbitone (○), allylbarbitone (●), dimethylbutylbarbituric acid (▲) and thiopentone (△) on the displaceable binding of [^{14}C]-amobarbitone (5 μM) to acetylcholine receptor membranes (1 μM ACh binding sites) was determined by centrifugation assay (see Methods). Maximum displaceable binding was defined as the difference in total binding in the presence and absence of excess amobarbitone (3 mM). For Figures 1–3, free [^{14}C]-amobarbitone concentrations never varied from total [^{14}C]-amobarbitone concentrations by more than 5–8%, the results are representative of at least two experiments with each barbiturate and each concentration determined in triplicate (standard deviation of the replicates in all figures was generally 2–5% of the mean and never greater than 10%). The concentration-response curves were drawn using $n_H = 1$ and the IC_{50} values given in Table 1.

secobarbitone) produced a maximum inhibition (I_{max}) of >90–95%. Thiopentone and DMBB inhibited [^{14}C]-amobarbitone binding by 75% and 80%, respectively, at the limits of their solubility in buffer (C_{sat}, Table 1). Their low aqueous solubility prevented us from establishing unequivocally their respective I_{max}. However, there was no evidence of a plateau in the percentage inhibition of either barbiturate at C_{sat}. Furthermore, the extrapolated best fit estimates of I_{max} for both barbiturates did not differ within experimental error from 100% inhibition.

Analysis of concentration-inhibition relationships for class one agents

The IC_{50} values for the class one barbiturates were obtained by fitting their respective data to Equation 1, as described in Methods. The Hill coefficient, n_H, ranged from 0.89 to 1.24 with an average standard deviation of 0.063. For purposes of comparison the values of the IC_{50}s given in Table 1 were derived from Equation 1 assuming a common value of $n_H = 1$, but values obtained allowing n_H to vary independently did not differ significantly. The values in Table 1 were then used in Equation 2 to calculate their respective K_I values.

Amobarbitone ($K_I = 13\,\mu$M) was the most potent inhibitor of [^{14}C]-amobarbitone binding. This K_I value was seven fold lower than that of the next most potent barbiturate, pentobarbitone ($K_I = 109\,\mu$M). The values for the next eight agents were grouped in a narrow concentration range (100–600 μM) with the rank ordering of pentobarbitone ≈ secobarbitone > thiopentone > DMBB > butabarbitone ≈ phenobarbitone > aprobarbitone > allylbarbitone. Although barbitone was also capable of completely inhibiting [^{14}C]-amobarbitone binding, it was much less potent ($K_I = 2.8$ mM) than the other nine barbiturates.

Analysis of concentration-inhibition relationships for class two agents

Four barbiturates (metharbitone, methohexitone, mephobarbitone and hexobarbitone) had minimal effect on displaceable [^{14}C]-amobarbitone binding (Figure 2). At their respective C_{sat}s (Table 1), methohexitone, mephobarbitone and hexobarbitone inhibited [^{14}C]-amobarbitone binding by approximately 30%, 20% and 20%, respectively (Figure 2). Because the magnitude of inhibition was so small at C_{sat}, more detailed analyses could not be undertaken.

Metharbitone (>0.6 mM) was unique in producing a slight, concentration-dependent increase in [^{14}C]-amobarbitone binding (Figure 2) which apparently plateaued approximately 15–20% above control values at 6–8 mM.

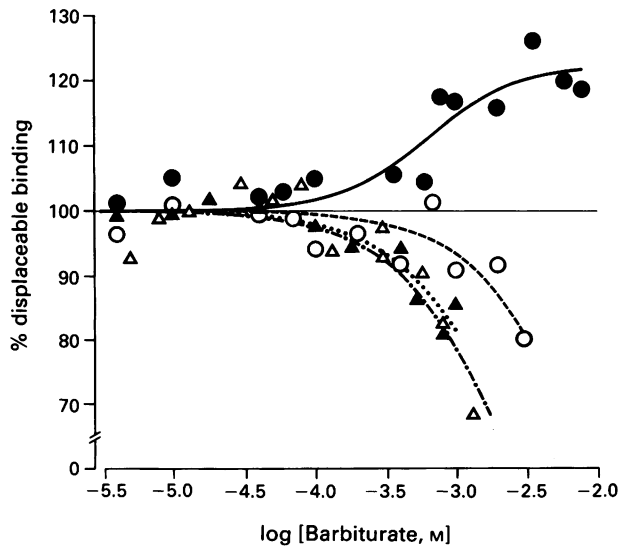

Figure 2 The N-methylated barbiturates are weak modulators of displaceable [^{14}C]-amobarbitone binding: the concentration-response curves for hexobarbitone (○), mephobarbitone, (▲), metharbitone (●), and methohexitone (△) were drawn by eye. The experimental conditions were as described in Figure 1.

Discussion

This study further characterizes the structural requirements of a [^{14}C]-amobarbitone binding site on the *Torpedo* acetylcholine receptor. It provides an accurate and self-consistent set of apparent inhibition constants for ten barbiturates and establishes that four others are without substantial effects.

Because the variation in the apparent inhibition constants (K_Is) of the ten class one agents might be accounted for simply by hydrophobicity, or lipid solubility, rather than specific structure-activity relationships, we first compared the K_Is with their octanol/water partition coefficients, $\lambda_{o/w}$ (Table 2). Linear regression yielded a rough correlation between these variables {log(K_I) versus log($\lambda_{o/w}$), $r^2 = 0.49$} which was in keeping with the observation that the longer the 5′ side chain, the higher the barbiturate potency (i.e., secobarbitone > aprobarbitone) (Table 2). However, the value of this correlation is limited, because the slope of the correlation deviated from the expected value of one. For example, amobarbitone and phenobarbitone were approximately 16 and 3.5 times, respectively, more potent, and thiopentone approximately 2.4 times less potent than their predicted values.

Rearrangements of the 5 or 5′ chain of a barbiturate also produced results different from those predicted from hydrophobicity. For example, amobarbitone and pentobarbitone are formula isomers, (5-ethyl, 5′(3-methylbutyl) barbituric acid and 5-ethyl,5′(1-methylbutyl) barbituric acid, respectively) with almost identical octanol/water partition coefficients. Yet this simple change in the position of a secondary methyl group made the K_I of pentobarbitone an order of magnitude higher than that of amobarbitone. Similarly, the addition of a methyl to the 1 position of amobarbitone to yield DMBB (5-ethyl, 5′(1,3 dimethylbutyl) barbituric acid) produced the expected increase in the octanol/water partition coefficient but a twenty fold decrease in affinity for the [^{14}C]-amobarbitone binding site. Similar deviations can also be seen with 5 chain substitutions. Thus, secobarbitone has an allyl group whereas pentobarbitone has an ethyl group on the 5 chain {5-allyl, vs 5-ethyl, 5′(1-methylbutyl) barbituric acid}, yet they have similar binding affinities despite a three fold difference in octanol/water partition coefficients.

Thiopentone differs from pentobarbitone only in having a thiocarbonyl group at the 2 position of the pyrimidine ring instead of a carbonyl group (i.e. 5-ethyl, 5′(1-methylbutyl)-2-thio barbituric acid). Despite enhancing the octanol/water partition coefficient four fold, this substitution decreased the binding affinity slightly.

The most radical changes were produced by substituting a methyl group for a hydrogen on the 1 position of the pyrimidine ring. Although N-methylation increases octanol/water partition coefficients by nearly an order of magnitude (Table 2), all four N-methyl barbiturates examined failed to cause significant displacement of [^{14}C]-amobarbitone (Table 1 and Figure 2). This loss of potency is not simply a function of the decreased aqueous solubility of the N-methyl analogues (Figure 3), because phenobarbitone and barbitone have IC_{50}s of 0.55 and 2.8 mM respectively, whereas their N-methyl analogues, mephobarbitone and methabitone, caused little inhibition at twice these concentrations.

Why do the N-methyl barbiturates bind so weakly, if at all, to the amobarbitone site? The simplest explanation is steric hindrance. For example, the fit of the whole pyrimidine ring into a narrow cleft on a binding site could be prevented by

Table 2 Comparison of inhibition constants for the barbiturate site on acetylcholine receptors with other properties

Agent	K_I^a (μM)	*Occupancy at [GA]*[b] (%)	*[^{3}H]-BMC*[c] (μM)	*[^{35}S]-TBPS*[d] (μM)	$\lambda^e_{o/w}$
Amobarbitone	11	0.97			129[f]
Aprobarbitone	390	0.72			41[f]
Barbitone	2,371	0.81	>500		5[g]
Butabarbitone	293	0.58			45[h]
Allylbarbitone	504	0.46			45[i]
DMBB	233	0.14	375	37	177[i]
Hexobarbitone		0		182	110[f]
Mephobarbitone		0	368	129	107[f]
Metharbitone		0		125	
Methohexitone		0			3,333[h]
Pentobarbitone	98	0.57	156	67	135[f]
Phenobarbitone	246	0.85	195	170	25[g]
Secobarbitone	108	0.37	143	14	389[f]
Thiopentone	132	0.10			390[j]

All data are corrected to the concentration of the unionised form.
[a] K_I, calculated from Table 1 using pKs summarized in Firestone *et al.*, 1986.
[b] Occupancy of the barbiturate site at the concentration causing general anaesthesia in tadpoles (Lee-son *et al.*, 1975).
[c] [^{3}H]-BMC, concentration causing allosteric inhibition of [^{3}H]-bicuculline methochloride ([^{3}H]-BMC) binding to rat synaptic membranes (Wong *et al.*, 1984; Olsen *et al.*, 1986).
[d] [^{35}S]-TBPS, concentration causing inhibition of [^{35}S]-*t*-butylbicyclophosphorothionate (Ticku & Rastogi, 1986).
[e] $\lambda_{o/w}$, octanol/water partition coefficient for unionized barbiturates.
Sources: [f] Yih & van Rossum, 1977; [g] Kakemi *et al.*, 1967a,b; [h] Backes *et al.*, 1984; [i] Hansch & Anderson, 1967; [j] Korten & Miller, 1979.

N-methyl substitution. Such a mechanism would also explain the lower than expected affinity of thiopentone.

Metharbitone alone of the barbiturates examined increased, rather than decreased, [^{14}C]-amobarbitone binding, suggesting that it acts by an entirely different mechanism. For example, under our control conditions, approximately 75–80% of the acetylcholine receptors exist in the resting state (Boyd & Cohen, 1980), which has high affinity for amobarbitone, whilst the remainder are in the desensitized state with negligible affinity for amobarbitone (Dodson *et al.*, 1987). Thus, metharbitone could act by converting all of the acetylcholine receptors into the resting state, perhaps by binding to a separate allosteric site.

The nicotinic and GABA receptors belong to the same superfamily, having high sequence homology especially in their transmembrane regions (Schofield *et al.*, 1987; Barnard *et al.*, 1988). Although specific barbiturate binding has not been directly demonstrated on the GABA receptor (because of the low density of binding sites), there are extensive parallels between the barbiturate interactions with the two receptors

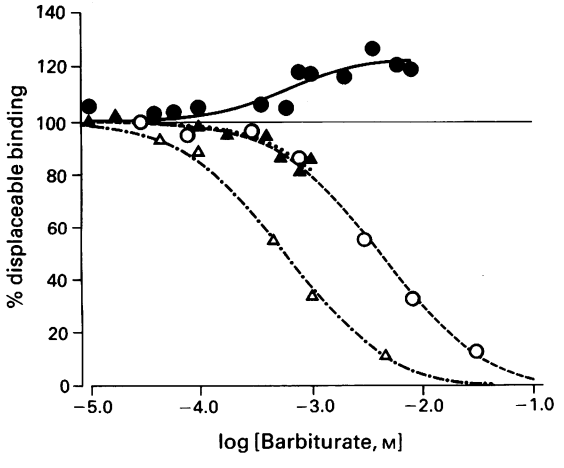

Figure 3 Comparison of phenobarbitone and barbitone with their N-methylated analogues: the effect of various concentrations of phenobarbitone (△) and barbitone (○) and their respective N-methyl analogues, mephobarbitone (▲) and metharbitone (●), on displaceable [^{14}C]-amobarbitone binding. See Figures 1 and 2.

(Barker *et al.*, 1980; Skolnick *et al.*, 1982; Heidmann *et al.*, 1983; Olsen *et al.*, 1986; Ticku & Rastogi, 1986; Dodson *et al.*, 1987; Schwartz, 1988).

The affinity of barbiturates for a possible allosteric site on the GABA receptor has been inferred from studies of their effects either on the binding of the picrotoxin-like caged convulsant [^{35}S]-*t*-butylbicyclophosphorothionate ([^{35}S]-TBPS) to its allosteric site or on the binding of the GABA antagonist [^{3}H]-bicuculline methochloride ([^{3}H]-BMC) (sources and results of these studies are given in Table 2). Although the affinity for the barbiturate site on both receptors is similar for our class one agents, the N-methylated barbiturates discriminate strongly between them. Thus, barbiturate sites on the ACh and the GABA receptors are similar but not identical, a conclusion also reached by Roth *et al.* (1989). They found that whilst the (+)-pentobarbitone enantiomer had equal apparent affinity for both receptors, the enantiomers of pentobarbitone bound to the acetylcholine receptor with the reverse stereoselectivity of that reported by Ticku & Rastogi (1986) for the GABA receptor. Another difference was that [^{14}C]-amobarbitone inhibition curves of group one agents on the acetylcholine receptors had a uniform slope (i.e., $n_H = 1$, Figure 1), whereas action on the GABA receptor exhibited a wide range of Hill coefficients (Leeb-Lundberg & Olsen, 1982).

The barbiturate site on the acetylcholine receptor does not appear to be specifically involved in general anaesthesia (Dodson *et al.*, 1987). For example, hexobarbitone, mephobarbitone and methohexitone are all potent anaesthetics (Table 2), yet barely inhibit [^{14}C]-amobarbitone binding (Figure 2). Even after excluding the N-methyl barbiturates, which may be rapidly demethylated *in vivo* (Butler, 1953), the rank order for displacing [^{14}C]-amobarbitone still differs markedly from that for inducing anaesthesia. This does not preclude a possible connection with a more subtle measure of anaesthetic potency, such as amnesia. Indeed, there may be a nicotinic component in the neuronal processes involving memory (Levin *et al.*, 1989).

In conclusion, the fourteen diverse barbiturates surveyed could be categorized into two distinct classes according to their ability to displace [^{14}C]-amobarbitone from its allosteric site on the *Torpedo* acetylcholine receptor. Ten of the barbiturates could be classed as competitive inhibitors of [^{14}C]-amobarbitone binding. However, a second class of four barbiturates had only minor effects (<30%) on [^{14}C]-amobarbitone binding. All of this second group of barbiturates

were N-methylated on the 1 position of the pyrimidine ring. These results support the existence of a barbiturate binding site on the acetylcholine receptor with specific structural requirements for binding that are different from those predicted from simple hydrophobic interactions.

This work was supported by Research Grant GM 35997 (B.D.) and Research Grant GM 15904 (K.W.M.) from the National Institute of General Medical Sciences.

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(Received March 22, 1990
Revised June 21, 1990
Accepted June 25, 1990)

Br. J. Pharmacol. (1990), **101**, 715–721

The effects of 5-HT on articular sensory receptors in normal and arthritic rats

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1 The effects of intra arterial (i.a.) injections of 5-hydroxytryptamine (5-HT, 1–100 μg) on the discharge of (a) identified articular high threshold mechanoreceptors and (b) unidentified chemosensitive receptors in the ankle joint have been studied electrophysiologically in anaesthetized normal and arthritic rats. Recordings were made from a fine branch of the medial plantar nerve.

2 5-HT increased the mechanical responsiveness of high threshold nociceptive mechanoreceptors with C and Aδ fibre afferents in both normal and adjuvant-arthritic rats. Receptors in arthritic joints were more sensitive to 5-HT than were those from normal joints.

3 5-HT produced a complex response from both types of articular receptors following i.a. injection. Two separate components were identified: (a) a fast transient burst of activity was obtained within 10 s of this injection in 66% of units from normal animals and 45% from arthritics, followed by (b) a delayed slow longer-lasting excitation seen in 62% of the units examined from normals and 77% of units from arthritic rats.

4 Increased mechanoreceptor resonsiveness produced by 5-HT was reduced or abolished by the 5-HT_3 receptor antagonists studied (MDL 72222, ICS 205-930, or GR 38032F, in single doses of 100 $\mu g\,kg^{-1}$, i.a.).

5 Fast excitation showed marked tachyphylaxis and was antagonized by MDL 72222, ICS 205-930 or GR 38032F. It was unaffected by ketanserin (100 $\mu g\,kg^{-1}$, i.a.). Delayed excitation was reduced or abolished by ketanserin but was unaffected by the 5-HT_3-receptor antagonists.

6 Administration of MDL 72222, ICS 205-930 or GR 38032F caused short lasting (< 5 min) reductions in background activity from both types of unit recorded in arthritic rats, as well as in normal rats in which activity had increased following administration of 5-HT. Ketanserin caused similar reductions in background activity in chemosensitive units, but had no effect on mechanoreceptors.

7 At least two types of receptor are involved in the actions of 5-HT on articular sensory receptors with fine afferent fibres. Increased mechano-responsiveness involves a 5-HT_3-receptor as does fast excitation. Delayed excitation probably involves a 5-HT_2-receptor. Endogenous 5-HT appears not to play a crucial role in sensitization of high threshold mechanoreceptors in this model of chronic inflammation and arthritis, although its local release may potentiate the actions of other inflammatory mediators on sensory receptors in the ankle joint.

Introduction

Adjuvant-induced polyarthritis in rats has been used extensively as a model for the study of chronic inflammatory pain (see Colpaert, 1987). Electrophysiological studies with this model have shown that high threshold C-fibre mechanoreceptors (putative nociceptors) have lower thresholds in the ankle joints of these animals in comparison with normal rats (Guilbaud *et al.*, 1985). These results suggest that the behavioural changes seen in adjuvant polyarthritis can partly be accounted for in terms of altered properties of articular sensory receptors. The enhanced receptor sensitivity can be reduced by lysine acetylsalicylate, suggesting that locally produced cyclo-oxygenase metabolites may be responsible for at least part of the sensitization (Guilbaud & Iggo, 1985). It is still uncertain, however, the extent to which other inflammatory mediators found in tissue exudates may contribute to the sensitization of peripheral sensory receptor mechanisms.

Keele & Armstrong (1964) demonstrated that 5-hydroxytryptamine (5-HT) has the ability to cause pain when applied to a blister base, and 5-HT was later shown to lower thresholds for chemically-induced pain in man (Sicuteri *et al.*, 1965) and to enhance pseudoaffective responses to bradykinin in animals (Nakano & Taira, 1976). Sensory nerve endings associated with small myelinated and non-myelinated axons have been found to be activated and sensitized by 5-HT in skin (Fjallbrant & Iggo, 1961; Beck & Handwerker, 1974) and muscle (Mense, 1981), as are cutaneous SAII mechanoreceptors with rapidly conducting afferent fibres (Fjallbrant & Iggo, 1961).

Although the pain evoked by application of 5-HT to a blister base has been demonstrated to be antagonized by ICS 205-930, and therefore probably involves a 5-HT_3-receptor (Donatsch *et al.*, 1984; Richardson *et al.*, 1985), in most cases the pharmacological identity of the 5-HT-receptor associated with sensory endings has not been established. The present study was undertaken to examine the effects of 5-HT on sensory receptors in the rat ankle joint, and to characterize the 5-HT receptors mediating these effects by the use of selective antagonists (Fozard, 1984; Bradley *et al.*, 1986; Brittain *et al.*, 1987). We also investigated whether 5-HT plays a role in the sensitization of high threshold mechanoreceptors in arthritic joints by using a rat model of adjuvant-induced mono-arthritis in which the arthritis is mild and confined to one ankle (Grubb *et al.*, 1988).

Methods

Induction of arthritis

Male Wistar rats weighing 200–250 g were anaesthetized with ether during subdermal injection of 0.15 ml of Freund's complete adjuvant (1.0 mg ml^{-1} heat killed *Myobacterium tuberculosis* in paraffin oil, Sigma) around the left ankle joint.

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Experiments were performed on anaesthetized animals following a period of two to nine weeks, during which time a localized arthritis consisting of swelling (approx. 50% increase in circumference of the left ankle joint) and redness of the injected ankle had developed and was maintained.

Surgical procedures

A total of 10 arthritic and 12 normal male Wistar rats weighing between 200 and 300 g was used in these experiments. Animals were anaesthetized with urethane (25% w/v, 0.6 ml $100\,g^{-1}$ body wt. i.p.). The trachea was cannulated and arterial blood pressure monitored via a cannula in the left carotid artery. A cannula was also inserted into the right femoral artery for the injection of drugs into the abdominal aorta at the level of the iliac bifurcation. Drugs were dissolved in 0.9% w/v aqueous NaCl solution (saline) and injected in volumes of 0.1 ml followed by a 0.2 ml saline wash. Accessibility to articular receptors via the vasculature was tested by use of a single low dose of capsaicin (1 μM i.a.) which caused a transient increase in neural discharge of all the afferents studied.

Electrophysiological recording

Neural recordings were made from the primary articulocutaneous ramus (PACR) of the left tibial nerve with platinum-iridium wire electrodes, by employing techniques described in detail elsewhere (Guilbaud *et al.*, 1985). Spontaneously active units for which no mechano-sensitive receptor fields could be found (termed 'chemosensitive' because of their excitation by 5-HT and capsaicin) and high threshold slowly-adapting mechanoreceptors with axons in the PACR were examined. Neural recordings were stored on videotape for subsequent analysis of individual afferent units by use of a pulse height voltage discriminator linked to a microcomputer.

Mechanoreceptors were identified by their response to mechanical stimulation, by use of smooth tipped glass probes of 0.5–1.0 mm diameter. Thresholds of individual units were determined with a series of calibrated Von Frey hairs; these high threshold mechanoreceptors probably function as nociceptors (Wyke, 1981; Guilbaud *et al.*, 1985). Mechanical stimuli in any given trial were delivered at fixed intervals by an electromechanical indentation generator; ramp and plateau waveforms were used routinely, with indentations of 200–600 μm and 2 s duration repeated at 60–120 s intervals. The indentation probe consisted of a sealed metal tube (1 mm diameter) smoothed over at the tip with epoxy-resin, containing a silver wire core, insulated except at the tip, used as the cathode for localized electrical stimulation when measuring conduction velocities. Afferent fibre conduction velocity was measured by localized electrical stimulation, via the probe at the level of the receptor, and determining the time take for the action potential to reach the recording electrodes.

Data analysis

Neural discharge (counts per second) was plotted against time for each test, and the change in frequency from the pre-injection control period calculated. In order to standardize results from experiments with different absolute discharge frequencies, mean values for blocks of 10 s duration were used to calculate peak response as a percentage of the mean discharge in the pre-injection 10 s control period. Mechanoreceptor responses were quantified in counts per mechanical stimulus and expressed as a percentage of the pre-injection response.

Statistics

Mean values are given $\pm$ s.e.mean. Statistical analysis of differences between means was carried out by the Wilcoxon two-sample test (two-tailed) and the null hypothesis rejected if $P < 0.05$.

Drugs

The following compounds were used in this study, with concentrations being expressed in terms of the salt: 5-hydroxytryptamine creatinine sulphate complex, dopamine hydrochloride (Sigma); MDL 72222 ((1αH, 3α, 5αH-tropan-3-yl) 3,5-dichlorobenzoate methanosulphonate salt, kindly donated by Merrell Dow Research Institute Strasbourg); ICS 205-930 ((3α-tropanyl)-3yl)-1H-indole-3-carboxylic acid ester, kindly donated by Sandoz, Basel); GR 38032F (1,2,3,9-tetrahydro-9-methyl-3-[(2methyl-1H-imidazol-1-yl)methyl]-4H-carbazol-4-one hydrochloride dihydrate, kindly donated by Glaxo Group Research, Ware); ketanserin, tartaric acid salt (kindly donated by Janssen Pharmaceuticals, Beerse). Capsaicin (8-methyl-n-vanylyl-nonenamide, Sigma) was prepared by diluting a stock solution (1 mg ml^{-1} in 10% ethanol: 10% Tween 80: 80% saline) in saline.

Results

Two types of sensory activity were investigated: mechanoreceptors for which receptive fields were found in the joint capsule (Guilbaud *et al.*, 1985), and 'chemosensitive' units, previously described by Grubb *et al.* (1988), which were excited by 5-HT and capsaicin but for which no receptor fields for mechanical stimuli were found (Table 1). Both mechanosensitive and chemosensitive units were excited by capsaicin (1–10 μg, i.a.) in all experiments. Low threshold, rapidly adapting mechanoreceptors with receptive fields in the tissues adjacent to the joint capsule were not considered in these studies.

Normal rats – mechanoreceptors

Mechanoreceptors with afferent fibres in the PACR and with receptive fields in the ankle joint tissues of normal rats had high mechanical threshold, were slowly adapting with punctate receptive fields of approximately 1 mm diameter and were therefore similar to those described by Guilband *et al.*, (1985). Units had mean von Frey thresholds of 81 $\pm$ 6.8 mN, and the conduction velocities of their afferents (2.1–10.5 ms^{-1}) indicated that they were C or Aδ fine afferent fibres. Only one mechanosensitive unit showed any background (spontaneous) discharge (0.2 i.p.s.) before the addition of 5-HT – the number of these high threshold units found in individual animals was small, and their lack of resting discharge and high mechanical

Table 1 Summary of the number of mechanosensitive and chemosensitive units responding to 5-hydroxytryptamine (5-HT) and the minimal effective doses for these effects in normal and arthritic rats

			No. of units displaying each type of response		
	Units	n	*Rapid excitation*	*Delayed response*	*Minimal effective dose* (μg)
Normals	Mechanosensitive	6	4	3	100
	Chemosensitive	16	12	12	1
Arthritic	Mechanosensitive	10	3	5	1
	Chemosensitive	12	7	12	1

thresholds made them difficult to find. A summary of all units responding to 5-HT is given in Table 1.

Effects of 5-HT on responsiveness to mechanical stimulation In the six units examined 5-HT (1–100 μg) evoked a dose-dependent increase in responsiveness to the standard mechanical stimulus (illustrated for 100 μg in Figure 1). The minimal effective dose which gave reproducible responses in all four of the units tested in this way was found to be 5 μg. As illustrated in Figure 2 a mean peak increase of 56% ($n = 4$) in response to subsequent mechanical stimuli was observed following injection of 5 μg 5-HT, and this effect lasted for 38 $\pm$ 34 s. Larger doses of 5-HT had a more prolonged action, as can be seen in Figure 1 where the response to a mechanical stimulus was still elevated three minutes after the injection of 100 μg 5-HT. Repeated injections of 5-HT produced a sensitization to the drug in four mechanosensitive units.

Excitatory effects of 5-HT on spontaneous activity of mechanosensitive units Close arterial injection of 1–100 μg 5-HT evoked a discharge in three previously silent mechanoreceptors and enhanced the discharge of one unit from a very low initial level of discharge. The effect was reproducible in two of these units at the highest dose used (100 μg 5-HT). A biphasic response observed following injection of 5-HT consisted of a transient burst of activity (hereafter called a 'fast' response) with rapid onset (<10 s), followed by a delayed (>20 s), longer-lasting increase in discharge, hereafter called a 'slow' response (see Figure 3).

Normal rats – chemosensitive afferents

In the 12 normal animals examined in this study 16 recordings consisting of between one and three different action potential spikes were obtained from units with a low level of activity before the addition of 5-HT. Their action potential spike characteristics were similar to those of identified C-fibre afferents, and their mean rate of discharge was 1.4 $\pm$ 0.3 i.p.s.

Excitatory effects of 5-HT on chemosensitive units All the units with an ongoing discharge were excited by an initial or subsequent injection of 5-HT (1–100 μg, i.a.); a further three units became spontaneously active following the drug administration.

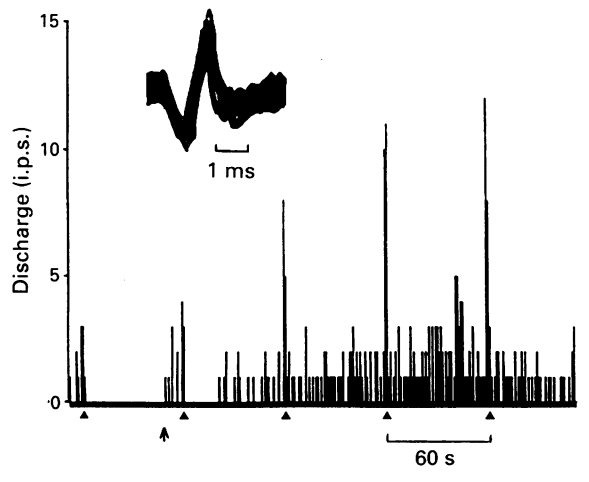

Figure 1 Effects of 100 μg 5-hydroxytryptamine (5-HT) on the activity of a high threshold mechanoreceptor with afferent fibre conduction velocity of 4 ms^{-1} from a normal animal. The graph (bin width 1 s) illustrates afferent discharge and shows fast and slow excitation. Mechanical stimuli (arrowheads) were repeated once every minute and neural responsiveness was increased following an injection of 5-HT (arrow). The inset shows 100 superimposed oscilloscope sweeps of the single afferent unit whose discharge was counted.

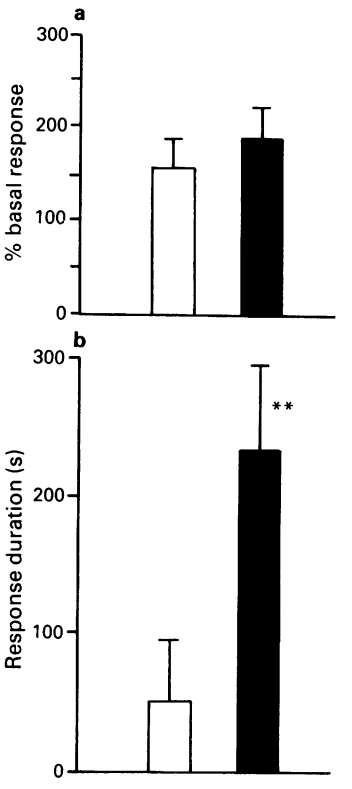

Figure 2 Effects of 5-hydroxytryptamine (5-HT) on mechanoreceptor responsiveness in both normal and arthritic rats. (a) The mean peak increase in mechanoreceptor responsiveness is shown as a percentage of preinjected control produced by 5 μg 5-HT (i.a.) in four normal rats (open column) and by 1 μg 5-HT (i.a.) in six arthritic rats (solid column). (b) Illustrates mean duration of increased responsiveness produced by the same injections of 5-HT (normal rats, open column; arthritics, solid column). Bars represent s.e. mean. ** $P < 0.01$ (Wilcoxon, two-tailed).

Two main components could be recognised in the response to 5-HT. An early, brief burst of activity, which was seen in 75% of active units, followed by a slow sustained increase in discharge in 75% of units. Responses in individual units were either monophasic or biphasic; fast responses occurred within 10 s following injection of 5-HT and lasted for a maximum of 30 s. Tachyphylaxis developed to repeated injections of 5-HT (20–100 μg at 10 min intervals; data not illustrated). However, with lower doses (1–10 μg) and a 15 min interval between injections, a relatively consistent response was obtained in four recordings. The slow response generally took longer than 15 s to develop and lasted for over 4 min in some cases. Depression of activity following the initial excitation was also seen in a small number of units when background activity was elevated.

Arthritic rats – mechanoreceptors

A characteristic feature of arthritic preparations, as previously described (Guilbaud *et al.*, 1985), was the large number of mechanoreceptors found in the joint. On average approximately three times as many mechanoreceptors were found in the ankle joint tissues of arthritic rats in comparison with control animals. These units had lower thresholds than normal for activation with von Frey hairs (52.7 $\pm$ 4.6 mN, $P < 0.05$) and generally had overlapping receptive fields; their conduction velocities (0.5–7.8 ms^{-1}) indicated that they were C/Aδ fibres. In contrast to the lack of spontaneous mechanoreceptor activity in normal rats, nine of the 10 mechanosensitive units studied showed spontaneous discharge, averaging 1.2 $\pm$ 0.4 i.p.s., before the administration of 5-HT.

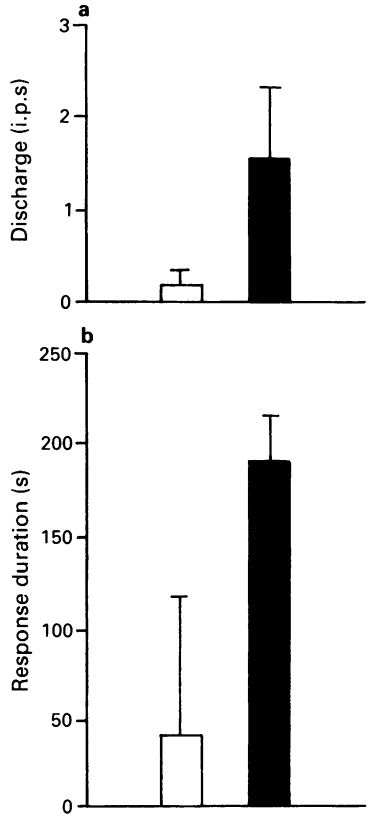

Figure 3 Comparison of 5-hydroxytryptamine (5-HT)-induced slow excitation of mechanosensitive units in normal and arthritic rats. (a) Shows the peak increase in discharge produced by 100 μg 5-HT (i.a.) in two responding units of six tested from normal rats (open column) and the mean peak increase produced by 1 μg 5-HT in four out of five units from arthritics (solid column). The mean basal rate of discharge in arthritic rats was 1.1 $\pm$ 0.8 i.p.s., whereas in control rats no activity was present before the injection of 5-HT. (b) Shows the duration of effects for the same injections as in (a). Open column, represents data from the two individual units which were excited by 5-HT in normal rats. Solid column represents mean of four units from arthritic rats. Bars show s.e.mean.

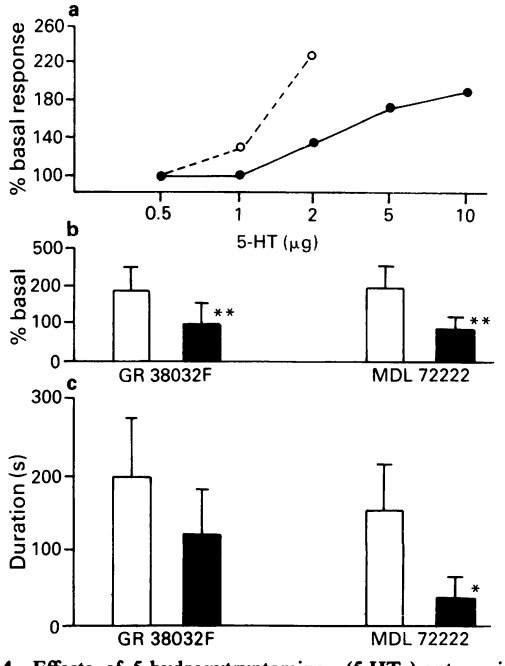

Figure 4 Effects of 5-hydroxytryptamine$_3$ (5-HT$_3$)-antagonists GR 38032F (100 μg kg^{-1}, i.a.), MDL 72222 (100 μg kg^{-1}, i.a.) and ICS 205-930 (100 μg kg^{-1} i.a.) on 5-HT-induced enhancement of mechanoreceptor responsiveness in both normal and arthritic rats. (a) Shows a shift to the right of the log dose-response curve caused by ICS 205-930 (100 μg kg^{-1} i.a.) in a high threshold mechanoreceptor with afferent fibre conduction velocity of 2.7 ms^{-1} from an arthritic rat. (○) and (●) responses before and after addition of antagonist, respectively. The dose of 5-HT is shown in μg with the peak response obtained given as a percentage of the preinjection control. (b) Illustrates the effect of GR 38032F (n = 5) and MDL 72222 (n = 5) on the mean peak increase in mechanoreceptor responsiveness produced by an effective standard dose of 5-HT (1–100 μg i.a.). Columns represent peak responses before (open) and after (solid) injection of antagonist. (c) The duration of response obtained for the same injections as in (a). Bars represent s.e.mean. Significantly different mean values are shown as: * $P < 0.05$ and ** $P < 0.01$.

Effects of 5-HT on responsiveness to mechanical stimulation In all 10 units examined a dose-dependent increase in responsiveness to the standard mechanical stimulus was obtained following the injection of 5-HT (1–100 μg). In seven units the effective threshold dose for production of consistent responses was found to be 1 μg. A mean increase of 75% (n = 6) in response to subsequent mechanical stimuli was produced following injection of the threshold dose. This effect lasted for 240 $\pm$ 48 s, a duration which is significantly greater ($P < 0.01$) than that produced by 5 μg 5-HT in normal rats (Figure 2). Sensitization of mechanoreceptor responses to 5-HT was observed in two units.

Excitatory effects of 5-HT on activity of mechanosensitive units Increases in spontaneous activity of six mechanosensitive units was seen following injection of 5-HT (1–100 μg). The biphasic response seen in normal animals was much less conspicuous, being obtained in only one recording. One unit gave a fast and slow response, and another unit displayed only the fast response. Three units responded with only a slow increase in spontaneous activity. In four of the units the slow response consisted of a mean peak increase of 1.6 $\pm$ 0.6 i.p.s. above basal discharge (1.1 $\pm$ 0.7 i.p.s.) which lasted for 185 $\pm$ 34 s. This effect of 5-HT contrasts markedly with the small response obtained following injection of 100 μg 5-HT in the normal rat (Figure 3). Spontaneous activity in a seventh unit was depressed following 5-HT administration.

Arthritic rats – chemosensitive units

Chemosensitive units Spontaneously active units lacking any identifiable mechanosensitive receptive fields were more numerous in arthritic rats than in controls. In experiments on 10 animals the effects of 5-HT on spontaneous discharge were examined in 12 recordings consisting of between one and three different units with action potentials characteristic of identified C-fibre afferents. Their mean rate of spontaneous discharge before the administration of 5-HT was 1.4 $\pm$ 0.2 i.p.s.

Excitatory effects of 5-HT on chemosensitive units All the units examined were responsive to injections of 5-HT (1–100 μg). A biphasic response was seen, as in normal rats. A fast excitatory response was seen in 58% of units, followed in all the units studied by a slow long lasting increase in discharge.

Effects of 5-HT-receptor antagonists in normal and in arthritic rats

The 5-HT receptor antagonists, MDL 72222, ICS 205-930 and ketanserin, were administered at 100 μg kg^{-1} i.a., doses previously found to be active in abolishing chemoreceptor responses to 5-HT in the cat (Kirby & McQueen, 1984). In the present experiments the 5-HT$_3$-receptor antagonists selectively blocked the 5-HT$_3$-receptor-mediated Bezold-Jarisch-like reflex evoked by 5-HT, and ketanserin selectively antagonized 5-HT-induced hypotension.

Table 2 Summary of the effects of 5-hydroxytryptamine (5-HT)-receptor antagonists on spontaneous discharge of chemosensitive units

	MDL 72222 ($100\,\mu g\,kg^{-1}$)		*ICS 205-930* ($100\,\mu g\,kg^{-1}$)		*GR 38032F* ($100\,\mu g\,kg^{-1}$)		*Ketanserin* ($100\,\mu g\,kg^{-1}$	
	n	% *reduction in discharge*	n	% *reduction in discharge*	n	% *reduction in discharge*	n	% *reduction in discharge*
Normals	4/4	53 (43–68)	3/3	62 (33–100)	4/4	53 (64–99)	5/9	63 (43–86)
Arthritic	1/4	100			4/4	36.5 (13–50)	6/9	58 (76–25)

Mean values are given – figures in parentheses show range of effect.
n = number of units.

Mechanoreceptor responsiveness

The 5-HT_3-receptor antagonists, MDL 72222, ICS 205-930 and GR 38032F, administered intra-arterially, each antagonized the 5-HT-induced sensitization of mechanoreceptors to mechanical stimuli in both normal and arthritic rats. In five units, treatment with MDL 72222 ($100\,\mu g\,kg^{-1}$) markedly reduced the increased responsiveness produced by 5-HT; injection of ICS 205-930 ($100\,\mu g\,kg^{-1}$) produced a clear rightward shift in the 5-HT dose-response curve in one unit. In studies on five units, GR 38032F ($100\,\mu g\,kg^{-1}$) abolished the response in one unit and produced a marked reduction in the response in the other four units (see Figure 4).

The 5-HT_2-receptor antagonist, ketanserin ($100\,\mu g\,kg^{-1}$), did not affect the 5-HT-evoked increase in mechanoreceptor responsiveness, either in normal or in arthritic rats when tested in six units.

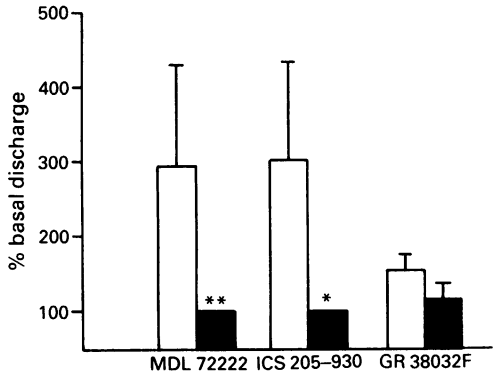

Figure 5 Effects of MDL 72222 ($100\,\mu g\,kg^{-1}$ i.a., $n = 6$), GR 38032F ($100\,\mu g\,kg^{-1}$ i.a., $n = 4$) and ICS 205-930) ($100\,\mu g\,kg^{-1}$ i.a., $n = 4$) on 5-hydroxytryptamine (5-HT)-induced fast excitation in chemosensitive units from both normal and arthritic rats. Each column shows the mean peak response as a percentage of basal discharge to a standard effective dose of 5-HT (5–$100\,\mu g$ i.a.) before (open) and after (solid) injection of antagonist. Bars represent s.e.mean. * $P < 0.05$ and ** $P < 0.01$.

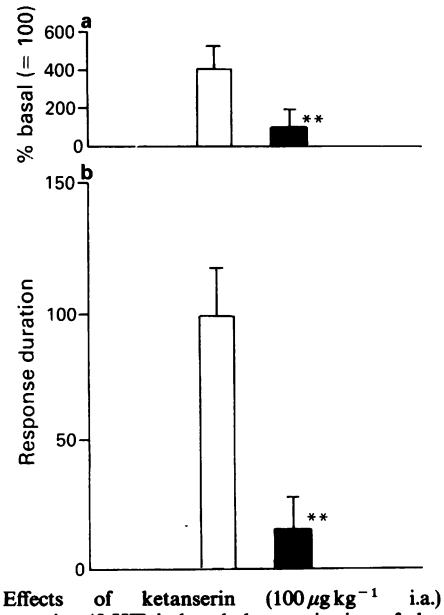

Figure 6 Effects of ketanserin ($100\,\mu g\,kg^{-1}$ i.a.) on 5-hydroxytryptamine (5-HT)-induced slow excitation of chemosensitive afferent units from normal and arthritic rats. (a) The mean peak discharge expressed as a percentage of preinjection control produced in response to a standard dose of 5-HT (1–$100\,\mu g$ i.a.) before (open column) and after (solid column) injection of ketanserin ($100\,\mu g\,kg^{-1}$, i.a., $n = 14$). (b) The mean duration of the effects shown in (a). Bars represent s.e.mean. ** $P < 0.01$.

Spontaneous activity of mechanosensitive units

In spontaneously active mechanosensitive units from arthritic rats, MDL 72222 ($100\,\mu g\,kg^{-1}$, $n = 1$) or GR 38032F ($100\,\mu g\,kg^{-1}$, $n = 2$) caused reductions in ongoing activity of 70% and 65% respectively when injected on their own. Reductions of activity lasted for no longer than 5 min in each case. In two mechanosensitive units, ketanserin ($100\,\mu g\,kg^{-1}$) had no effect on spontaneous activity.

An examination of the effects of the various 5-HT receptor antagonists on the fast and slow components of 5-HT-induced increases in spontaneous activity was complicated by the inconsistent nature of the fast response and its marked susceptibility to tachyphylaxis. The slow response, however, was consistently observed in all cases, and in arthritic animals neither MDL 72222 ($100\,\mu g\,kg^{-1}$, $n = 1$), ICS 205-903 ($100\,\mu g\,kg^{-1}$, $n = 1$) nor GR 38032F ($100\,\mu g\,kg^{-1}$, $n = 1$) had any affect on it. In only one out of two units did ketanserin cause a shift to the right of the 5-HT dose-response curve.

Chemosensitive units

In the case of spontaneously active chemosensitive units, MDL 7222, ICS 205-930 and GR 38032F all reduced ongoing discharge in arthritic rats as well as in normal animals previously exposed to 5-HT (see Table 2). Reductions in activity produced by antagonists lasted for 1–3 min. Ketanserin also markedly reduced ongoing discharge in arthritic and normal rats (Table 2).

Analysis of the effects of 5-HT-receptor antagonists on the fast response to injection of 5-HT were complicated by inconsistency of the response and its susceptibility to tachyphylaxis. However, it was markedly reduced or abolished following injections of the 5-HT_3-receptor antagonists MDL 72222 ($n = 6$), ICS 205-930 ($n = 4$) and GR 38032F ($n = 4$) (see Figure 5).

The slow response to injection of 5-HT was unaffected by the 5-HT_3-receptor antagonists, but in 14 out of 15 recordings was blocked or markedly reduced by ketanserin ($100\,\mu g\,kg^{-1}$) (see Figure 6).

Discussion

This investigation has shown that exogenous 5-HT can both excite and increase the responsiveness of sensory receptors

with fine (C, possibly some Aδ) afferents located in the ankle joint tissues of normal and arthritic rats. High threshold nociceptive mechanoreceptors were affected by 5-HT in both normal and arthritic animals, the effect of the amine on arthritic joints being more marked. The units recorded from arthritic joints had spontaneous activity, in contrast with the general lack of activity in units from normal joints. These findings are in good agreement with those obtained previously for the inflamed ankle joint (Guilbaud et al., 1985). Furthermore, background discharge originating from chemosensitive units for which no mechanosensitive receptive fields could be found was greater in arthritic rats. These units were activated by 5-HT or capsaicin and responded to 5-HT with two components – fast brief excitation followed by a slow prolonged increase in activity. The effects of exogenous 5-HT on joint sensory receptors are quire similar to those on cat carotid chemosensors, where fast and slow excitatory effects involving 5-HT_3- and 5-HT_2-receptors, respectively, have been obtained (Kirby & McQueen, 1984).

Action of 5-HT on mechanoreceptors

Single close-arterial bolus injections of 5-HT increased the responses of high threshold mechanoreceptors to a standard mechanical stimulus for as long as six minutes. This duration of action is similar to that obtained for 5-HT-induced sensitization of high threshold mechanoreceptors in muscle to excitation induced by bradykinin (Mense, 1981), as well as for the action of 5-HT on SAII cutaneous mechanoreceptors (Fjallbrant & Iggo, 1961). MDL 72222, ICS 205-930 or GR 38032F prevented this action, but did not otherwise affect the response to mechanical stimuli. Ketanserin was without effect. These results suggest that the sensitization demonstrated may involve the action of 5-HT at a 5-HT_3-receptor located on the mechanoreceptor terminals within the joint tissues.

Fast excitation

Brief excitation of mechanosensitive units and chemosensitive units occurred within 10s of the injection of 5-HT in both normal and arthritic joints. This action was blocked or reduced by the 5-HT_3-receptor antagonists. Fast depolarization evoked by 5-HT has been observed in several isolated neuronal preparations. For example, in cat and rabbit superior cervical ganglion (Haefely, 1974; Wallis & North, 1978), rabbit nodose ganglion (Higashi & Nishi, 1982) and guinea-pig coeliac ganglion (Wallis & Dun, 1988) 5-HT produced a rapid depolarization which was prone to tachyphylaxis and was sensitive to MDL 72222 or ICS 205-950 (Azami et al., 1985; Round & Wallis, 1986; 1987; Wallis & Dun, 1988).

Slow excitation

The most consistent response to 5-HT was a slow dose-dependent long-lasting increase in discharge that was seen in the majority of the chemosensitive units examined, as well as in mechanosensitive units from normal and arthritic rats. The 5-HT_3-receptor antagonists had no effect on this slow excitation, whereas in the case of chemosensitive units ketanserin reduced or abolished it. The delayed nature of this effect could mean that 5-HT is acting indirectly to increase afferent activity. In our preparation, slow excitation was dose-dependent and outlasted the hypotensive effect of 5-HT, thus making it unlikely to be secondary to changes in blood pressure. Alternative mechanisms could include the involvement of a second messenger system in the afferent nerve terminal or the release of other algogenic substances from surrounding tissues by 5-HT. Evidence for a direct effect is suggested from studies on isolated neuronal preparations where a slow response produced by 5-HT has also been described (Kiraly et al., 1983; Dun et al., 1984).

The finding that long-lasting mechanoreceptor sensitization involves a 5-HT_3-receptor, whereas delayed excitation does not, suggests that separate mechanisms may be involved in receptor sensitization and 5-HT-induced excitation. This may relate to differing transduction pathways for mechanically- or chemically-evoked activation of sensory nerve endings.

Involvement of 5-HT in sensitization of sensory receptors during inflammation

The ability of 5-HT to sensitize high threshold articular mechanoreceptors suggests that endogenous 5-HT could play a role in the increased responsiveness of these receptors in chronically inflamed joints. Our results indicate that a 5-HT_3-receptor may be involved in this process. However, in arthritic rats the administration of antagonists selective for 5-HT_3- and 5-HT_2-receptors did not reduce mechanoreceptor sensitivity significantly, which they should have done if endogenous 5-HT acting at these receptors was a significant cause of sensitization. Low levels of spontaneous activity in mechanosensitive units recorded from arthritic joints were, on the other hand, reduced markedly by the addition of 5-HT_3-receptor antagonists. Similar results were obtained for chemosensitive units following administration of both 5-HT_3- and 5-HT_2-receptor antagonists. These observations suggest that while endogenous 5-HT may contribute to ongoing neural activity seen in inflamed joints, it is not a major factor in the sensitization of afferents.

In mechanoreceptors recorded from arthritic joints, with already enhanced mechanosensitivity, responsiveness to 5-HT was much greater than in normal joints, providing clear evidence that sensitivity of these sensors to 5-HT is increased in inflamed joints, and showing that the acute release of endogenous 5-HT could further boost sensitivity. The induction of high threshold mechanoreceptor sensitization and fast excitation by 5-HT, via a 5-HT_3-receptor, is consistent with the observation that pain produced by application to 5-HT to a blister base is mediated through a 5-HT_3-receptor (Donatsch et al., 1984; Richardson et al., 1985), assuming that the discharge recorded from the ankle joint afferents is involved in nociception. A role for 5-HT_2-receptors in 5-HT-induced pain has not previously been described, and further studies may be warranted in view of our results showing that 5-HT_2- and 5-HT_3-antagonists reduced afferent discharge in arthritic rats.

Finally, a role for 5-HT in the development of acute inflammatory pain has been suggested by Eschalier et al. (1989), who have shown that the administration of ICS 205-930 inhibits and reverses carrageenan-induced hyperalgesia in rats. It may be that endogenous 5-HT, released from platelets (Page, 1988), mast cells (Johnson & Erdos, 1973) or nerve fibres (Williams, 1967; Verhofstad et al., 1981) is responsible for development of sensitization during the acute inflammatory response and become less important for chronic sensitization. However, further (acute) release of 5-HT may cause additional short-lasting sensitization of sensory receptors in chronic arthritis.

This work was supported by a grant from the Arthritis Research Council. G.J.B. is an SERC-CASE research scholar.

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(Received March 30, 1990
Revised July 6, 1990
Accepted July 12, 1990)

Br. J. Pharmacol. (1990), **101**, 722–726

Endothelin and a Ca^{2+} ionophore raise cyclic GMP levels in a neuronal cell line via formation of nitric oxide

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1 The vasoconstrictor peptide endothelin-1 caused a fast, transient rise in guanosine 3':5'-cyclic monophosphate (cyclic GMP) levels in a neuronal cell line (mouse neuroblastoma x rat glioma hybrid cells 108CC15). The mechanism of activation of guanylate cyclase by endothelin-1 was investigated. The endothelin-1-induced rise depended on the release of internal Ca^{2+}.

2 The stimulation of cyclic GMP synthesis induced by endothelin-1 was suppressed after preincubating the cells in medium containing haemoglobin (IC_{50} 3 μM). Similarly, pretreatment of the cells with the L-arginine analogues, L-canavanine (IC_{50} 60 μM) or N^G-monomethyl-L-arginine (IC_{50} 2.5 μM), inhibited the cyclic GMP response to endothelin-1. Therefore, endothelin-1 activates guanylate cyclase most probably via formation of nitric oxide, which is released from L-arginine.

3 The Ca^{2+} ionophore ionomycin induced a transient rise in cyclic GMP levels, which was also suppressed by preincubation in the presence of either haemoglobin or the L-arginine analogues L-canavanine or N^G-monomethyl-L-arginine. Therefore, we conclude that ionomycin can activate guanylate cyclase by a mechanism involving nitric oxide formation, similar to that induced by endothelin-1.

4 The alkaloid veratridine, which activates Na^+ channels and also causes influx of Ca^{2+} induced a transient rise of cyclic GMP levels in the neuronal cell line. This stimulation was blocked by pretreating the cells with L-canavanine, N^G-monomethyl-L-arginine or haemoglobin.

5 Loading the cells with the Ca^{2+} chelator BAPTA suppresed the cyclic GMP response to application of endothelin-1, ionomycin, or veratridine. Thus, in the neuronal cell line a rise in cytosolic Ca^{2+} activity seems to be sufficient to stimulate the nitric oxide forming enzyme which synthesizes the activator of soluble guanylate cyclase.

Introduction

Several hormones and neurotransmitters raise guanosine 3':5'-cyclic monophosphate (cyclic GMP) levels in neural cell lines (Snider & Richelson, 1984; Reiser *et al.*, 1984; McKinney, 1987). Among the neurotransmitter receptors coupled to soluble guanylate cyclase are those for acetylcholine (muscarinic), histamine, 5-hydroxytryptamine and various peptides like neurotensin and bradykinin (Waldman & Murad, 1987). The functional consequences of increases in cyclic GMP levels in neural tissue are largely unknown. Apart from the well established function of cyclic GMP in the visual transduction process, there is little evidence of a role of cyclic GMP in regulation of ion channels (Stryer, 1986).

Furthermore, the mechanism of activation of guanylate cyclase by the diverse neurotransmitter receptors is still a matter of controversy. It has been suggested that arachidonic acid metabolites formed by lipoxygenase activity are involved in activation of guanylate cyclase (Snider *et al.*, 1984; McKinney & Richelson, 1986; Friedl, 1986; McKinney, 1987). However, recently it has been established that endothelium-derived relaxing factor (EDRF, Furchgott & Vanhoutte, 1989), which has been identified as nitric oxide (Palmer *et al.*, 1987), is active in a variety of systems in which soluble guanylate cyclase is stimulated (Moncada *et al.*, 1989). Thus, nitric oxide or a related nitroso compound seems to be a second messenger of widespread significance (Knowles *et al.*, 1989).

Here, evidence is presented that the vasoconstrictor peptide endothelin-1 (Yanagisawa *et al.*, 1988) induces a rise in cyclic GMP levels in a neuronal cell line mediated by nitric oxide or a related nitroso compound. Moreover, it is shown that the Ca^{2+} ionophore ionomycin causes a transient rise in cyclic GMP levels, which is also due to formation of nitric oxide. Veratridine, which indirectly induces influx of Ca^{2+} into cells is demonstrated to stimulate cyclic GMP synthesis by the same mechanism.

Methods

Measurement of cyclic GMP levels

Mouse neuroblastoma x rat glioma hybrid cells, clone 108CC15 of passage numbers between 14 and 32, were cultured as described (Hamprecht *et al.*, 1985). Cells were seeded at a density of 3 to 4×10^5 cells in plastic Petri dishes (diameter 50 mm) and grown for 2 or 3 days. To start the experiment, the growth medium was removed and the cells were washed twice with 2 ml incubation medium containing (mM): NaCl 145, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1.0, Na_2HPO_4 2.0, glucose 20 and HEPES 20, adjusted to pH 7.4 with Tris, osmolarity 320–350 mOsmol l^{-1}. Cells were preincubated at 37°C in 2 ml incubation medium for a period of 25 to 35 min allowing the cells to equilibrate. This preincubation has been found to enhance the sensitivity of the hybrid cells to peptides stimulating cyclic GMP levels (Friedl, 1986). Reactions were started by adding 20 μl of a concentrated stock solution of the compounds to be tested, dissolved in incubation medium or in H_2O. Veratridine was added as a 0.4 mM solution to give final concentrations between 0.1 and 0.2 mM. Usually after 20 s, reactions were stopped by adding 1 ml ethanol. Cellular content of cyclic GMP was determined with incubations carried out in duplicate by radioimmunoassay as described by Reiser *et al.* (1984), and referred to cellular protein, determined by the Lowry method using bovine serum albumin as standard. Oxyhaemoglobin was prepared by adding a molar excess of dithionite to a solution of haemoglobin, bubbling through with O_2 and desalting by Sephadex G-25 (Pharmacia, Uppsala, Sweden) chromatography. The stability of oxyhaemoglobin was monitored spectrophotometrically after the experiment (absorption maxima at 576, 540 and 410 nm).

Materials

Bradykinin triacetate, the bradykinin BK_2 antagonist [$Thi^{5,8}$, D-Phe^7]-bradykinin (H-Arg-Pro-Pro-Gly-Thi-Ser-D-Phe-Thi-

Arg-OH), haemoglobin (bovine), veratridine and canavanine were from Sigma (München, F.R.G.); endothelin (endothelin-1) from Bachem Biochemica (Heidelberg, F.R.G.); ionomycin and N^G-monomethyl-L-arginine were from Calbiochem (Frankfurt, F.R.G.); BAPTA (*bis*(O-aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid)/acetoxymethylester was from Molecular Probes (Eugene, OR, U.S.A.). D888 (desmethoxyverapamil) was kindly provided by Dr Traut from Knoll A.G. (Ludwigshafen, F.R.G.). All other chemicals, of analytical grade, were purchased from E. Merck (Darmstadt, F.R.G.) or Sigma (Deisenhofen, F.R.G.).

Results

Endothelin-1 induced a rise in cyclic GMP levels in the neuroblastoma x glioma hybrid cells. The cyclic GMP levels rose to a maximum within 30 s after addition of the peptide and declined sharply thereafter (Figure 1a). The baseline value was reached again 60 s after the beginning of the challenge. The concentration-response curve (Figure 1b) shows that endothelin-1 raises cyclic GMP levels at concentrations above 10 nM, with a half-maximal value at 55 nM. The maximum was reached at concentrations above 200 nM.

The capacity of the cells to respond to endothelin-1 was lost at extracellular Ca^{2+} concentrations below 100 μM (Figure 1c) when cells were preincubated in media with various Ca^{2+} concentrations for 30 min. However, after only 5 min preincubation in medium with 10 and 50 μM Ca^{2+} the stimulation by 100 nM endothelin-1 was still 51 and 76% of that under control conditions, respectively (data not shown).

In a series of experiments the neuronal cells were preloaded with the Ca^{2+} chelator BAPTA. The cells were preincubated for 30 min in the presence of varying concentrations of the membrane permeant analogue BAPTA acetoxymethylester, which is cleaved intracellularly. Thus, free BAPTA accumulates in the cells. Subsequent challenges with endothelin-1 resulted in reduced rises in cyclic GMP levels. Half-maximal inhibition was seen at 2.5 μM BAPTA in the incubation medium (not illustrated).

When the cells were pretreated for 20 to 30 min in incubation medium containing the Ca^{2+} ionophore ionomycin at concentrations below 1 μM (Figure 1d(iv)) the cells could no longer be stimulated by endothelin-1. The comparable inhibition of bradykinin-stimulated cyclic GMP increase by pretreatment with ionomycin is also displayed in Figure 1d(ii). Addition of the organic Ca^{2+} channel antagonist D888 (desmethoxyverapamil) did not affect the cyclic GMP response to endothelin-1 at concentrations up to 50 μM (Figure 1d(iii)). The neuroblastoma x glioma hybrid cells display functional receptors for the neuropeptide bradykinin (Reiser & Hamprecht, 1985; Reiser *et al.*, 1990). [Thi^{5,8}, D-Phe^7]-bradykinin, a BK_2 receptor antagonist, at 10 μM suppressed the effect of bradykinin almost completely, but did not affect the stimulation by endothelin-1 (Figure 1d(iv)).

Methylene blue, an agent which inhibits soluble guanylate cyclase (Waldman & Murad, 1987), blocked the rise in cyclic GMP levels induced by 100 nM endothelin-1 (Figure 2a), half-maximally at 4 ± 1 μM (mean ± s.d., $n = 3$). Oxyhaemoglobin blocked the rise of cyclic GMP levels induced by endothelin-1 (Figure 2b) with half-maximal effect at 3 ± 1 μM. Preincubating the hybrid cells with the arginine analogue L-canavanine suppressed the ability of endothelin-1 to raise the cyclic GMP levels (Figure 2c) with an IC_{50} value of 60 ± 36 μM ($n = 3$). N^G-monomethyl-L-arginine blocked the rise in cyclic GMP levels caused by endothelin-1 (Figure 2c) with an IC_{50} of 2.5 ± 1.4 μM ($n = 4$).

The Ca^{2+} ionophore ionomycin at concentrations above 100 nM caused a transient rise in cyclic GMP levels in the hybrid cells. The maximum rise was obtained 20 s after addition of ionomycin (Figure 3, a–c). Figure 3 also demonstrates that the stimulation by ionomycin could be suppressed by L-

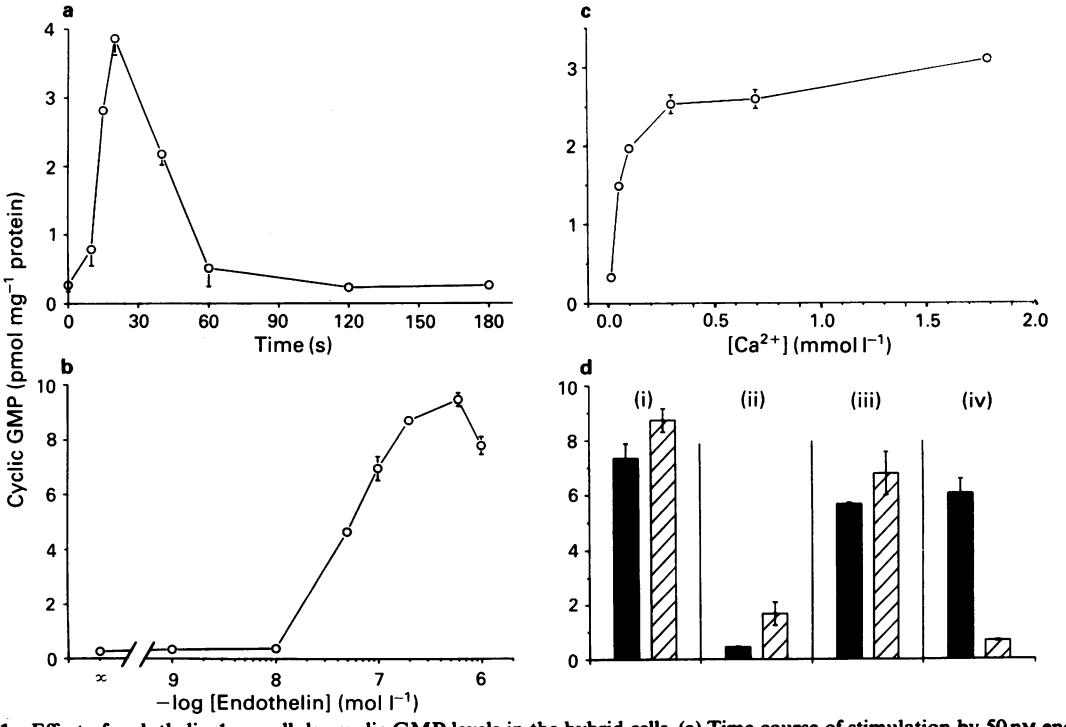

Figure 1 Effect of endothelin-1 on cellular cyclic GMP levels in the hybrid cells. (a) Time course of stimulation by 50 nM endothelin-1. (b) Concentration-effect curve. Cells were challenged with various concentrations of endothelin-1 for 20 s. In (a) and (b) after a 30 min preincubation in incubation medium, endothelin-1 was added and cellular cyclic GMP levels were determined as described in Methods. (c) Influence of extracellular Ca^{2+} concentration on stimulation of cyclic GMP levels by endothelin-1. Cells were preincubated for 30 min in medium containing various Ca^{2+} concentrations. Then the cells were challenged with 100 nM endothelin-1 for 20 s. (d) Stimulation of cyclic GMP levels by 100 nM endothelin-1 (solid columns) or 100 nM bradykinin (hatched columns). Before exposure to the peptides the cells were preincubated for 30 min in incubation medium (i) without addition for control, or supplemented with (ii) 100 nM ionomycin, (iii) 50 μM D888, or (iv) 10 μM bradykinin BK_2 antagonist [Thi^{5,8}, D-Phe^7]-bradykinin (H-Arg-Pro-Pro-Gly-Thi-Ser-D-Phe-Thi-Arg-OH). (a) and (b) are taken from the same experiment. Here and in Figures 2 and 3 typical results are shown, which are representative of at least two experiments with comparable results. Basal content of cyclic GMP was 0.3 ± 0.2 pmol mg^{-1} protein.

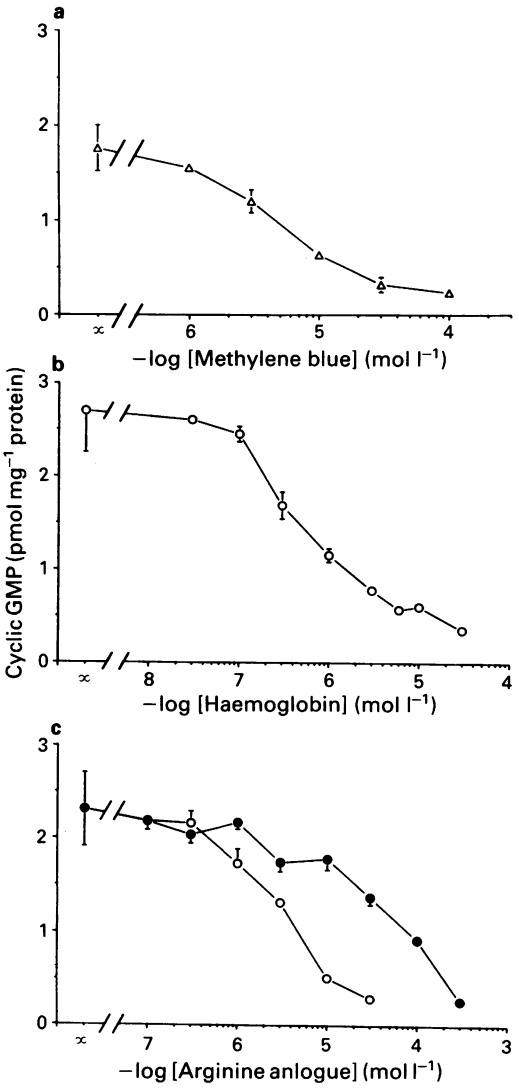

Figure 2 Inhibition by methylene blue (a), oxyhaemoglobin (b) and the arginine analogues (c) N^G-monomethyl-L-arginine (○) and canavanine (●), of cyclic GMP responses of hybrid cells induced by 100 nM endothelin-1. Various concentrations of methylene blue were added during the preincubation. Haemoglobin and L-arginine analogues were given for the last 2.5 min of the preincubation period, and subsequently the cells were challenged with endothelin-1 for 20 s.

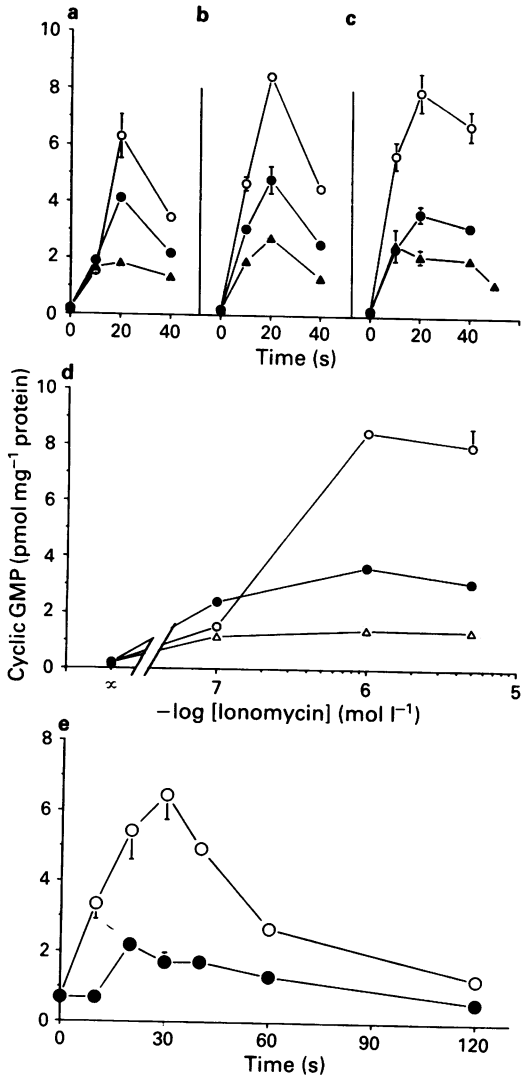

Figure 3 Influence of ionomycin (a–d) and veratridine (e) on cyclic GMP levels in the hybrid cells. Cells were challenged for the periods of time indicated on the abscissa scale in incubation medium containing ionomycin at concentrations of 0.1 μM (a), 1 μM (b) and 5 μM (c) in the absence (○) or presence of 100 μM canavanine (●) or 10 μM N^G-monomethyl-L-arginine (△). (d) Rise of cyclic GMP levels induced by various concentrations of ionomycin (challenge incubation period 20 s) without addition (○) or with 1 μM (●) or 10 μM haemoglobin (△). (e) Time course of stimulation of cyclic GMP level by 0.2 mM veratridine. Cells were tested after 30 min preincubation in medium without addition (○) or with addition of 6 μM of N^G-monomethyl-L-arginine (●) for the last 2.5 min of the preincubation period.

canavanine or by N^G-monomethyl-L-arginine. Half-maximal inhibitory activity was seen at 6 ± 5 μM ($n = 3$) for N^G-monomethyl-L-arginine and at 60 ± 50 μM ($n = 3$) for L-canavanine (data not shown). The rise in cyclic GMP levels caused by ionomycin was also inhibited by incubating the cells in the presence of oxyhaemoglobin (Figure 3d) with half-maximal inhibition at 0.6 μM ($n = 2$).

Veratridine (0.2 mM) induced a rise in cyclic GMP levels in the neuronal cell line with the maximum reached after 30 s (Figure 3e). The decline towards the basal levels was slower than following stimulation with endothelin-1 such that there was still a significant elevation of cyclic GMP levels at 60 s. As can also be seen in Figure 3e, N^G-monomethyl-L-arginine (6 μM) blocked the veratridine-induced rise in cyclic GMP levels. Half-maximal inhibition was obtained at 3 ± 2 μM N^G-monomethyl-L-arginine ($n = 3$) and 50 ± 45 μM canavanine ($n = 3$). The effect of 0.2 mM veratridine was also suppressed by haemoglobin (IC_{50} 0.5 μM) or by pretreating the cells with BAPTA (IC_{50} 4 μM; data not shown).

Discussion

In a neuronal cell line endothelin-1 activates guanylate cyclase with a time course similar to the effects of bradykinin (Reiser *et al.*, 1984) on BK_2 receptors and of 5-hydroxytryptamine on 5-HT_3 receptors (Reiser & Hamprecht, 1989; Reiser, 1990). Endothelin-1 does not act on the bradykinin receptors since a BK_2 receptor antagonist suppressed the stimulation by bradykinin but not by endothelin. Blockade by haemoglobin or by methylene blue indicates that the mediator between activated endothelin-1 receptor and soluble guanylate cyclase resembles EDRF, which has been identified as nitric oxide (Palmer *et al.*, 1987). Haemoglobin seems to scavenge an activating substance that is released into the extracellular space after challenge with endothelin-1. Thus a diffusible factor released from the cells could act in a paracrine manner, as suggested for cerebellar granule cells exposed to N-methyl-D-asparate (Garthwaite *et al.*, 1988).

Nitric oxide or a nitroso compound has been reported to be formed enzymatically from terminal guanidino nitrogen of L-arginine (Schmidt *et al.*, 1988; Palmer *et al.*, 1988; Moncada *et al.*, 1989). This synthesis of nitric oxide can be suppressed by structural analogues of L-arginine, such as N^G-monomethyl-L-

arginine and L-canavanine (Schmidt *et al.*, 1988). Since both analogues effectively block the rise of cyclic GMP levels caused by endothelin-1, formation of nitric oxide from L-arginine appears to be involved in the action of endothelin-1 in the hybrid cells.

Endothelin-1 raises cytosolic Ca^{2+} activity transiently for about 1 min in the neuronal hybrid cells loaded with the Ca^{2+}-sensitive dye fura-2 (Reiser & Donié, 1990) as in glial cells (Ashley *et al.*, 1989). The rise in cytosolic Ca^{2+} activity is mediated by release from internal stores through inositol 1,4,5-trisphosphate as we have demonstrated by the following findings: (i) Endothelin-1 caused a rise of cytosolic Ca^{2+} activity even in the absence of external Ca^{2+} and (ii) depletion of internal Ca^{2+} stores by ionomycin abolished the Ca^{2+} effect of endothelin-1 (Reiser & Donié, 1990).

The stimulation of guanylate cyclase by endothelin-1 depends on a rise of cytosolic Ca^{2+} activity, since buffering of cytosolic Ca^{2+} by BAPTA and depletion of internal stores by pretreatment with the Ca^{2+} ionophore, ionomycin, suppressed the cyclic GMP response (Figure 1d(ii)). At the low concentrations used the Ca^{2+} ionophore primarily depletes internal Ca^{2+} stores and prevents their refilling (Pollock *et al.*, 1987; Reiser *et al.*, 1989; 1990). The inhibition seen at low extracellular Ca^{2+} concentrations (Figure 1c) seems to be due to a depletion of internal Ca^{2+} stores, since the size of inhibition decreases with shortening the length of the preincubation period in Ca^{2+}-depleted medium.

A rise in cytosolic Ca^{2+} activity appears to be the crucial step for activation of guanylate cyclase in the neuronal hybrid cells, since buffering intracellular Ca^{2+} by the chelator BAPTA (Grynkiewicz *et al.*, 1985) suppressed the stimulation either by endothelin-1, ionomycin or veratridine. Cyclic GMP synthesis is activated similarly by bradykinin via BK_2 receptors which primarily release Ca^{2+} from internal stores (Reiser *et al.*, 1990), or by 5-hydroxytryptamine via 5-HT_3 receptors (Reiser & Hamprecht, 1989), which induce influx of extracellular Ca^{2+} (Reiser *et al.*, 1989).

Here we show that the Ca^{2+} ionophore, ionomycin, causes a transient rise of cyclic GMP levels. The time course is comparable to that of the rise of cytosolic Ca^{2+} activity induced by ionomycin in the hybrid cells (Reiser *et al.*, 1989). The mechanism of activation of guanylate cyclase by ionomycin seems to employ nitric oxide, like the activation by the hormones bradykinin, 5-hydroxytryptamine (Reiser, 1990) or endothelin-1 (shown here). If the Ca^{2+} ionophore has no direct effect on any enzymatic activity in the neuronal cells, we can conclude that a rise in cytosolic Ca^{2+} activity seems to be sufficient to activate the nitric oxide forming enzyme. The Ca^{2+}-requirement of the cytosolic enzyme which forms nitric oxide (Knowles *et al.*, 1989) is due to the influence of calmodulin (Bredt & Snyder, 1990). Further support for our interpretation comes from the ability of veratridine to increase cyclic GMP levels in the hybrid cells (Figure 3). The alkaloid veratradine, which activates voltage-dependent Na^+ channels (Ulbricht, 1969) and induces oscillatory depolarizations of the membrane of the hybrid cells (Reiser & Hamprecht, 1983), indirectly causes a rise in Ca^{2+} influx in neuroblastoma cells (Freedman *et al.*, 1984).

Thus, all the agents studied here, which raise cyclic GMP levels, namely endothelin-1, ionomycin and veratridine, cause a rise in cytosolic Ca^{2+} activity. The increase in cytosolic Ca^{2+} activity seems to be a prerequisite for the cascade of nitric oxide formation and subsequent activation of guanylate cyclase. The cascade can be interrupted either by chelating cytosolic Ca^{2+} or by blocking nitric oxide formation.

The expert technical assistance of Britta Baumann is gratefully acknowledged. This work was supported by a grant from the Deutsche Forschungsgemeinschaft (Re 563/2-2).

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(Received May 31, 1990
Revised July 3, 1990
Accepted July 5, 1990)

Br. J. Pharmacol. (1990), **101**, 727–733

Actions of capsaicin on peripheral nociceptors of the neonatal rat spinal cord-tail *in vitro*: dependence of extracellular ions and independence of second messengers

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1 We have tested the hypothesis that capsaicin-induced activation, desensitization and impairment of peripheral nociceptor function is mediated by separate mechanisms. This was investigated by use of an *in vitro* preparation of the neonatal rat spinal cord with the functionally attached tail in which the cord and tail were separately superfused with physiological solution. Activation of peripheral fibres by noxious (capsaicin, bradykinin, 5-hydroxytryptamine, heat, pinch) and innocuous (light brush) stimuli was assessed by recording the depolarization of a spinal ventral root (L_3–L_5).

2 Brief administration of capsaicin produced dose-related depolarizing responses (EC_{50} = 280 nM). These responses could be reproduced for many hours following the repeated application of capsaicin at a sub-maximal concentration. Prolonged application of 0.5–2.0 μM capsaicin induced a selective desensitization to subsequent brief administrations of capsaicin. Prolonged administration at 20–50 μM produced an additional non-selective reduction in responses to all noxious stimuli without changing innocuous brush responses.

3 Removal of extracellular calcium from the tail superfusate did not reduce the response to capsaicin or prevent capsaicin-induced desensitization. However, high concentrations of capsaicin no longer induced a non-specific reduction of responses to other noxious stimuli. The response to a brief administration of capsaicin was unaffected by calcium channel blocking drugs including nifedipine, cadmium or ω-conotoxin. On the other hand high extracellular calcium increased the incidence of the non-selective reduction of responses to all noxious stimuli produced by high concentrations of capsaicin.

4 Replacement of extracellular sodium with choline blocked peripheral nerve conduction but did not prevent the desensitization produced by capsaicin. In addition, high concentrations of capsaicin were less effective in reducing the responsiveness to other noxious stimuli.

5 Neither capsaicin-evoked responses nor capsaicin-induced desensitization were affected by the administration of forskolin, dibutyryl cyclic AMP, nitroprusside, dibutyryl cyclic GMP, β-12,13 phorbol dibutyrate, trifluoperazine, indomethacin, staurosporine or mepacrine, in the tail superfusate.

6 These data suggest that capsaicin-induced activation, desensitization and impairment of peripheral nociceptors may be separable phenomena. Extracellular calcium is not required for capsaicin-induced activation or desensitization but calcium as well as sodium are important for capsaicin-induced impairment of nociceptive responses. Desensitization may occur independently of peripheral fibre activation and cannot be attributed to a central mechanism. Finally neither capsaicin-induced activation nor desensitization require the participation of a second messenger.

Introduction

Capsaicin (8-methyl N-vanillyl-6-noneamide), an irritant and algogenic compound obtained from hot red peppers, has highly selective effects on a subset of mammalian sensory neurones including polymodal nociceptors and warm thermoceptors (Fitzgerald, 1983; Szolcsanyi, 1985; 1990; Buck & Burks, 1986; Bevan *et al.*, 1987; Winter, 1987). Local applications of capsaicin in man produce a painful sensation by activation of primary afferent C- and probably Aδ-neurones. This is due to a membrane depolarization, observed in sensory neurones and fibres *in vitro*, with an increased membrane permeability to a number of cations including sodium, potassium and calcium (Hayes *et al.*, 1984a; Heymen & Rang, 1985; Bevan *et al.*, 1987; Marsh *et al.*, 1987; Wood *et al.*, 1988). With repeated exposure to capsaicin both the C-fibre activation and the algesic effect desensitize. Furthermore, exposure to high concentrations of capsaicin produces a prolonged block of conduction in some C-fibres (Petsche *et al.*, 1983; Baranowski *et al.*, 1986; Marsh *et al.*, 1987; Waddell & Lawson, 1989) and may induce a selective sensory neurotoxicity (Jancso *et al.*, 1985; Wood *et al.*, 1988). One or other of these actions may be involved in the antinociceptive and anti-inflammatory effect seen after an acute administration of capsaicin (Hayes & Tyers, 1980; Hayes *et al.*, 1984b; Campbell *et al.*, 1989a).

In the present experiments we have tested the hypothesis that the various effects of capsaicin on nociceptive neurones are separable phenomena and that the impairment of nociceptor function, which may relate to the systemic actions of capsaicin, requires specific conditions. In order to evaluate and in particular, quantify the effects of capsaicin on functioning peripheral nociceptors which respond to physiological stimuli, we have used an *in vitro* preparation of the spinal cord with the functionally attached tail obtained from the neonatal rat. In the neonate, peripheral cutaneous nociceptors are present from birth and possess functional characteristics similar to those found in the adult (Fitzgerald, 1985; 1987). In the *in vitro* preparation, nociceptors can be activated by a variety of noxious stimuli to produce a spinal nociceptive response (Yanagisawa *et al.*, 1984; Yanagisawa & Otsuka, 1984). This response can be used as an indirect, but convenient indicator of the activity of peripheral nociceptive fibres. In addition this *in vitro* preparation facilitates mechanistic studies since the extracellular environment may be easily manipulated by changing the composition of the tail superfusate. Some aspects of this study have been published as abstracts (Bettaney *et al.*, 1988; 1989; Dray *et al.*, 1989).

Methods

The intact spinal cord and the functionally connected tail were removed from 1–2 day old rats following decapitation. The most superficial layer of the skin was carefully removed

from the distal four fifths of the tail with fine forceps as described by Otsuka & Yanagisawa (1988). This procedure was thought to expose cutaneous fibres and their endings to allow activation by bradykinin and to facilitate activation by capsaicin. Damage to the underlying tissue was avoided as this severely compromised responsiveness to peripheral stimuli. We cannot be certain however that our tests were made in an environment entirely free of tissue damage. Histology was not routinely performed, since electron microscopy would have been necessary to confirm the structural integrity of fine afferent nerve endings and quantification of this would have presented considerable difficulty. However bradykinin, which was used routinely as an algesic chemical stimulus, is not known to activate axons of nociceptors. More likely it stimulates nociceptors via the signal transducing elements, at the terminations of primary afferent fibres. The efficacy of bradykinin thus suggested that nociceptors were preserved in our viable preparations. This would strongly indicate minimal nerve fibre damage. In addition the effects of our peripheral stimuli were robust and reproducible over many hours. This also would be unlikely in the face of significant tissue damage.

The preparation was placed in a chamber such that the cord and tail could be separately superfused (2–4 ml min^{-1}) with a physiological salt solution (composition mM: NaCl 138.6, KCl 3.35, $CaCl_2$ 1.26, $MgCl_2$ 1.16, $NaHCO_3$ 21.0, $NaHPO_4$ 0.58, glucose 10; at 24°C and gassed with 95% O_2/5% CO_2). Peripheral nociceptive fibres were activated by superfusion of the tail with noxious chemicals (capsaicin, bradykinin or 5-hydroxytryptamine (5-HT), by superfusate heated to 48°C, or by pinching the tail with fine forceps at a pressure that was uncomfortable to the experimenter. Innocuous stimulation of peripheral fibres was produced by lightly brushing the denuded tail with a fine sable-hair paint brush. Each stimulus was applied for 10 s with an intervening period of 15 min between stimuli. Bradykinin doses were separated by at least 40–60 min to avoid tachyphylaxis.

The activation of peripheral fibres was assessed by measuring the depolarization produced in a spinal ventral root (L_3–L_5) with a low impedance glass pipette which was placed in an electrolyte-filled well containing the selected ventral root. The ventral root potential was recorded d.c. with respect to the spinal cord which was earthed. The signals were amplified by conventional means and displayed simultaneously on an oscilloscope and on a rectilinear chart recorder.

In the first series of experiments the effect of changing extracellular calcium concentration in the tail superfusate was assessed with respect to capsaicin-induced activation of peripheral fibres, capsaicin-induced desensitization and the non-selective impairment of peripheral nociceptors induced by high doses of capsaicin. Calcium-free superfusate contained no added calcium and 1 mM EGTA. High calcium solutions contained 10–20 mM calcium. We also tested whether calcium channel blockers affected responses to capsaicin.

In the second series of experiments the influence of removing extracellular sodium from the tail superfusate on the peripheral effects of capsaicin was determined. In such superfusates sodium salts were replaced by choline salts.

The final series of experiments examined whether the stimulation of peripheral nociceptors by capsaicin was mediated via the activation of a second messenger system. In these experiments we used activators and inhibitors of various second messenger systems.

The following drugs were used: capsaicin (Sigma, 10 mM stock solution in dimethylsulphoxide (DMSO) made up to the desired concentration in physiological salt solution), bradykinin (synthesized at the Sandoz Institute), forskolin, sodium nitroprusside, dibutyryl adenosine 3′:5′-cyclic monophosphate (dibutyryl cyclic AMP), dibutyryl guanosine 3′:5′-cyclic monophosphate (dibutyryl cyclic GMP), trifluoperazine, indomethacin, mepacrine, 5-hydroxytryptamine (all from Sigma), nifedipine and ω-conotoxin (Research Biochemicals Incorporated), β-phorbol 12,13 dibutyrate (Avanti Polar Lipids), staurosporine (Fluka).

Statistics: where necessary data were compared statistically by Student's t test.

Results

Brief administrations of capsaicin to the tail produced a short-lived (45–100 s) depolarization of a ventral root. The amplitude of the depolarization was related to the dose of capsaicin (Figure 1: EC_{50} = 280 nM). The dose-response curve shown in Figure 1 was obtained by comparing the amplitude of the depolarizing response produced by a single low concentration of capsaicin with that produced by a subsequent supramaximal dose. In this way any possible desensitization due to repetitive capsaicin administration was avoided, but only a single dose-effect point could be obtained from each cord-tail preparation.

In subsequent experiments, reproducible responses could be obtained over many hours following a brief application of a submaximal concentration of capsaicin (0.3–0.7 μM) administered to the tail (Figure 2). In addition noxious heat stimulation was used in most experiments and this also evoked stable and reproducible responses. In many experiments bradykinin was used as a noxious chemical stimulus while in others 5-HT, noxious pinch or innocuous brush were also used as additional stimuli. If control responses to peripheral stimuli became unstable the preparation was discarded. Overall sensitivity to bradykinin (and 5-HT) appeared to depend on the successful removal of the skin from the tail without damaging the underlying tissue. This suggested that responsiveness may have depended on the preservation of exposed but functioning nociceptor nerve endings. In some preparations, where the skin was not removed at all (n = 3), poor responses of low amplitude with slow rise time were obtained to capsaicin (1 μM) and bradykinin (1 μM) did not evoke any response.

To study capsaicin-evoked desensitization and the non-selective impairment of peripheral nociceptors we used the following experimental paradigm. In normal calcium (1.26 mM) containing superfusate, a continuous administration of capsaicin (0.5–2.0 μM, n = 14) for 5 min evoked a response that was not sustained throughout the presence of capsaicin (Figure 2) but gradually waned back to the baseline. Following a wash period of 60 min or more, subsequent administration of a brief 10 s test dose of capsaicin was ineffective while responsiveness to other noxious (heat, n = 14; pinch, n = 4; bradykinin, n = 8; 5-HT, n = 4) or innocuous stimuli (n = 6) was unchanged (Figure 2). Sensitivity to capsaicin, particularly after the lowest desensitizing dose (0.5 μM, n = 3), returned after several hours, suggesting that the effect was unlikely to be due to neurotoxicity.

Similar prolonged administrations of higher doses of capsaicin (20–50 μM) also produced depolarization that was not sus-

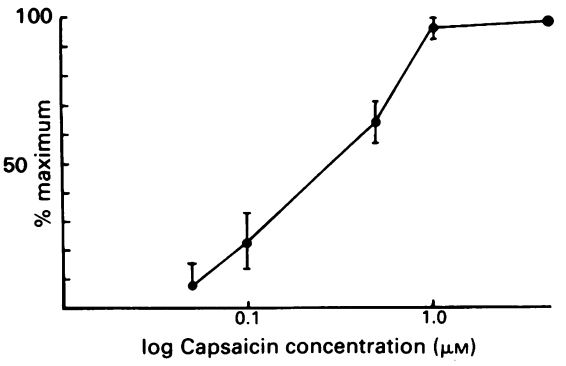

Figure 1 Dose-response curve showing percentage of maximal amplitude of spinal ventral root depolarizing response produced by incremental (log concentration, μM) doses of capsaicin applied to the tail. The EC_{50} = 280 nM. Each point represents the mean of 4–6 values; vertical bars show s.e.mean.

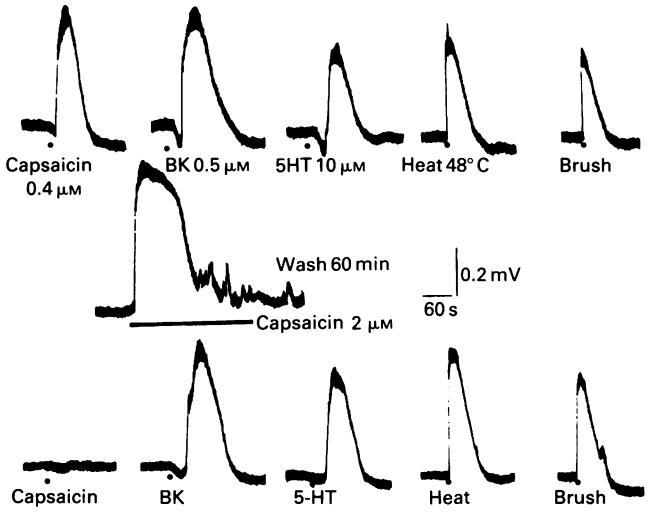

Figure 2 Selective desensitization of the response to capsaicin by low doses of capsaicin. The top traces show control response to capsaicin (0.4 μM), bradykinin (BK 0.5 μM), 5-hydroxytryptamine (5-HT 10 μM) noxious heat (48°C) and to innocuous brush. Each stimulus was applied for 10 s. A prolonged 5 min superfusion of the tail with capsaicin (2 μM) produced a maximal response which was not maintained during the superfusion (middle trace). Following a 60 min wash, the application of each agent was repeated. The response to capsaicin was completely desensitized whereas responses to other stimuli were unchanged. The calibration bars of 0.2 mV and 60 s are indicated on this and subsequent figures.

tained throughout the presence of capsaicin. Subsequently there was (7 of 12 experiments) a non-selective reduction (>50%) of responses to all noxious stimuli (heat, $n = 7$; bradykinin, $n = 4$; pinch, $n = 4$) (Figure 3), whereas responses

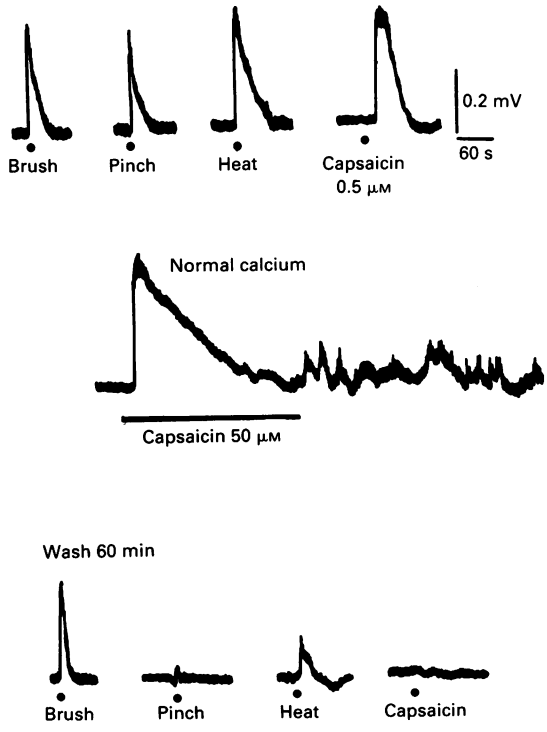

Figure 3 The non-selective impairment of responsiveness to noxious stimuli following administration of high doses of capsaicin in superfusate containing normal (1.2 mM) calcium. The top traces show control responses to light brushing, pinch, heat at 48°C and a submaximal dose of capsaicin (0.5 μM). During the continuous superfusion of the tail with 50 μM capsaicin the evoked response was not sustained. The bottom traces show the non-selective attenuation or abolition of all nociceptive responses but not the response to light brushing when stimuli were retested 60 min later.

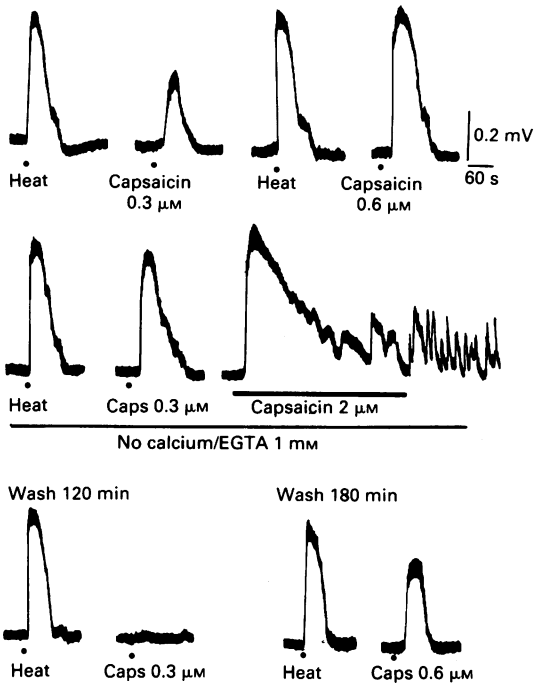

Figure 4 Responses to capsaicin in the absence of extracellular calcium. The top traces show reproducible responses to noxious heat and dose-related responses to capsaicin at 0.3 μM and 0.6 μM respectively. Superfusion of the tail with solution containing no added calcium and 1 mM EGTA was started 15 min before and continued throughout further testing of peripheral stimuli. During this period (middle traces) the responsiveness to noxious heat was unchanged but the response to the lower concentration of capsaicin (0.3 μM) was enhanced. Continuous administration of capsaicin for 5 min at 2 μM (indicated by the bar below the trace) evoked a response that was not sustained throughout the period of capsaicin administration. On returning to superfusion with normal calcium and washing for 120 min, the responses to heat was unchanged but that to capsaicin (Caps) was abolished. Partial recovery of capsaicin-sensitivity occurred during a further 180 min wash.

to innocuous brushing ($n = 6$) were unchanged. Responsiveness to noxious stimuli was still reduced or abolished 4–6 h later.

Calcium-free experiments

In these experiments, control responses were obtained to capsaicin and other peripheral nerve stimuli. The tail was then superfused with calcium-free solutions containing 1 mM EGTA. Superfusion started 15 min before, and was maintained throughout the period that peripheral stimuli were retested. The response produced by a brief administration of capsaicin was not reduced; quite the contrary, it was often enhanced (Figure 4), though the degree of enhancement was not studied in detail. Moreover in the absence of calcium, a desensitizing dose of capsaicin (1–2 μM, 5 min) consistently produced ($n = 8$) selective desensitization to capsaicin (Figure 4). Partial recovery of capsaicin sensitivity could be observed 3–6 h later. Higher doses of capsaicin (20–50 μM, 5 min, $n = 6$) administered in the absence of calcium also produced a selective desensitization to capsaicin. However, when normal calcium superfusate was restored, selective desensitization to capsaicin persisted and responses to other stimuli were not significantly affected (8 of 8 experiments; heat, $n = 8$; bradykinin, $n = 3$; light brush, $n = 3$). As in previous experiments desensitization could not be attributed to neurotoxicity since sensitivity to capsaicin was partially restored after several hours.

In other experiments superfusions of the tail with calcium channel blockers such as nifedipine (10 μM, 20 min superfusion,

4 of 4 experiments), ω-conotoxin (100 μg ml^{-1}, 20 min superfusion, 2 experiments) or cadmium (200 μM, 20 min superfusion, 5 of 7 experiments) did not change capsaicin-evoked responses.

High calcium experiments

Superfusion of the tail with solution containing 10–20 mM calcium did not significantly affect the acute response to a brief administration of capsaicin, pinch, noxious heat or light brushing. However following the prolonged administration of high concentrations of capsaicin (20–50 μM, 5 min) subsequent tests with brief applications of capsaicin, as well as other noxious stimuli, produced either an attenuated response or no response at all (capsaicin, $n = 8$; heat, $n = 8$; pinch, $n = 2$) (Figure 5) in each of 8 preparations. Nociceptive responses were not restored even after prolonged washing (4–6 h) of the tail in normal calcium-containing solution.

Sodium-free superfusion

During the prolonged (40–60 min) superfusion of the tail with sodium-free solution the responses to all peripheral stimuli were gradually attenuated and then abolished, due to failure of nerve conduction. When the superfusate was changed to one containing a normal sodium concentration, responsiveness to all stimuli returned within 60 min. However if, during the absence of sodium, a desensitizing dose of capsaicin was administered (2 μM, 5 min, $n = 6$), subsequent re-superfusion with normal sodium allowed the restoration of all peripherally evoked responses (heat, $n = 6$; bradykinin, $n = 3$; brush, $n = 3$) except that to capsaicin (Figure 6), indicating the occurrence of a selective capsaicin desensitization. Finally in zero sodium superfusion, high concentrations of capsaicin (20–50 μM, 5 min, $n = 5$) produced desensitization to subsequently administered capsaicin, after reperfusion with normal bathing solution. However there was no evidence of impaired responsiveness to other peripheral stimuli (heat, $n = 5$; brush, $n = 2$).

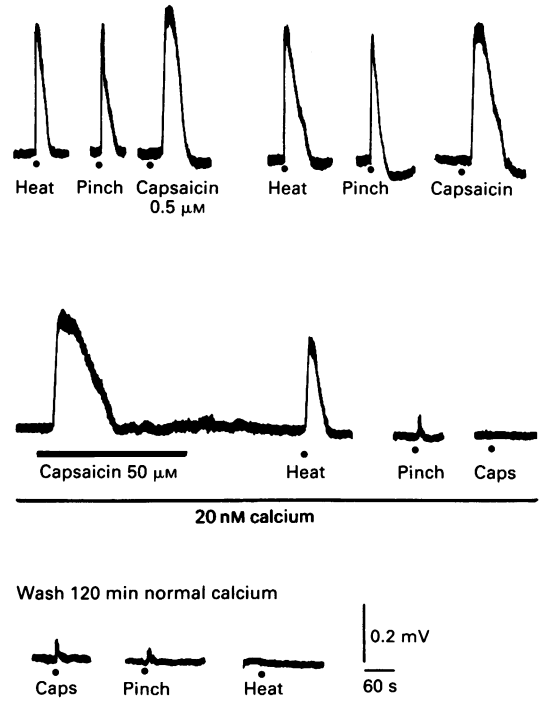

Figure 5 The effect of high extracellular calcium concentration on the action of capsaicin. The top traces show a series of reproducible responses to heat (48°C), pinch and capsaicin (0.5 μM), during superfusion of the tail with a normal concentration of calcium. During superfusion with 20 mM calcium, prolonged administration of 50 μM capsaicin evoked a response which was smaller in amplitude compared with the control responses evoked by the brief application of a submaximal dose of capsaicin. In addition, the effect of heat, pinch and subsequent doses of capsaicin (Caps) were attenuated or abolished. On returning to superfusate containing a normal calcium concentration, no recovery of responsiveness could be observed (bottom traces).

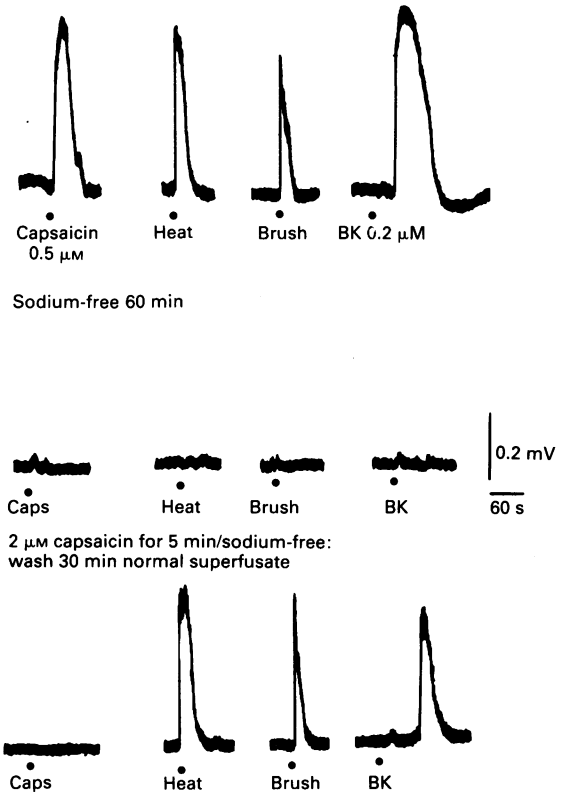

Figure 6 The effect of removing extracellular sodium on the desensitization produced by capsaicin. The top traces show control responses in normal superfusate. Sodium was replaced by choline in the tail superfusate. Nerve conduction failed following a 60 min superfusion and no response to any peripheral stimulus could be evoked. At this point a concentration of capsaicin that was usually sufficient to produce desensitization was administered (2 μM, 5 min). This was followed by resuperfusion with normal sodium-containing solution for 30 min after which time peripheral stimuli were retested. The bottom traces show that the response to each stimulus returned but capsaicin (Caps) remained ineffective. BK = Bradykinin.

Second messenger studies

These experiments were carried out to examine the possibility that a second messenger system was involved in the activation of peripheral fibre by capsaicin or with desensitization induced by capsaicin. When administered in the tail superfusate, activators of cyclic AMP-dependent kinase, [forskolin (5–10 μM, $n = 3$) and dibutyryl-cyclic AMP (100 nM–1 mM, $n = 3$)] or activators of cyclic GMP-dependent kinase, [nitroprusside (10–50 μM, $n = 6$) and dibutyryl cyclic GMP (0.1–1.0 mM, $n = 4$)] did not produce measurable responses nor did these substances change the ability of capsaicin to activate or desensitize peripheral fibres. The protein kinase C activator β-12,13 phorbol dibutyrate (PDBu, 1 μM, $n = 6$) produced a response which has been previously shown to be mediated by activation of capsaicin-sensitive fibres (Dray *et al.*, 1988). However, responses to capsaicin were unaffected when protein kinase C was inactivated either by prolonged administrations of PDBu, to down-regulate the enzyme or by the addition of the protein kinase C inhibitor, staurosporine (Tamaoki *et al.*, 1986). The responses to capsaicin were not affected by the calmodulin-dependent kinase inhibitor, trifluoroperazine (10 μM, $n = 4$), the phospholipase A_2 inhibitor,

mepacrine (10 μM, $n = 4$), nor by the cyclo-oxygenase inhibitor, indomethacin (1–10 μM, $n = 6$).

Discussion

The depolarizing responses evoked in a spinal lumbar ventral root of the neonatal rat, following the physiological activation of peripheral nociceptive fibres, was produced by the activation of motoneurones in the lumbar spinal segment (Akagi *et al.*, 1985). Since depolarization was recorded distal from the site of entry of the afferent input, this suggests that peripherally evoked depolarization spreads over several spinal segments, as shown in the adult spinal cord (Bagust & Kerkut, 1988). The effects of capsaicin on peripheral nociceptors were concentration-dependent and at a submaximal concentration were reproducible, showing little tendency to desensitize, providing that a sufficient interval was allowed between applications. The EC_{50} concentration for nociceptor activation was found to be similar to that reported for other measures of capsaicin-evoked activation of sensory neurones such as the uptake of calcium (Wood *et al.*, 1988). Prolonged administration of capsaicin, at concentrations $< 2.0\,\mu$M, produced a selective desensitization without significant impairment of responses to other noxious chemical (bradykinin, 5-HT) or physical stimuli (heat, pinch). Tenfold or higher concentrations of capsaicin were required to produce a non-selective impairment of responses to other noxious stimuli. However, even at the highest concentrations used (50 μM), large reductions in the responses to noxious stimuli were not always observed.

Since all major cutaneous receptor types seen in adults are also present in the neonatal rat at birth it is likely that the effects of capsaicin were due to an action on C-fibre nociceptors shown to respond to several types of noxious stimuli (Fitzgerald, 1987). It is possible that capsaicin could have affected A-neurones since these are functionally immature at birth (Fitzgerald, 1987) and may have some properties similar to C-neurones since their conduction velocities are similar (Fulton, 1986). This seems to be unlikely since even high doses of capsaicin, that produce striking neurotoxicity in small C-type neurones of neonates, produce relatively little effect on the A-neurone population (Lawson & Harper, 1984). Also, in the present study doses of capsaicin which reduced responses to noxious stimuli did not affect responses to innocuous brushing. This suggests that responses to innocuous stimuli were produced by activation of capsaicin-insensitive nerve fibres. It is unclear whether the capsaicin sensitivity of neonatal nociceptive fibres differs significantly from adult sensory fibres. Studies of neonatal and adult sensory neurones maintained in culture show that sensitivity differences to capsaicin are likely to be minimal (Winter *et al.*, 1988; Wood *et al.*, 1988). However, further studies on the receptive terminals of single C-fibre filaments seem necessary to address this issue directly.

In cultured sensory neurones, capsaicin-induced activation of nociceptors was likely to be accompanied by an increase in membrane permeability to cations including Na^+ and Ca^{2+} (Baccaglini & Hogan, 1983; Heyman & Rang, 1985; Bevan *et al.*, 1987; Wood *et al.*, 1988). The cellular accumulation of these ions during prolonged exposure to capsaicin may therefore be involved in several phenomena such as desensitization, block of fibre conduction and selective neurotoxicity (Marsh *et al.*, 1987; Wood *et al.*, 1988). Omission of extracellular calcium in the present study, did not reduce capsaicin-evoked responses, indeed the response to capsaicin was often increased. This could be explained by a generalized increase in neural excitability (Brink & Bronk, 1937) and responsiveness following the lowering of extracellular calcium concentrations (Schafer, 1987; Lang *et al.*, 1990). Alternatively calcium ions may normally impede the passage of sodium ions as shown during the capsaicin-mediated increase in membrane conductance in sensory neurones (Forbes & Bevan, 1988). This effect may contribute to the increased response to capsaicin that we observed, since an increased rate of entry of sodium into nerve fibres, in the absence of calcium, may allow more rapid and more effective depolarization of nociceptors as well as the activation of a greater proportion of peripheral fibres.

In the absence of calcium, there was no apparent change in the ability of capsaicin to produce desensitization. Other observations shown that the capsaicin-induced desensitization of ion fluxes ([^{14}C]-guanidinium, ^{86}rubidium) in sensory neurones may occur in the absence or presence of calcium (Minhas *et al.*, 1990). Thus extracellular calcium ions appear not to be important for capsaicin-induced desensitization but the participation of intracellular sources of calcium cannot be excluded. In the absence of calcium there was also little evidence of a capsaicin-induced reduction of the responses to other noxious stimuli. On the other hand, in high extracellular calcium, capsaicin-induced impairment of nociceptor function was exaggerated. It is possible that fibre conduction and metabolism may be disrupted by a capsaicin-induced accumulation of calcium as suggested by others (Marsh *et al.*, 1987). Since we did not observe a recovery of responsiveness to peripheral stimuli, it remains unclear whether this effect of capsaicin is part of a sequence of events which lead to neurotoxicity. Similar concentrations of capsaicin have induced morphological changes in vagal C-fibres (Marsh *et al.*, 1987).

Omission of sodium from the tail superfusate prevented conduction in peripheral nerves. Hence all stimuli appeared ineffective. Under these conditions capsaicin still produced a selective desensitization revealed when nerve conduction was restored following the re-introduction of sodium. In addition, in the absence of sodium, exposure to high concentrations of capsaicin did not appear to impair subsequent responsiveness to other noxious stimuli. This latter observation suggests that sodium as well as calcium accumulation in peripheral nociceptive fibres may be important for the non-specific disruption of nociceptor activity. This observation is also in keeping with the finding that capsaicin-induced morphological changes observed in cultured sensory neurones were reduced in the absence of extracellular sodium ions (Baccaglini & Hogan, 1983; Winter *et al.*, 1990).

The occurrence of desensitization, in the absence of nerve conduction to the spinal cord, suggested that capsaicin-induced desensitization was a local phenomenon and was unlikely to result from changes at the level of the spinal cord. In addition, fibre activation itself may not be critical for capsaicin-induced desensitization. Thus desensitization may result directly from capsaicin interacting with a recognition site on the nerve membrane without the necessity for signal transduction. Supporting this was the lack of interactions between a number of possible second messenger systems (cyclic AMP- or cyclic GMP-dependent kinases, protein kinase C, calmodulin-kinase, cyclo-oxygenase-dependent arachidonic acid products, phospholipase A_2) and capsaicin-evoked responses or capsaicin-induced desensitization. Similar conclusions were reached in studies on cultured sensory neurones (Wood *et al.*, 1989). Furthermore, single ion channel conductance was evoked by capsaicin in patches of sensory neurone membranes where cytoplasmic components were lacking (Forbes & Bevan, 1988).

Capsaicin-induced desensitization appears similar to desensitization occurring with other, better-characterized, ligand-receptor interactions. For example, desensitization of nicotinic receptors was independent of membrane voltage, extracellular calcium and second messenger systems (Cachelin & Colquhoun, 1989). In addition, β-adrenoceptor occupation was sufficient to produce desensitization and neither signal transduction, via G-protein coupling, nor activation of a second message (cyclic AMP) was required (Sibley & Lefkowitz, 1985). Such similarities support the possibility that capsaicin interacts with and desensitizes a 'receptor' or specific membrane binding site. In addition a capsaicin recognition site, has been proposed from studies using capsaicin analogues

(Hayes et al., 1984; Szolcsanyi & Jancso-Gabor, 1975; 1976) and by using a capsaicin-like photoaffinity probe (James et al., 1988). More directly, capsaicin has been shown to displace the binding of [^{3}H]-resiniferatoxin (Szallosi & Blumberg, 1989a), a highly potent, naturally occurring capsaicin analogue (Campbell et al., 1989b; Szallosi & Blumberg, 1989b; Dray et al., 1990), with a similar mechanism of action (Szallosi & Blumberg, 1989b; Winter et al., 1990).

In summary the present data show that capsaicin-induced activation and desensitization of peripheral nociceptors in the neonatal rat tail are separable phenomena and do not require extracellular calcium ions or a second messenger system. However, it is likely that an intracellular accumulation of calcium and sodium ions is involved in the non-selective impairment of nociceptor function produced by higher doses of capsaicin. In addition, the selective desensitization produced by capsaicin occurs in the periphery without requiring the prior activation of nociceptive fibres. Finally, the impairment of nociceptor function produced by capsaicin may be relevant to its acute antinociceptive properties.

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(Received April 23, 1990
Revised June 26, 1990
Accepted July 5, 1990)

Br. J. Pharmacol. (1990), **101**, 734–738

Electrophysiological and arrhythmogenic effects of platelet activating factor during normal perfusion, myocardial ischaemia and reperfusion in the guinea-pig

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1 Platelet activating factor (PAF) is often used to study the effects of platelet activation. While direct myocardial electrophysiological effects of PAF have been described in superfused myocardial tissue, little is known about its actions on the whole heart.

2 The cellular electrophysiological and arrhythmogenic effects of PAF (10^{-11} M, 10^{-10} M and 10^{-9} M) were studied during normal perfusion, global myocardial ischaemia and reperfusion in Langendorff-perfused guinea-pig hearts at 32°C.

3 PAF (10^{-9} M) increased the incidence of ventricular fibrillation during ischaemia and reduced action potential duration (APD) during normal perfusion and early myocardial ischaemia (10^{-9} M and 10^{-10} M). PAF also reduced refractory period (RP) during normal perfusion (10^{-9} M) and early ischaemia (10^{-9} M and 10^{-10} M). PAF prevented recovery of APD (10^{-9} M) and RP (10^{-9} M and 10^{-10} M) during reperfusion. PAF at a concentration of 10^{-11} M had no electrophysiological effects.

4 PAF (10^{-9} M) increased the QRS width of the electrocardiogram during late ischaemia while 10^{-10} M PAF raised pacing threshold during late ischaemia.

5 Perfusion pressure was increased, and developed tension decreased by 10^{-9} M PAF.

6 These results demonstrate that PAF has direct myocardial electrophysiological effects in the whole heart which occur during normal perfusion and are capable of augmenting the effects of myocardial ischaemia, but are independent of the presence of platelets.

Introduction

Recent evidence suggests that platelet activation and aggregation contributes to the deleterious effects of myocardial ischaemia (Mehta & Mehta, 1979; Fitzgerald *et al.*, 1986; Rösen *et al.*, 1987; Wainwright *et al.*, 1989). Release of platelet activating factor (PAF) from activated cells and also from the myocardium during ischaemia has been documented in man and in experimental animals (Lotner *et al.*, 1980; Annable *et al.*, 1985; Zimmerman *et al.*, 1985; Montrucchio *et al.*, 1986; 1989; Sisson *et al.*, 1987). PAF has been determined to be a phospholipid (Demopoulos *et al.*, 1979) and is now commonly used as a pharmacological agent to study the effects of platelet activation and aggregation (McManus *et al.*, 1981; Petty & Scrutton, 1989; Slattery & Beaumont, 1989). Studies on intact animals and isolated hearts have shown the ability of PAF to increase the effects of myocardial ischaemia (Mickelson *et al.*, 1988; Chakrabarty *et al.*, 1988). Others have described the effects of exogenous PAF on myocardial contractility and vascular smooth muscle (Benveniste *et al.*, 1983; Bessin *et al.*, 1983; Feuerstein *et al.*, 1984; Levi *et al.*, 1984; Sybertz *et al.*, 1985; Baranes *et al.*, 1986; Riedel *et al.*, 1987; Ezra *et al.*, 1987; Robertson *et al.*, 1987; Sirén & Feuerstein, 1989), while experiments performed on isolated, superfused papillary muscles suggest that PAF has direct myocardial electrophysiological effects, causing reductions in action potential duration, and increases in action potential amplitude and upstroke velocity (Camussi *et al.*, 1984; Tamargo *et al.*, 1985; Alloatti *et al.*, 1986). Little is known about the cellular electrophysiological actions of PAF in intact hearts and an understanding of these is important if the effects of platelet activation are to be differentiated from those produced by PAF alone. We investigated the cellular electrophysiological and arrhythmogenic effects of three concentrations of PAF (10^{-11} M, 10^{-10} M and 10^{-9} M) during normal perfusion, global myocardial ischaemia and reperfusion using isolated, Langendorff-perfused guinea-pig hearts.

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Methods

Hearts were removed rapidly from 34 male guinea-pigs (Dunkin-Hartley, 500–600 g) which had been killed by cervical dislocation and were mounted for perfusion by the Langendorff technique. The hearts were perfused with oxygenated aqueous Krebs-Henseleit buffer prewarmed to 32°C, containing (mM): NaCl 118, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.1, $NaHCO_3$ 24, $CaCl_2$ 2.5, glucose 9 and pyruvate 2 (pH 7.4) at a constant flow rate of 7 ml min^{-1}. Hearts were supported horizontally in the organ bath and superfused with aqueous buffer (as above) at 32°C to avoid alterations in temperature associated with myocardial ischaemia. Heart rate was maintained constant at 3.3 Hz (just above the spontaneous sinus rate) by right ventricular pacing at twice the diastolic pacing threshold. Action potentials, refractory periods, electrocardiograms, perfusion pressure and developed tension were recorded as described previously (Penny & Sheridan, 1983). Action potentials were analysed with an on-line microcomputer, designed to provide real-time analogue reconstruction of digitised data prior to analysis (Sheridan *et al.*, 1983; Flores *et al.*, 1989). Transmembrane action potentials were recorded by the floating microelectrode technique from epicardial cells in the apical region of the right ventricle.

Measurements were made in 10 control hearts, in 8 hearts perfused with 10^{-11} M PAF, in 8 hearts perfused with 10^{-10} M PAF, and 8 hearts perfused with 10^{-9} M PAF. All hearts were perfused initially with Krebs-Henseleit buffer for a control period of 20 min when electrophysiological measurements were made. Perfusion was changed to buffer containing PAF which was allowed to flow at the control rate for 10 min when electrophysiological measurements were repeated.

Myocardial ischaemia was induced by reducing global coronary flow to 0.7 ml min^{-1} (10% of control) for 30 min,

and reperfusion was initiated by restoring flow to control for 10 min. The incidence and time of onset of spontaneously occurring ventricular tachycardia (VT) and ventricular fibrillation (VF) were noted during ischaemia and reperfusion.

Electrophysiological measurements are expressed as the mean ± standard error of the mean for each group. Comparisons are made between the groups by Student's non-paired t test or X^2-analysis, and within groups using the paired t test, with $P < 0.05$ defining the probability value indicating statistical significance. Results were analysed according to the Lambeth Conventions (Walker *et al.*, 1988).

Platelet activating factor (1-O-alkyl-2-acetyl-sn-glyceryl-3-phosphorylcholine) was obtained from Sigma Chemical Co. Ltd., Poole, Dorset and was prepared by dissolving initially 0.4 mg in 200 ml of 0.9% w/v NaCl containing 0.25% w/v bovine serum albumin. Subsequent dilutions were made using Krebs-Henseleit buffer.

Results

Effects of 10^{-9} M PAF

PAF increased the incidence of ventricular fibrillation (VF) during ischaemia (88% vs 40%, $P < 0.05$, compared to controls) but had no effect on the onset time for VT (20 ± 3 min vs 21 ± 3 min in controls) or on the incidence of ventricular tachycardia during ischaemia, (Figure 1). PAF tended to prevent spontaneous recovery from VF during reperfusion (25% vs 70%, $0.05 < P < 0.10$). No differences were noted in the incidence of reperfusion arrhythmias since the hearts which developed VF during ischaemia remained in VF throughout reperfusion making comparisons difficult (Walker *et al.*, 1988).

PAF reduced action potential duration (APD) and refractory period (RP) during normal perfusion, from 159 ± 5 ms to 129 ± 5 ms, $P < 0.001$ and from 143 ± 3 ms to 129 ± 5 ms, $P < 0.05$, respectively. These effects were maintained during early ischaemia, such that APD was less with 10^{-9} M PAF than in control hearts at 2 and 5 min of ischaemia, while RP was less at 2 min of ischaemia. PAF also prevented recovery of APD and RP during reperfusion (Figures 2a and b). PAF increased conduction time during normal perfusion (from 14 ± 2 ms to 20 ± 2 ms, $P < 0.05$), and increased the QRS width of the electrocardiogram during late ischaemia (43 ± 3 ms vs 30 ± 0 ms, $P < 0.02$ at 30 min) compared to control hearts. PAF had no effect on action potential amplitude, maximum velocity of the upstroke of the action potential ($\dot{V}_{max}$) or pacing threshold during normal perfusion, myocardial ischaemia or reperfusion compared to control hearts. No arrhythmias were seen during normal perfusion with PAF.

Perfusion pressure was increased by 10^{-9} M PAF during normal perfusion (from 64 ± 7 mmHg to 72 ± 7 mmHg, $P < 0.05$) and developed tension was reduced by PAF (from 4.4 ± 0.6 g to 2.0 ± 0.3 g, $P < 0.05$).

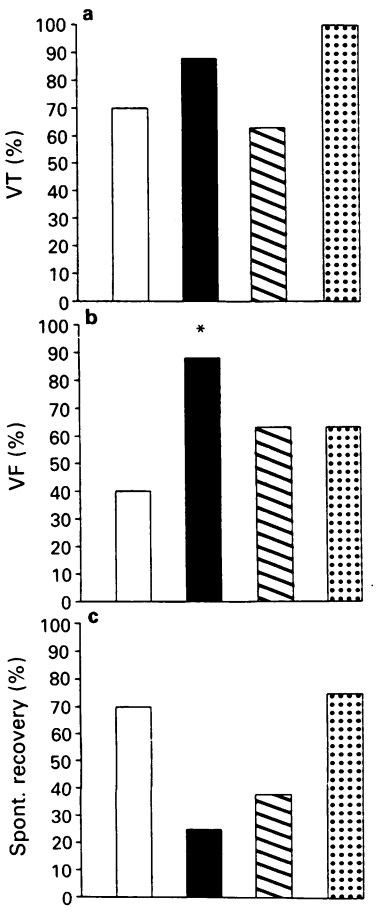

Figure 1 Percentage incidence of ventricular tachycardia (VT, a) and ventricular fibrillation (VF, b) during ischaemia in control hearts (open columns), and hearts treated with 10^{-9} M (solid columns), 10^{-10} M (hatched columns) and 10^{-11} M (stippled columns) PAF. Percentage spontaneous recovery from arrhythmias during reperfusion is illustrated in (c) (*$P < 0.05$ vs control).

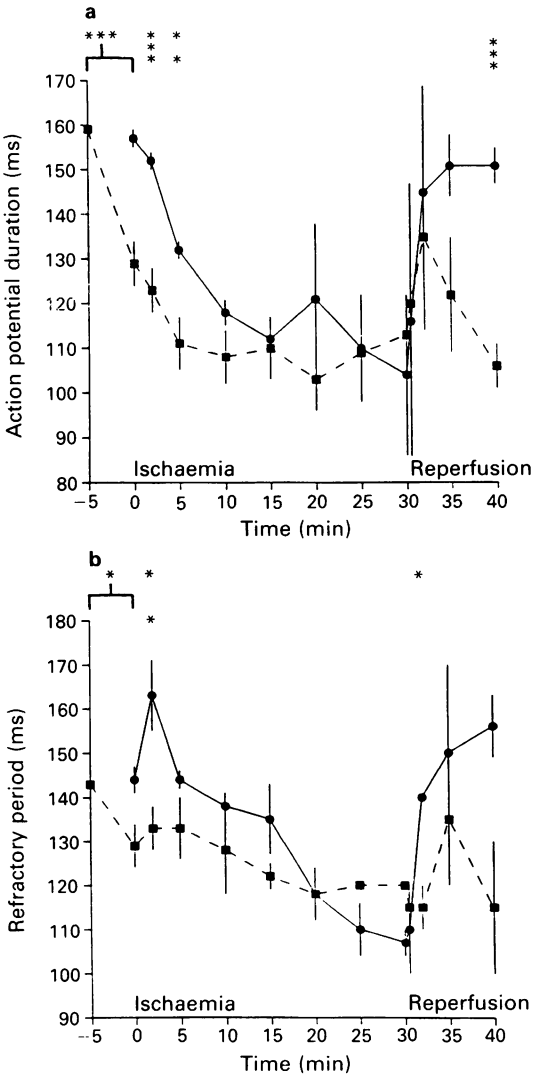

Figure 2 Changes in action potential duration (a) and refractory period (b) in control hearts (●) and hearts treated with 10^{-9} M PAF (■) during normal perfusion, myocardial ischaemia and reperfusion. (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

Effects of 10^{-10} M PAF

PAF reduced APD during normal perfusion (from 156 ± 5 ms to 138 ± 3 ms, $P < 0.01$) and early ischaemia (Figure 3a). RP was reduced during the first 2 min of ischaemia, but this was reversed by 30 min of ischaemia when RP was greater with PAF compared to control hearts. During reperfusion, RP was initially increased by PAF (at 30 s) and later reduced (10 min) compared to controls (Figure 3b). PAF increased pacing threshold during late ischaemia (20–30 min) compared to controls (Figure 3c). PAF had no effect on action potential amplitude, conduction time, $\dot{V}_{max}$ or QRS width during normal perfusion, myocardial ischaemia or reperfusion compared to controls. No arrhythmias were seen during normal perfusion with PAF.

Perfusion pressure and developed tension were unchanged by 10^{-10} M PAF (69 ± 7 mmHg vs 63 ± 5 mmHg and 3.1 ± 0.2 g vs 3.0 ± 0.3 g).

PAF had no effect on the onset time for VT (17 ± 1 min vs 21 ± 3 min) and had no significant effects on the incidence of arrhythmias during ischaemia and reperfusion. The results suggest an apparent trend for 10^{-10} M PAF to increase the incidence of VF during ischaemia (63% vs 40%) and to prevent spontaneous recovery from VF during reperfusion (38% vs 70%), Figure 1.

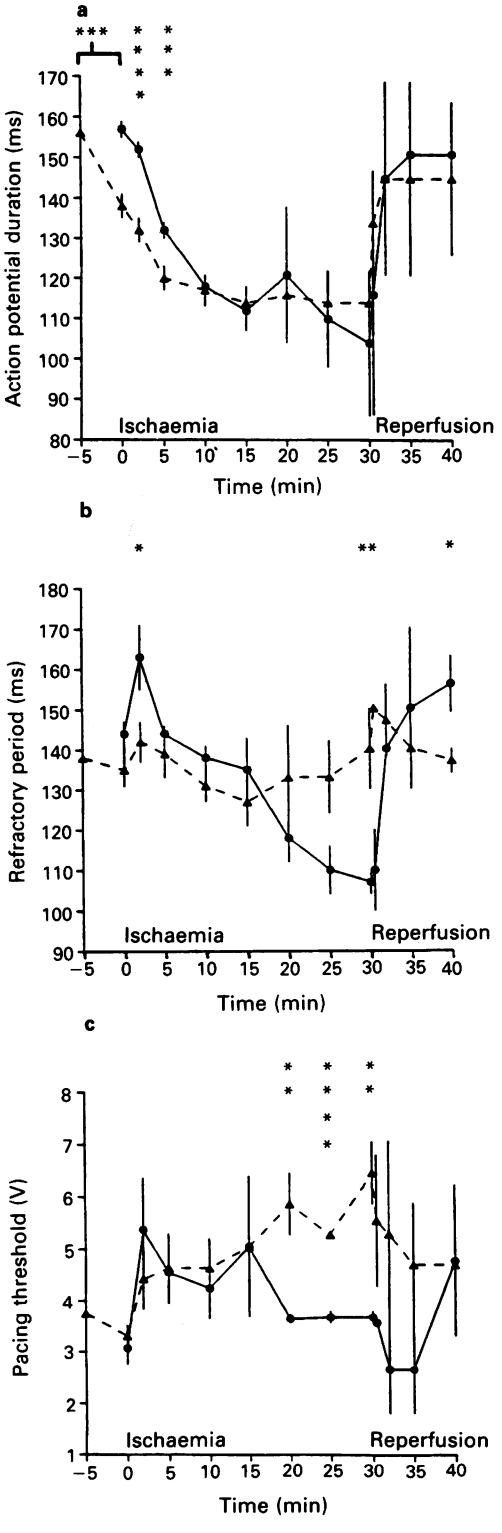

Figure 3 Changes in action potential duration (a), refractory period (b) and pacing threshold (c) in control hearts (●) and hearts treated with 10^{-10} M PAF (▲) during normal perfusion, myocardial ischaemia and reperfusion. (*$P < 0.05$, **$P < 0.02$, ***$P < 0.01$; ****$P < 0.001$).

Effect of 10^{-11} M PAF

At 10^{-11} M, PAF had no electrophysiological effects during normal perfusion, myocardial ischaemia or reperfusion compared to control hearts. Perfusion pressure and developed tension also remained unchanged (62 ± 6 mmHg vs 65 ± 6 mmHg and 3.2 ± 0.5 g vs 3.2 ± 0.6 g).

PAF had no effect on the onset time for VT (17 ± 2 min vs 21 ± 3 min) and had no significant effects on the incidence of arrhythmias during ischaemia and reperfusion. Spontaneous recovery from VF occurred to a similar extent in both control and PAF-treated hearts (75% vs 70%) (Figure 1). No arrhythmias were seen during normal perfusion.

Discussion

These experiments were performed to determine the cellular electrophysiological effects of PAF in the whole heart in the absence of circulating platelets at concentrations within the ranges reported to be released during ischaemia. PAF reduced action potential duration and refractory period during normal perfusion and increased conduction time but had no other electrophysiological or arrhythmogenic effects during normal perfusion. During early ischaemia PAF reduced action potential duration and refractory period compared to control hearts and raised QRS width and pacing threshold during the later stages of ischaemia. PAF at the highest concentration studied increased the incidence of VF during ischaemia. PAF also increased perfusion pressure indicating an increase in total coronary vascular resistance, and decreased developed tension during normal perfusion.

Infusion or injection of PAF into intact dogs, pigs and rats has been shown to decrease coronary blood flow, myocardial contractility, blood pressure, cardiac output, heart rate, and myocardial oxygen consumption; to increase myocardial oxygen extraction and vascular resistance; and to produce metabolic acidosis and ST segment changes (Bessin *et al.*, 1983; Feuerstein *et al.*, 1984; Sirén & Feuerstein, 1984; Sybertz *et al.*, 1985; Ezra *et al.*, 1987). Injection or perfusion of guinea-pig isolated hearts with PAF at concentrations up to 10^{-8} M produced similar effects: reductions in coronary flow and myocardial contractile force, increases in perfusion pressure and coronary vascular resistance and also impaired atrioventricular conduction (Benveniste *et al.*, 1983; Levi *et al.*, 1984; Baranes *et al.*, 1986). Similar effects were seen in rabbit isolated hearts perfused with platelets and PAF (Montrucchio *et al.*, 1989), but in the absence of platelets, PAF had fewer effects.

The results obtained from our study support these earlier observations since 10^{-9} M PAF increased perfusion pressure, indicating coronary vasoconstriction and increased total coronary vascular resistance, and reduced myocardial contractility. Unlike Levi *et al.* (1984) we observed no conduction block or ventricular arrhythmias during normal perfusion with PAF.

This may reflect methodological differences between the studies since the hearts were unpaced in their experiments and exposed to sequentially increasing concentrations of PAF. In hearts perfused at a constant pressure of 40 mmHg (20 mmHg less than the baseline pressures we observed), Levi *et al.* (1984) also observed an increase in heart rate which may have contributed to the conduction block they observed.

Experiments performed on isolated myocardial tissues (atria, papillary and pectinate muscles) have also demonstrated the negative inotropic effects of PAF (Levi *et al.*, 1984; Robertson *et al.*, 1987). Studies on isolated papillary muscles have reported cellular electrophysiological effects produced by PAF which have been confirmed by our study using whole hearts. Camussi *et al.* (1984) reported a negative inotropic effect of 10^{-10} M PAF associated with a reduction in action potential duration in guinea-pig papillary muscles at 30°C, which was later confirmed for concentrations between 10^{-11} M and 10^{-7} M by Tamargo *et al.* (1988). In their studies, which were performed at 34°C, 10^{-11} M PAF shortened action potential duration, while concentrations from 10^{-10} M to 10^{-7} M increased action potential amplitude and $\dot{V}_{max}$. The absence of changes in these parameters in our preparation may reflect differences between perfused and superfused tissues or may be related to temperature-dependent actions since no changes were observed by us at 32°C or by Camussi *et al.* (1984) at 30°C. Alloatti *et al.* (1986) studied the effects of PAF (10^{-11} M to 10^{-6} M) on human cardiac muscle at 35°C and reported negative inotropic effects and reductions in action potential duration with no effect on amplitude or $\dot{V}_{max}$. The effects of PAF in other organs and its role during anaphylaxis and inflammation have been reviewed recently by Braquet *et al.* (1987).

Potential mechanisms explaining the cardiac effects of PAF have been proposed by Tamargo *et al.* (1988) and include the suggestion that PAF might produce its mechanical and electrophysiological effects by increasing calcium uptake into the sarcoplasmic reticulum and reducing calcium efflux possibly through calcium-dependent potassium channels or by changing the availability of calcium intracellularly, or myocardial sensitivity to the intracellular calcium concentration. Our results support these suggestions — the reductions in action potential duration are consistent with changes in calcium fluxes while the lack of any effect of PAF on $\dot{V}_{max}$ suggests that changes in sodium conductance are not involved. Changes in the responses of vascular smooth muscle and cardiac muscle to calcium ion concentrations would also explain the ability of PAF to reduce contractility, induce vasoconstriction and increase coronary vascular resistance. Further work remains to be done to confirm these hypotheses.

The results of our experiments have demonstrated that the effects of PAF on action potential duration and refractory period are maintained during the first 5 min of ischaemia, but thereafter no differences in these parameters were noted between the groups. Differences between control and PAF-treated groups were noted during later ischaemia for QRS width (with 10^{-9} M) and pacing threshold (with 10^{-10} M) suggesting that PAF may be acting via different mechanisms to produce cellular changes affecting action potential duration and refractory period during normal perfusion and early ischaemia, and global changes affecting the heart as a whole (QRS width and pacing threshold) which only become apparent during later ischaemia.

Recent studies have reported the ability of PAF to increase the deleterious effects of myocardial ischaemia (Mickelson *et al.*, 1988; Chakrabarty *et al.*, 1988). Studies from our laboratory using intact rabbits, have shown that administration of PAF following coronary artery ligation rapidly produces hypotension and ventricular fibrillation (Chakrabarty *et al.*, 1988). These effects could be reversed substantially by pretreatment with antiplatelet antiserum which reduced platelet counts to less than 5% of control, indicating significant platelet-dependence of the response observed. Perfusion of isolated, infarcted rabbit hearts with PAF increased coronary perfusion pressure and reduced left ventricular developed pressure and produced release of thromboxane B_2 and leukotrienes (Mickelson *et al.*, 1988). Thus, both direct and platelet-mediated effects appear to be important in producing the adverse effects of PAF.

The results obtained here suggest that the ability of PAF to increase the incidence of VF during ischaemia and to prevent recovery during reperfusion reflect direct cellular and global, electrophysiological and mechanical effects. The enhanced arrhythmogenesis observed with PAF in intact, anaesthetized rabbits is thus probably mediated initially through these direct myocardial electrophysiological and mechanical effects which are maintained during the early stages of ischaemia. Further release of PAF and other mediators (histamine, 5-hydroxytryptamine, thromboxane, leukotrienes) from stimulated cells and the ischaemic myocardium will then be able to maintain the adverse effects observed with PAF during continued ischaemia. The deleterious effects of PAF reported in intact animals and its ability to increase arrhythmogenesis during ischaemia are thus likely to be the result of the involvement of platelet-dependent and -independent mechanisms. The development of specific PAF-antagonists (Braquet *et al.*, 1987) and their ability to attenuate the effects of ischaemia (Wainwright *et al.*, 1989; Chakrabarty *et al.*, 1989; Montrucchio *et al.*, 1989) suggests the importance of endogenous PAF release during ischaemia in the presence of platelets. Further work is required to determine their mechanism of action.

In conclusion, these experiments have demonstrated that platelet activating factor at concentrations of 10^{-10} M and 10^{-9} M has direct myocardial electrophysiological effects which occur during normal perfusion and augment the electrophysiological and arrhythmogenic effects of ischaemia, and which occur independently of the presence of platelets. It also produces vasoconstriction (at 10^{-9} M) which may contribute to the exacerbation of ischaemia.

This work was supported by the British Heart Foundation.

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(Received June 12, 1990
Accepted July 18, 1990)

Br. J. Pharmacol. (1990), **101**, 739–745

Bradykinin-induced plasma exudation in guinea-pig airways: involvement of platelet activating factor

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1 We studied the effect of bradykinin on plasma exudation in the airways of the anaesthetized guinea-pig *in vivo*. Tissue content of extravasated Evans blue dye was used as an index of protein exudation in the larynx, trachea, main bronchi and intrapulmonary airways (i.p.a.).

2 Bradykinin increased the content of Evans blue in all tissues studied in a dose-related manner. The response was greatest in the main bronchi and i.p.a., less in the trachea and least in the larynx. A dose of 47 nmol kg^{-1} was the lowest tested which caused significant ($P < 0.001$) plasma exudation with increases in leakage above control values of 256% in the larynx, 405% in the trachea, 394% in the main bronchi and 485% in intrapulmonary airways.

3 Leakage was significantly ($P < 0.05$) increased above control values by 1 min after bradykinin (47 nmol kg^{-1}) in the main bronchi and intrapulmonary airways and was maximal in all airways 5 min after bradykinin. Although reduced by 15 min, the tissue content of dye was still significantly ($P < 0.05$) increased 2 h after bradykinin.

4 The prolonged tissue dye retention was due to a later phase of slow and maintained exudation preventing full clearance of dye after the initial response.

5 The initial phase of leakage was partially attenuated by the platelet activating factor (PAF) receptor antagonists WEB 2086 or BN 52021, by indomethacin or by inhibiting sensory nerve activation by opioid anaesthesia: it was not affected by mepyramine and cimetidine nor by the sulphidopeptide leukotriene receptor antagonists FPL 55712 or ICI 198,615. Adrenoceptor blockade of the anti-leakage effects of endogenously-released catecholamines significantly ($P < 0.05$) enhanced leakage.

6 The later phase of plasma leakage was completely inhibited by the PAF antagonists.

7 We conclude that, in guinea-pig airways *in vivo*, the initial phase of bradykinin-induced plasma exudation is mediated in part by PAF, sensory nerves and prostaglandins, whereas the later, prolonged phase of leakage is mediated exclusively by PAF. If bradykinin is generated in asthma, its potent and prolonged effects on plasma leakage may contribute significantly to airway oedema and may be involved in the development of bronchial hyperresponsiveness.

Introduction

Bradykinin, a nonapeptide metabolite of the kallikrein-kinin system, is an inflammatory mediator released locally at sites of tissue injury (Regoli & Barabe, 1980). One component of the inflammatory response to bradykinin is increased microvascular leakage of plasma proteins in a number of tissues, including the airways (Saria *et al.*, 1983). The mechanism of action of bradykinin in inducing airway leakage has not, however, been determined but may be via activation of several secondary mechanisms. For example, bradykinin-induced bronchoconstriction may be due to prostaglandin release (Collier & Shorley, 1960; Piper & Vane, 1969) or to stimulation of nerves (Kaufman *et al.*, 1980; Fuller *et al.*, 1987; Ichinose *et al.*, 1990). In addition, bradykinin causes cultured endothelial cells to generate a number of inflammatory mediators including prostaglandins and platelet-activating factor (PAF) (Jose *et al.*, 1981; McIntyre *et al.*, 1985; Stewart *et al.*, 1989). The purpose of the present study was, therefore, to characterize bradykinin-induced plasma exudation in the airways of the anaesthetized guinea-pig *in vivo* and determine the contribution of secondary mechanisms to the response by using specific drugs to inhibit histamine, products of arachidonic acid metabolism, PAF and sensory nerve activation. Changes in microvascular permeability to plasma proteins were quantified by a technique in which an intravenously-injected dye, Evans blue, binds to serum albumin and is extractable after extravasation into tissues (Evans *et al.*, 1987).

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Methods

Male Dunkin-Hartley outbred guinea-pigs (Charles River U.K. Ltd., Kent) 250–425 g body weight were housed in a temperature-controlled (21°C) room with food (Special Diet Services Ltd., Essex) and water freely available. They were pre-medicated with diazepam (6.5 mg kg^{-1}) by intraperitoneal injection (i.p.) and anaesthetized with Hypnorm (2.4 ml kg^{-1} by intramuscular injection). We used this anaesthetic combination because, in our hands, its effects are predictable over an extended period which reduces mortality. It also attenuates leakage due to sensory nerve activation (see below) and eliminated this effect from the studies not involved in investigating neural mechanisms. In the latter set of experiments, some animals were anaesthetized with urethane (2 g kg^{-1}, i.p.). Body temperature (rectal) was maintained at 34°C by placing the animals under a lamp. The jugular veins were exposed and covered with saline-damped gauze and Parafilm (American Can Co., Greenwich, U.S.A.) to limit fluid loss. Drug injections into the jugular veins (i.v.) were given by passing the needle through the pectoralis major to prevent bleeding on withdrawal.

Measurement of plasma exudation

The tissue content of Evans blue in response to experimental intervention was determined after perfusing the systemic circulation of dye (Evans *et al.*, 1987). The left ventricle was incised, a blunt-ended 13-gauge needle inserted into the aorta and the ventricles cross-clamped. Intravascular dye was expelled from

the incised right atrium using saline (pH 5.5, 21°C) at 100 mmHg pressure until the perfusate was clear (approximately 200 ml infused). The larynx, trachea, main bronchi and lungs were removed. The intrapulmonary airways were exposed by gently scraping away the parenchyma. Tissues were blotted between filter papers to remove excess fluid, their wet weights recorded and Evans blue dye extracted in formamide at 37°C for 16 h. The absorbance of extractable Evans blue dye was determined (PU 8620 Series spectrophotometer, Philips, Cambridge) at its maximum of 620 nm wavelength and, by interpolation from a standard curve of Evans blue in the range 0.5–10 $\mu g\,ml^{-1}$, its concentration calculated as ng dye mg^{-1} wet weight tissue.

Experimental protocol

All studies were carried out under diazepam-Hypnorm anaesthesia except where indicated otherwise. Leakage of Evans blue dye was determined in response to i.v. bradykinin at concentrations in the range 0.094–940 nmol kg^{-1} (Saria *et al.*, 1983). At time 0, 1 ml kg^{-1} saline was given i.v. (control) followed 2 min later by 30 mg kg^{-1} Evans blue dye i.v. After a further 1 min, bradykinin was injected and the animal perfused 5 min later. A dose of 47 nmol kg^{-1} was used in subsequent studies because it was threshold in the least responsive airway studied (i.e. larynx, see Figure 1).

Three time courses in response to bradykinin were examined: (1) initial leakage, accumulation and retention of Evans blue dye, (2) duration of 'initial phase' dye leakage, (3) 'late phase' tissue accumulation of Evans blue dye. The effect of drug intervention was examined in (1) and (3).

(1) To determine the time course of tissue accumulation and retention of Evans blue dye, animals were given saline (the vehicle for FPL 55712 and WEB 2086) at time 0, Evans blue at 2 min, bradykinin (47 nmol kg^{-1}) at 3 min and perfused 1–120 min later. Longer times were not studied as the animals would require additional anaesthetic.

Perfusion at 5 min was found to identify a maximal response and the effects of drugs on bradykinin-induced leakage were examined using this time point. Indomethacin (30 mg kg^{-1} in alkaline buffer) or alkaline buffer alone were given i.p. 13 h and 1 h before injection of saline at time 0. Mepyramine and cimetidine (both at 10 mg kg^{-1}) were injected i.p. 30 min before saline at time 0, with equivalent volumes of saline (1 ml kg^{-1}) given to control animals. The sulphidopeptide leukotriene (LT) receptor antagonists FPL 55712 (5 mg kg^{-1}) or ICI 198,615 (0.03 μmol kg^{-1}), or the PAF receptor antagonists WEB 2086 (1–1000 $\mu g\,kg^{-1}$) or BN 52021 (1 mg kg^{-1}) were given i.v. at time 0 (i.e. instead of saline). The vehicle solutions (at appropriate dilutions in saline: see below) for ICI 198,615, WEB 2086 or BN 52021 were administered to control animals.

The duration of effect of WEB 2086 (100 $\mu g\,kg^{-1}$) or BN 52021 (1 mg kg^{-1}), given at time 0, on the prolonged retention of dye was examined in animals perfused 1 h and 2 h after bradykinin.

To determine the contribution of sensory nerve activation to bradykinin-induced leakage, the effect of urethane anaesthesia was compared with diazepam-Hypnorm anaesthesia. We have shown previously (Belvisi *et al.*, 1988) that leakage due to the non-adrenergic, non-cholinergic (NANC) component of vagus nerve stimulation is significantly attenuated with diazepam-Hypnorm: leakage under urethane anaesthesia should be relatively greater. To prevent urethane-induced release of endogenous catecholamines reducing the magnitude of leakage (Boschetto *et al.*, 1988), phentolamine (2.5 mg kg^{-1}) and propranolol (1 mg kg^{-1}) were given i.v. 30 min before saline at time 0. The adrenoceptor antagonists were also given to a group of animals anaesthetized with diazepam-Hypnorm. To avoid increased leakage being obscured by the near-maximal effects of 47 nmol kg^{-1} bradykinin in certain tissues, a lower dose of 9.4 nmol kg^{-1} was used in this part of the study.

(2) To determine the duration of 'initial phase' dye leakage, saline was given at time 0 and again after 2 min (i.e. instead of Evans blue) followed 1 min later by bradykinin (47 nmol kg^{-1}). Evans blue dye was then injected 30 s–120 min after bradykinin. Irrespective of the time of dye injection, animals were perfused 5 min after Evans blue dye.

(3) To determine the 'late phase' tissue accumulation of dye after the initial leakage had resolved, saline was given at time 0, bradykinin at 5 min and, based on the time course of resolution of initial leakage, Evans blue dye was given at 30 min. Animals were then perfused at 45, 60 and 120 min. Baseline control animals were given saline instead of bradykinin at 5 min. WEB 2086 (100 $\mu g\,kg^{-1}$) was administered 2 min before Evans blue (i.e. 28 min after bradykinin) and its effect on bradykinin-induced leakage examined in animals perfused at 60 and 120 min.

For comparison with bradykinin under the present experimental conditions, the effect of PAF on leakage was also examined. Animals were given Evans blue followed by saline or PAF (C_{16}; 50 ng kg^{-1}) and were perfused 5 or 45 min later. Leakage was measured only in the trachea in these animals.

Drugs and chemicals

The following drugs and chemicals were used: Evans blue dye, bradykinin (triacetate salt), formamide, urethane, indomethacin (Sigma Chemical Co. Ltd., Dorset); diazepam (Pharma Hameln G.m.b.H., W. Germany); Hypnorm (fentanyl citrate and fluanisone; Janssen Pharmaceuticals Ltd., Oxford); phentolamine mesylate BP (CIBA Laboratories, Horsham); propranolol hydrochloride (Imperial Chemical Industries PLC, Macclesfield); cimetidine (Smith, Kline & French Labs., Ltd., Welwyn Garden City); C_{16}-PAF (Bachem AG, Bubendorf, Switzerland); saline (0.9% sodium chloride; Travenol Laboratories, Thetford). Bradykinin was aliquoted into 2 ml stock solutions containing 940 nmol ml^{-1} w/v in saline which were stored at −20°C. Evans blue dye, 30 mg ml^{-1} in saline, was filtered through an Acrodisc membrane (Gelman Sciences, Michigan, U.S.A.) of 0.2 μm diameter. Urethane was prepared as 25% w/v in saline giving an injection volume of 8 ml kg^{-1}. The following drugs were kind gifts and were prepared in solution as follows: 3-(4-(2-chlorophenyl)-9-methyl-6H-thieno-(3, 2-F)(1,2,4)-triazolo-(4,3-a)(1,4)-diazepine-2-yl)-1-(4-morpholinyl)-1-propanone (WEB 2086, Boehringer Ingleheim GmbH, W. Germany), 10 mg ml^{-1} w/v in 10% v/v ethanol in distilled water; 7-[3-(4-acetyl-3-hydroxy-2-propylphenoxy)-2-hydroxypropoxy]-4-oxo-8-propyl-4H-1-benzopyran-2-carboxylic acid (FPL 55712, Fisons Pharmaceuticals, Loughborough, Leics.), 5 mg ml^{-1} in saline and mepyramine maleate (Rhone-Poulenc Ltd., Dagenham, Essex), 10 mg ml^{-1} in saline. BN 52021 (3-t-butyl-hexahydro-4,7b,11-trihydroxy-8-methyl-9H-1, 7a-epoxymethano-1H,6aH-cyclopenta-[c]furo[2,3-b]furo[3', 2':3,4]cyclopenta[1,2-d]furan-5,9,12(4H)trione) (Dr. P. Braquet, Laboratoires Henri Beaufour, Paris, France) was dissolved in the solvent provided (20 mg kg^{-1}) and diluted in saline. ICI 198,615 ([1-[[2-methoxy-4-[[(phenylsulphonyl)carbonyl]phenyl]methyl]-1H-indazol-6-yl] carbamic acid cyclopentyl ester) (Stuart Pharmaceuticals, Division of ICI Americas Inc., Wilmington, Delaware, U.S.A.) was dissolved in equal volumes of 1 N NaOH and polyethylene glycol 400 and diluted with phosphate-buffered saline (10 mM, pH 7.4) (Krell *et al.*, 1987). Drug solutions were prepared on each day of experimentation with dilutions made to give injection volumes of 1 ml kg^{-1}. Indomethacin was prepared as a solution of 3.3 mg ml^{-1} w/v in alkaline phosphte buffer (pH 7.8) of composition (mM) KH_2PO_4 20 and Na_2HPO_4 120, stored at 4°C, and warmed to room temperature before use.

Statistical analyses

Distribution of data for the concentration of extractable Evans blue dye approximated a normal distribution but showed positive skew and the Mann-Whitney U-test (two-

tailed) was used to test the null hypothesis (Armitage & Berry, 1971). For ease of presentation, data in Results are means ± one standard error (s.e.mean). The null hypothesis was rejected at $P < 0.05$. Inhibition by a drug of bradykinin-induced increase in tissue Evans blue content was considered to be complete when the dye concentration in drug-treated animals was significantly different from that in bradykinin-treated animals but not to that in control (baseline) animals.

Results

All results are from animals anaesthetized with diazepam-Hypnorm except where stated otherwise. Intravenously-injected bradykinin in animals previously given i.v. Evans blue dye caused each of the airways to become blue in colour when compared with control animals. The colouration was presumably due to dye which had leaked into the interstitial tissue as a result of increased microvascular permeability and plasma leakage. Quantification of the response to bradykinin demonstrated a dose-related increase in tissue content of Evans blue dye (Figure 1). The lowest dose examined which caused a significant ($P < 0.05$) increase in tissue dye content above control (i.e. threshold) in larynx was 47 nmol kg^{-1} whilst in trachea, main bronchi and intrapulmonary airways it was 9.4 nmol kg^{-1}. The response was greater in the lower airways (i.e. main bronchi and i.p.a.) than in the larynx and trachea. The values used as baseline controls include those from 3 animals given the alcohol : water : saline diluent for WEB 2086 as there was no significant difference in dye content between animals given diluent and those given saline alone.

Dye accumulation in response to 47 nmol kg^{-1} bradykinin was significantly ($P < 0.05$) increased compared with controls by 1 min after injection in main bronchi and intrapulmonary airways (Figure 2). The response in all airways studied was maximal at 5 min (referred to as 'initial phase' below) and was significantly increased above controls at all time points studied up to 2 h after injection (referred to as 'retention' below).

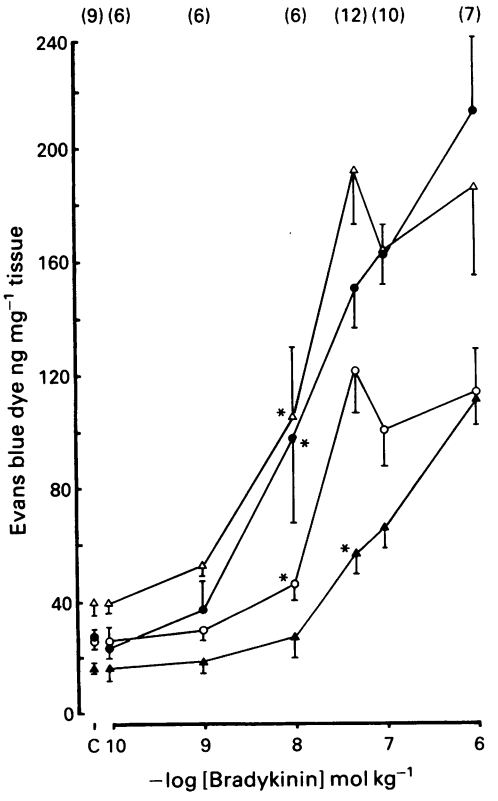

Figure 1 Effect of bradykinin on plasma exudation in guinea-pig airways: (△) main bronchi; (●) intrapulmonary airways; (○) trachea; (▲) larynx. Bradykinin increased the tissue content of the plasma marker Evans blue; * minimum dose tested causing significant ($P < 0.05$) leakage compared with control (C). Numbers of animals for each mean value indicated in parentheses; s.e.mean shown by vertical bars.

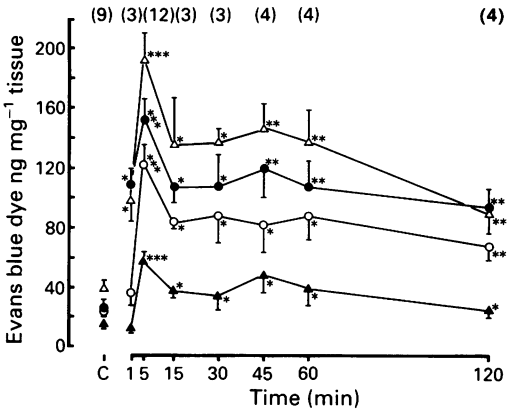

Figure 2 Time course of effect of bradykinin (47 nmol kg^{-1}, i.v.) on plasma exudation in guinea-pig airways: (△) main bronchi; (●) intrapulmonary airways; (○) trachea; (▲) larynx. Tissue content of the plasma marker Evans blue was significantly increased compared with controls (C) for up to 2 h: * $P < 0.05$; ** $P < 0.01$, *** $P < 0.001$. Numbers of animals for each mean value indicated in parentheses; s.e.mean shown by vertical bars.

PAF significantly ($P < 0.05$) increased leakage of dye in the trachea, an effect which had resolved completely by 45 min after injection: mean dye contents, ng mg^{-1} ($n = 3$ for each value), were 29.4 ± 5.1 for saline and 126.5 ± 15.3 for PAF, whilst at 45 min the values were 20.6 ± 6.3 and 29.5 ± 6.3 respectively.

Effect of antagonists on bradykinin-induced leakage

The PAF antagonist WEB 2086 inhibited the initial phase of bradykinin (47 nmol kg^{-1})-induced dye leakage in a dose-related manner (Figure 3): in the larynx complete inhibition was achieved at a dose of 1 μg kg^{-1}; in the remaining airways 100 and 1000 μg kg^{-1} were inhibitory (by 51–57%, depending on the airway) but neither achieved complete blockade. WEB 2086 (100 μg kg^{-1}) alone had no significant effect on baseline control values (Figure 3). A structurally dissimilar PAF antagonist BN 52021 (1 mg kg^{-1}) also partially and significantly ($P < 0.05$) inhibited the initial phase of bradykinin-induced leakage in the two airways studied: by 36% in the trachea and by 49% in the main bronchi (Table 1).

WEB 2086 (100 μg kg^{-1}) or BN 52021 (1 mg kg^{-1}) also inhibited the retention of Evans blue dye: at 1 h, inhibition was significant ($P < 0.05$); at 2 h, inhibition was complete in

Table 1 Effect of BN 52021 on bradykinin-induced plasma exudation in guinea-pig airways

		Airway	
Administration	n	*Trachea*	*Main bronchi*
Vehicle + saline	3	16.1 (0.6)	39.7 (7.2)
Vehicle + bradykinin	4	103.4* (12.7)	152.4* (15.2)
BN 52021 + bradykinin	4	72.1* (5.6)	97.9*† (6.0)

Values are mean (s.e.mean) tissue content of the plasma marker, Evans blue dye (ng mg^{-1} wet wt. tissue) after bradykinin (47 nmol kg^{-1}, i.v.) alone or with BN 52021 (1 mg kg^{-1}). * $P < 0.05$ compared with vehicle + saline; † $P < 0.05$ compared with vehicle + bradykinin.

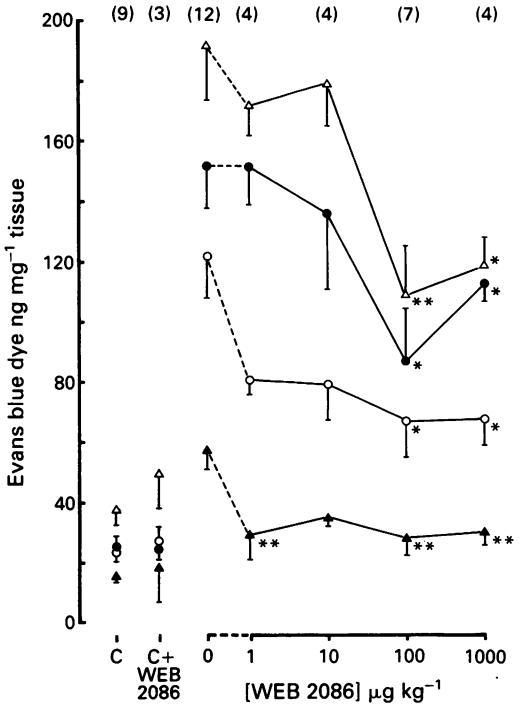

Figure 3 Inhibitory effect of the PAF antagonist WEB 2086 on bradykinin (47 nmol kg^{-1}, i.v.)-induced plasma exudation in guinea-pig airways: (△) main bronchi; (●) intrapulmonary airways; (○) trachea; (▲) larynx. WEB 2086 had no significant effect on control values (C vs. C + WEB 2086) but significantly inhibited leakage of the plasma marker Evans blue: * $P < 0.05$, ** $P < 0.01$ compared with bradykinin (i.e. 0 μg kg^{-1} WEB 2086). Numbers of animals for each mean value indicated in parentheses; s.e.mean shown by vertical bars.

all airways studied. The inhibitory effect of the two antagonists is shown for the main bronchi in Figure 4.

Neither the anti-histamines mepyramine and cimetidine in combination (10 mg kg^{-1} each) nor leukotriene antagonists FPL 55712 (5 mg kg^{-1}) or ICI 198,615 (0.03 μmol kg^{-1}) significantly affected the initial phase of dye leakage induced by bradykinin (Table 2, Figure 5). The alkaline buffer used as solvent for indomethacin also had no significant inhibitory effect, although the values at each airway level were lower than those with bradykinin alone (Figure 5). Indomethacin, when compared with the values for the buffer, showed significant ($P < 0.05$), but not complete, inhibition of initial phase leakage in the larynx (by 63%) and intrapulmonary airways (by 29%).

Data for the effects of anaesthesia and adrenoceptor blockade are given in Table 3. Whichever anaesthetic was used, bradykinin (9.4 nmol kg^{-1}) induced significant leakage of dye

Table 2 Effect of antihistamine drugs on bradykinin-induced plasma exudation in guinea-pig airways

		Airway			
Administration	n	*Larynx*	*Trachea*	*Main bronchi*	*I.p.a.*
Baseline control	9	16.3 (1.2)	24.2 (2.6)	39.0 (4.4)	26.0 (2.8)
Bradykinin	5	47.7** (9.9)	83.3** (6.3)	189.2** (16.8)	166.2** (16.2)
Anti-histamines + bradykinin	5	48.1** (4.6)	113.0** (15.8)	173.9** (23.9)	161.1** (12.6)

Values are mean (s.e.mean) tissue content of the plasma marker, Evans blue (ng mg^{-1} wet wt. tissue) after bradykinin (47 nmol kg^{-1}, i.v.) alone or with the antihistamines mepyramine and cimetidine (10 mg kg^{-1} each, i.p.). ** $P < 0.01$ compared with baseline control. I.p.a. = intrapulmonary airways.

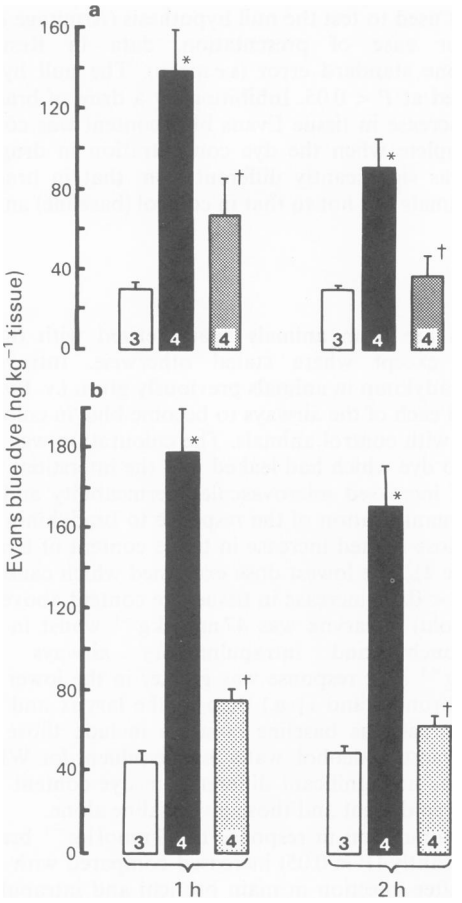

Figure 4 Inhibitory effect of the PAF antagonists WEB 2086 (100 μg kg^{-1}, i.v.) and BN 52021 (1 mg kg^{-1}, i.v.) on bradykinin (47 nmol kg^{-1}, i.v.)-induced tissue retention (at 1 and 2 h) of the plasma marker Evans blue in guinea-pig main bronchi: in (a) and (b), open columns, control; solid columns, bradykinin. In (a) stippled columns, WEB 2086 + bradykinin; in (b) stippled columns, BN 52021 + bradykinin. * $P < 0.05$ compared with control; † $P < 0.05$ compared with bradykinin. Number of animals for each mean indicated in the histograms; s.e.mean shown by vertical bars.

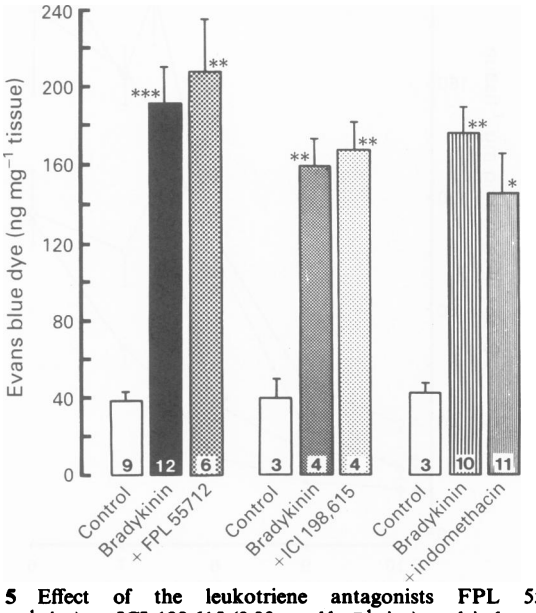

Figure 5 Effect of the leukotriene antagonists FPL 55712 (5 mg kg^{-1}, i.v.) or ICI 198,615 (0.03 μmol kg^{-1}, i.v.), and indomethacin (30 mg kg^{-1}, i.p.) on bradykinin (47 nmol kg^{-1}, i.v.)-induced plasma exudation of the plasma marker Evans blue in guinea-pig main bronchi. For each drug, the vehicle was given to both control and bradykinin-treated animals. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with control. Numbers of animals for each mean value indicated in the histograms; s.e.mean shown by vertical bars.

Table 3 Effect of anaesthesia and adrenoceptor blockade on bradykinin-induced plasma exudation in guinea-pig airways

Anaesthesia	*Administration*	*Airway*			
		Larynx	*Trachea*	*Main bronchi*	*I.p.a.*
Diazepam + Hypnorm	Baseline control ($n = 5$)	16.3	24.2	39.0	26.0
		(1.2)	(2.6)	(4.4)	(2.8)
	Bradykinin ($n = 6$)	27.5	45.6**	105.7*	97.4*
		(7.1)	(5.8)	(24.3)	(29.7)
	Phent. + Prop. + bradykinin ($n = 6$)	13.7	35.5	98.5*	85.1***
		(2.7)	(6.7)	(20.1)	(10.8)
Urethane	Baseline control ($n = 5$)	14.3	26.0	41.4	27.5
		(2.7)	(3.4)	(7.7)	(3.7)
	Bradykinin ($n = 5$)	15.1	42.2	102.5*	60.8*
		(4.5)	(8.6)	(12.5)	(14.0)
	Phent. + Prop. + bradykinin ($n = 7$)	11.2	54.6*	172.8**†	162.6**†
		(1.5)	(14.2)	(19.1)	(17.1)

Values are mean (s.e.mean) tissue content of the plasma marker, Evans blue (ng mg^{-1} wet wt. tissue) after bradykinin (9.4 nmol kg^{-1}, i.v.) with or without pretreatment with phentolamine (Phent., 2.5 mg kg^{-1}, i.v.) and propranolol (Prop., 1 mg kg^{-1}, i.v.). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with baseline control for each anaesthetic; † $P < 0.05$ compared with bradykinin alone with urethane anaesthesia and also compared with appropriate values for Phent. + Prop. + bradykinin with diazepam plus Hypnorm.

in main bronchi and intrapulmonary airways: similar dye leakage was seen in the trachea, although only with diazepam-Hypnorm did the values reach significance. Phentolamine and propranolol did not significantly affect the bradykinin response with diazepam-Hypnorm. However, adrenoceptor blockade in urethane-anaesthetized animals not only significantly augmented bradykinin-induced leakage, but also significantly ($P < 0.05$) increased it above that in diazepam-Hypnorm-anaesthetized animals with adrenoceptor blockade.

Duration of initial phase dye leakage

Evans blue dye given at a number of time points after bradykinin with animals consistently perfused 5 min later demonstrated that the initial phase of dye leakage was of short duration (Figure 6): leakage of dye became rapidly reduced within 1 min, was significantly ($P < 0.05$) reduced after 5 min with baseline established between 15–30 min and with no further significant leakage up to 2 h after bradykinin.

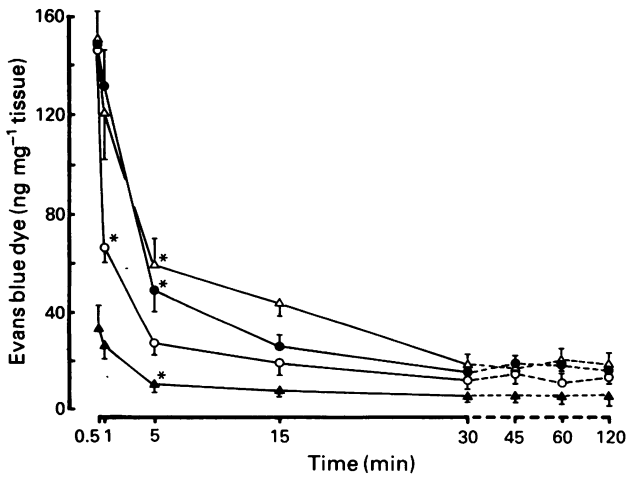

Figure 6 Time course of resolution of initial phase leakage after bradykinin (47 nmol kg^{-1}, i.v.)-induced plasma exudation in guinea-pig airways: (△) main bronchi; (●) intrapulmonary airways; (○) trachea; (▲) larynx. The plasma marker Evans blue was given at different times after bradykinin and its mean tissue content determined in animals killed 5 min after dye injection; s.e.mean shown by vertical bars. * first time point significantly different ($P < 0.05$) from values at 0.5 min. $n = 3$ animals at each time point.

Late phase dye accumulation and the effect of WEB 2086

Evans blue dye given 30 min after bradykinin (by which time the initial phase of dye leakage had resolved; see above) demonstrated significant ($P < 0.05$) accumulation of dye 30 and 90 (but not 15) min later (i.e. 1 and 2 h, but not 45 min, after bradykinin) (Figure 7). WEB 2086, 100 μg kg^{-1}, completely inhibited dye accumulation at both 1 and 2 h.

Discussion

Our results demonstrate that bradykinin causes a long-lasting increase in tissue Evans blue dye content throughout the lower airways in guinea-pigs. Evans blue binds to plasma proteins, particularly albumin (Rawson, 1943; LaVeen & Fishman, 1947), a property which has been used to quantify plasma leakage in many tissues including the airways (Chung *et al.*, 1990). In airways, leakage of Evans blue correlates with that of radiolabelled albumin (Rogers *et al.*, 1989) which indicates that bradykinin increased the permeability of airway endothelium to relatively high-molecular weight plasma proteins (60 KDa).

The increase in airway permeability in response to brady-

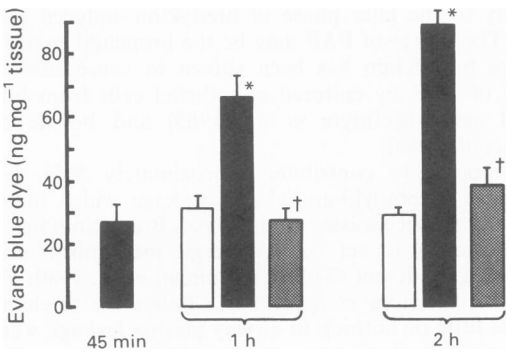

Figure 7 Effect of the PAF antagonist WEB 2086 on bradykinin (BK, 47 nmol kg^{-1}, i.v.)-induced tissue accumulation of the plasma marker Evans blue dye in guinea-pig main brochi: open columns, control; solid columns, bradykinin; stippled columns, bradykinin + WEB 2086. Evans blue and WEB 2086 (100 μg kg^{-1}, i.v.) were injected 30 min after bradykinin and the mean dye content determined 13, 30 and 90 min later (i.e. 45 min, 1 h and 2 h after BK); s.e.mean shown by vertical bars. * $P < 0.05$ compared with control; † $P < 0.05$ compared with bradykinin. $n = 4$ animals for each value.

kinin was rapid in onset and maximal at 5 min, an observation which is characteristic of the response to a number of mediators (Chung *et al.*, 1990). Unlike the response to other mediators which resolves in about 30 min, tissue content of dye, although declining, was maintained for at least 2 h. The prolonged response was not artifactual because PAF-induced leakage in the present study resolved within 45 min which is consistent with previous findings (Evans *et al.*, 1987; O'Donnell & Barnett, 1987).

The prolonged response to bradykinin may be due to continual leakage, reduced clearance of initial leakage, or to a biphasic response. The rapid decline in tissue dye content when Evans blue was injected at intervals after bradykinin indicated that the prolonged plasma exudation was due to an extended initial phase of leakage. Extravasated plasma is usually cleared from airway tissue by passage into the airway lumen and by lymphatic drainage (Chung *et al.*, 1990). However, a slow and maintained leakage of plasma might prevent full clearance. By allowing the initial bradykinin-induced plasma leakage to subside for 30 min before injecting Evans blue we found a slow accumulation of dye over the following 90 min. Thus, the prolonged plasma exudation in guinea-pig airways in response to bradykinin appears to be due to an initial and abrupt increase in vascular permeability with rapid bulk flow followed by a period of maintained leakage at a rate slightly less than the rate of dye clearance from the initial phase.

PAF is a potent mediator of airway plasma leakage (Evans *et al.*, 1987; 1989; O'Donnell & Barnett, 1987). The triazoldiazepine WEB 2086 (Casals-Stenzel *et al.*, 1987) and the ginkgolide mixture BN 52021 (Guinot *et al.*, 1986) are specific PAF receptor antagonists which block PAF-induced plasma leakage, with WEB 2086 being the more potent (Hellewell & Williams, 1989). In the present study, both WEB 2086 and BN 52021, the most active of the ginkgolides comprising BN 52063, partially inhibited the initial phase of bradykinin-induced leakage and completely blocked the later phase which suggests an increasing involvement of PAF with time. Maximal inhibition of the early phase of leakage was approximately 50% and included doses of WEB 2086 which we have shown to block completely equivalent PAF-induced leakage (Evans *et al.*, 1988). Similarly, inhibition approached 50% at a dose of BN 52021 shown previously to inhibit markedly PAF-induced bronchoconstriction (Braquet *et al.*, 1985). In contrast both WEB 2086 and BN 52021 completely abolished the later phase retention of dye. In addition, when given after the initial phase of leakage had resolved, WEB 2086 completely blocked late phase dye accumulation. Blockade was presumably a result of partially inhibited initial phase leakage with blockade of later accumulation allowing full clearance of the initial tissue content of plasma. Thus, PAF appears to contribute exclusively to the later phase of bradykinin-induced plasma leakage. The source of PAF may be the bronchial endothelial cells since bradykinin has been shown to cause substained synthesis of PAF by cultured endothelial cells from human umbilical vein (McIntyre *et al.*, 1985) and bovine aorta (Stewart *et al.*, 1989).

PAF appears to contribute approximately 50% to the initial phase of bradykinin-induced leakage which indicates that additional mechanisms are involved. Bradykinin has been shown previously to act via cholinergic mechanisms and to stimulate vagal afferent C-fibres (Kaufman *et al.*, 1980; Fuller *et al.*, 1987; Ichinose *et al.*, 1990). Cholinergic mechanisms contribute little or nothing to airway plasma leakage whereas NANC neural mechanisms potently induce airway exudation in rodents (Barnes *et al.*, 1990). This 'neurogenic' plasma exudation can be blocked in a number of ways including the use of anaesthetics such as Hypnorm which contain opiates (Belvisi *et al.*, 1988). Animals anaesthetized with Hypnorm should exhibit reduced leakage in response to bradykinin compared with those anaesthetized with urethane, a non-opioid containing anaesthetic, when the anti-leakage effects of urethane-induced release of endogenous catecholamines (Spriggs, 1965) are blocked by adrenoceptor antagonists (Boschetto *et al.*, 1988). Under phentolamine and propranolol blockade we found that in animals anaesthetized with urethane, bradykinin induced twice as much leakage as in animals anaesthetized using Hypnorm which indicates that activation of sensory nerves contributes to the early phase of bradykinin-induced airway leakage. Interestingly, morphine weakly inhibits bradykinin-induced bronchoconstriction in the guinea pig (Collier & Shorley, 1960).

Combined histamine H_1 and H_2 receptor blockade with mepyramine and cimetidine had no inhibitory effect on bradykinin-induced leakage, consistent with data in rat skin (Whalley, 1987), and suggests that histamine release is not involved in the leakage response. Similarly, the sulphidopeptide leukotriene receptor antagonist FPL 55712 (Chand, 1978) had no inhibitory effect at a dose that completely blocks plasma leakage induced by LTD_4 (Evans *et al.*, 1988). FPL 55712 is neither selective nor long-acting (Hamel *et al.*, 1982). However, a more selective and longer-acting compound ICI 198,615 (Krell *et al.*, 1987) did not inhibit bradykinin-induced leakage which indicates that leukotrienes do not contribute to the response. In contrast, indomethacin partially inhibited bradykinin-induced leakage in the larynx and intrapulmonary airways which indicates the involvement of products of cyclo-oxygenase metabolism in the response. This finding is consistent with those which have implicated the involvement of prostaglandins in bradykinin-induced bronchoconstriction (Collier & Shorley, 1960; Piper & Vane, 1969; Ichinose *et al.*, 1990). It is possible that bradykinin-induced PAF generation is involved in the response although we have shown previously that PAF-induced plasma leakage is unaffected by pretreatment with indomethacin (Evans *et al.*, 1987). However, endogenous release of PAF may generate prostaglandins because PAF antagonists will block bradykinin-induced PGI_2 generation by bovine endothelial cells *in vitro* (Stewart *et al.*, 1989). Of the prostaglandins possibly generated, thromboxane has not been shown to affect leakage (Chung *et al.*, 1990) and is unlikely to be involved. Vasodilator prostaglandins such as PGI_2 or PGE_2 potentiate plasma exudation in skin by increasing blood flow to sites of leakage (Williams, 1983). In the airways the effect of altered blood flow on plasma leakage is unclear. Unlike skin, where blood flow is low, bronchial blood flow is greater and exogenously-administered vasodilators do not potentiate the leakage induced by exogenously-administered inducers of leakage (Rogers *et al.*, 1988; Boschetto *et al.*, 1988). However, the action of locally-released prostaglandins may be different from that of vasodilators given intravenously. Prostaglandins released by bradykinin in the present study may, therefore, contribute to the response by augmenting leakage induced by sensory nerve activation and PAF. The source of the prostaglandins in the present study is unclear but may be from the bronchial endothelial cells because bradykinin has been shown previously to stimulate the production of prostaglandins by aortic endothelial cells *in vitro* (Jose *et al.*, 1981; Stewart *et al.*, 1989).

Plasma exudation has been emphasised as important in asthma (Persson, 1986). Bradykinin may be released by inflammatory cells (Newball *et al.*, 1979; Proud *et al.*, 1985) whilst reduced concentrations of the bradykinin-precursor kininogen in the plasma of asthmatics coupled with raised levels of esterase enzyme activity and plasma kinins (Abe *et al.*, 1967; Bortkiewicz, 1983) indicate a role for bradykinin in asthma. The results of the present study suggest that if bradykinin is generated in asthma its effects on plasma exudation are likely to be prolonged and contribute significantly to the airway inflammation which may underlie bronchial hyperresponsiveness (Hogg *et al.*, 1987). However, our results also indicate potential therapy using PAF antagonist drugs.

Note added in proof

Ichinose, M. & Barnes, P.J. report that a bradykinin B_2-receptor mediates the response (1990, *Am. Rev. Respir. Dis.*, **142**, in press).

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(Received May 2, 1990
Accepted July 13, 1990)

Br. J. Pharmacol. (1990), **101**, 746–752

Characterization of three inhibitors of endothelial nitric oxide synthase *in vitro* and *in vivo*

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1 Three analogues of L-arginine were characterized as inhibitors of endothelial nitric oxide (NO) synthase by measuring their effect on the endothelial NO synthase from porcine aortae, on the vascular tone of rings of rat aorta and on the blood pressure of the anaesthetized rat.

2 N^G-monomethyl-L-arginine (L-NMMA), N-iminoethyl-L-ornithine (L-NIO) and N^G-nitro-L-arginine methyl ester (L-NAME; all at 0.1–100 μM) caused concentration-dependent inhibition of the Ca^{2+}-dependent endothelial NO synthase from porcine aortae.

3 L-NMMA, L-NIO and L-NAME caused an endothelium-dependent contraction and an inhibition of the endothelium-dependent relaxation induced by acetylcholine (ACh) in aortic rings.

4 L-NMMA, L-NIO and L-NAME (0.03–300 mg kg^{-1}, i.v.) induced a dose-dependent increase in mean systemic arterial blood pressure accompanied by bradycardia.

5 L-NMMA, L-NIO and L-NAME (100 mg kg^{-1}, i.v.) inhibited significantly the hypotensive responses to ACh and bradykinin.

6 The increase in blood pressure and bradycardia produced by these compounds were reversed by L-arginine (30–100 mg kg^{-1}, i.v.) in a dose-dependent manner.

7 All of these effects were enantiomer specific.

8 These results indicate that L-NMMA, L-NIO and L-NAME are inhibitors of NO synthase in the vascular endothelium and confirm the important role of NO synthesis in the maintenance of vascular tone and blood pressure.

Introduction

Vascular endothelial cells synthesize nitric oxide (NO) from the terminal guanidino nitrogen atom(s) of L-arginine (Palmer *et al.*, 1988a). This synthesis of NO accounts for the biological actions of endothelium-derived relaxing factor on vascular strips (Palmer *et al.*, 1987; Ignarro *et al.*, 1987; Furchgott, 1990), in perfused hearts (Amezcua *et al.*, 1988; Kelm & Schrader, 1988) and on platelets (Radomski *et al.*, 1987a,b). The formation of NO by vascular endothelial cells in culture is inhibited by N^G-monomethyl-L-arginine (L-NMMA), but not by its D-enantiomer (Palmer *et al.*, 1988b). L-NMMA also inhibits the NO synthase in endothelial cell homogenates (Palmer & Moncada, 1989).

L-NMMA causes an endothelium-dependent increase in the tone of rings of both rabbit aorta (Palmer *et al.*, 1988b; Rees *et al.*, 1989a) and guinea-pig pulmonary artery (Sakuma *et al.*, 1988) and a rise in coronary perfusion pressure in the isolated perfused rabbit (Amezcua *et al.*, 1989) and guinea-pig (Levi *et al.*, 1990) heart. Furthermore, L-NMMA inhibits endothelium-dependent relaxation of rings of rabbit aorta (Palmer *et al.*, 1988b; Rees *et al.*, 1989a) and guinea-pig pulmonary artery (Sakuma *et al.*, 1988) and the release of NO from the perfused rabbit aorta (Rees *et al.*, 1989a). In addition, it inhibits the fall in coronary perfusion pressure induced by acetylcholine (ACh) in the isolated perfused heart of rabbit (Amezcua *et al.*, 1989) and guinea-pig (Levi *et al.*, 1990). These effects of L-NMMA are consistent with inhibition of vascular endothelial NO synthesis from L-arginine under basal and stimulated conditions.

Intravenous administration of L-NMMA induces an increase in mean arterial blood pressure in anaesthetized rabbit (Rees *et al.*, 1989b) and guinea-pig (Aisaka *et al.*, 1989a) and inhibits the hypotension induced by ACh (Rees *et al.*, 1989b) and bradykinin (Aisaka *et al.*, 1989b). Furthermore, the release of NO from perfused aortae taken from L-NMMA-treated rabbits is inhibited (Rees *et al.*, 1989b). Similar observations have been made in man, where an infusion of L-NMMA into the brachial artery significantly reduces forearm blood flow and into the dorsal hand veins inhibits ACh-induced vasodilatation. These effects can be reversed by L-arginine (Vallance *et al.*, 1989a,b). These data indicate that the formation of NO plays an important role in the regulation of blood flow and the control of blood pressure.

In the present study we have examined the effects of L-NMMA *in vitro* and *in vivo* and have characterized the effects of two other L-arginine analogues (Figure 1), N-iminoethyl-L-ornithine (L-NIO) and N^G-nitro-L-arginine methyl ester (L-NAME) as inhibitors of NO synthesis from L-arginine by the vascular endothelium. These compounds have recently been shown to inhibit NO synthase in adrenal

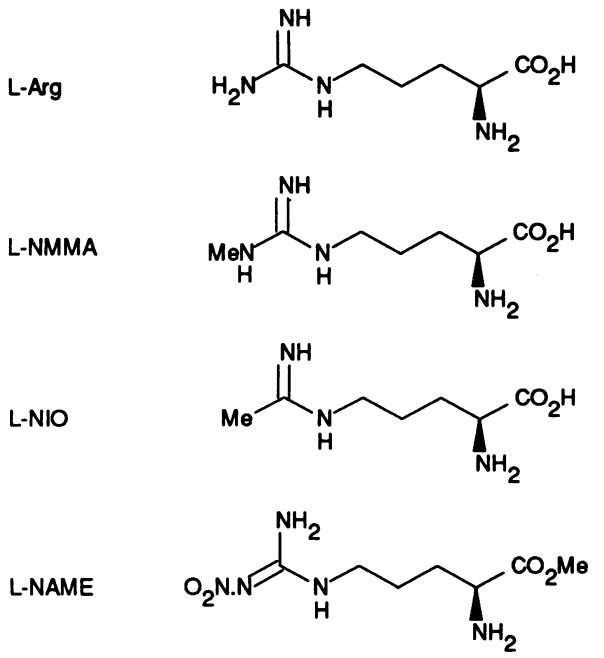

Figure 1 Structural formulae of L-arginine (L-Arg), N^G-monomethyl-L-arginine (L-NMMA), N-iminoethyl-L-ornithine (L-NIO) and N^G-nitro-L-arginine methyl ester (L-NAME).

glands (Palacios *et al.*, 1989) and brain synaptosomes (Knowles *et al.*, 1990). Some of these results were presented at the meeting 'Nitric oxide from L-arginine: a bioregulatory system' at the Royal Society, London, September 14–15, 1989.

Methods

Assay of endothelial nitric oxide synthase

Fresh porcine thoracic aortae, obtained from a local abattoir, were trimmed free of adhering fat and connective tissue and washed with phosphate buffered saline (pH 7.4). The aortae were cut longitudinally and the endothelium removed by scraping with a scalpel. The scrapings were taken up in ice-cold 0.1 M HEPES buffer, pH 7.4, containing 1 mM dithiothreitol (3 aortae/ml) and homogenized by sonication twice for 5 s. The homogenate was centrifuged at 150,000 *g* for 30 min at 4°C and the supernatant incubated with AG50-X8 (Na^+-form; 100 mg ml^{-1} of supernatant) for 5 min at 4°C to deplete endogenous L-arginine. The ion exchange resin was removed by centrifugation at 12,000 *g* for 2 min and the supernatant used as the source of NO synthase and soluble guanylate cyclase.

Nitric oxide synthase was assayed by the stimulation of guanylate cyclase (Knowles *et al.*, 1989) and by the formation of [^{3}H]-citrulline from L-arginine (Palmer & Moncada, 1989). For the determination of guanylate cyclase activity, endothelial homogenate (100 μl) was incubated with NADPH (100 μM), L-arginine (30 μM), Mg^{2+} (5 mM), guanosine 5'-triphosphate (GTP) (1 mM) and the inhibitors in a total volume of 200 μl for 20 min. Incubations were terminated by addition of 50 μl of perchloric acid (20% v/v in H_2O) and neutralised with 100 μl of 1.08 M K_3PO_4. The incubates were then centrifuged (12,000 *g* for 2 min) and aliquots of the supernatant diluted appropriately with 50 mM Tris buffer (pH 7.5) containing 4 mM EDTA. Guanosine 3':5'-cyclic monophosphate (cyclic GMP) was determined by radioimmunoassay.

For determination of [^{3}H]-citrulline formation, incubates contained 5 μCi [^{3}H]-L-arginine in 3 μM L-arginine, NADPH (100 μM) and 100 μl endothelial cytosol in a total volume of 250 μl 0.1 M HEPES buffer, pH 7.4. After incubation for 20 min at 37°C, the reaction was stopped by the addition of 50 μl perchloric acid as above. [^{3}H]-citrulline formation was determined by high performance liquid chromatography (h.p.l.c.) using a μ Bondapak C_{18} column (Waters) with a mobile phase of 2% acetonitrile in 25 mM sodium acetate (pH 4.35) containing sodium hexane sulphonate (15 mM). The radioactivity in the fractions containing [^{3}H]-citrulline was determined by liquid scintillation counting.

Organ bath studies

Male Wistar rats (250–300 g) were killed by a blow on the head and exsanguination. The thoracic aorta was removed, trimmed free of adhering fat and connective tissue and cut into 4 mm rings. The endothelium was removed from some rings by gently rubbing the internal surface with a pipe cleaner. The failure of ACh (1 μM) to induce relaxation of these rings was taken as an indication of endothelium removal.

The rings were mounted under 1.0 g resting tension on stainless steel hooks in 20 ml organ baths filled with Krebs buffer containing indomethacin (5 μM), gassed with 95% O_2 and 5% CO_2 at 37°C. Tension was recorded with Grass FTO3 isometric transducers on a 6-channel multipen recorder (Rikadenki). The tissues were allowed to equilibrate for 60 min during which the Krebs buffer was changed at 15 min intervals, before being contracted submaximally (approx. ED_{90}) by addition of phenylephrine (750 nM). Cumulative relaxation curves to ACh were obtained in each ring to assess the integrity of the endothelium. Rings showing <65% relaxation were discarded. After washout the tissues were allowed to equilibrate for a further 60 min during which the Krebs buffer was changed at 15 min intervals.

In those tissues used to study the effects of the inhibitors on basal tone, L-NMMA, L-NIO or L-NAME (100 μM for each) was added to the organ bath. In a separate series of experiments, cumulative contraction curves to these compounds were obtained in each ring in the presence of a threshold concentration of phenylephrine (10 nM). Those tissues used to study the effects of the inhibitors on ACh-induced relaxation were contracted submaximally by addition of phenylephrine (750 nM). When a stable contraction was obtained L-NMMA, L-NIO or L-NAME was added 10–15 min before a second cumulative relaxation curve to ACh was obtained.

In vivo *studies*

Male Wistar rats (200–300 g) were anaesthetized with sodium thiobutabarbitone (120 mg kg^{-1}, i.p.). A tracheotomy was performed and a catheter containing heparinised (10 units ml^{-1}) saline inserted into the right carotid artery for the measurement of mean arterial blood pressure. A catheter was also inserted into the femoral vein for continuous infusion of phenylephrine (150–300 μg kg^{-1}) and a 25 gauge butterfly needle inserted into the tail vein for administration of all other drugs. Anaesthesia was then maintained by bolus administration of sodium thiobutabarbitone (5–10 mg kg^{-1}, i.v.). Indomethacin (5 mg kg^{-1}, i.v.) was administered 5 min prior to the experimental protocol and all drugs were administered in 0.9% NaCl. The hypotension induced by the vasodilators was measured as the area comprising the fall and duration of the response and determined by computerized planimetry.

Chemicals

Acetylcholine bromide, NADPH, L- and D-arginine (free base), indomethacin, bradykinin acetate, phenylephrine hydrochloride, L-NAME hydrochloride (all Sigma), dithiothreitol (Boehringer, Mannheim), AG50-X8 (Bio Rad), sodium thiobutabarbitone (Inactin, Byk-Gulden, Konstanz, FRG), sodium hexane sulphonate (BDH) and L[^{3}H]-arginine (Amersham) were obtained as indicated. L-NMMA acetate, L-NIO hydrochloride, their D-enantiomers and D-NAME hydrochloride were synthesized as described previously (Patthy *et al.*, 1977; Scannell *et al.*, 1972).

Statistics

Results are expressed as mean ± s.e.mean for n separate experiments. Student's paired or unpaired t test, as appropriate, was used to determine the significance of differences between means and $P < 0.05$ was taken as statistically significant. The EC_{50}/IC_{50} responses were expressed as a percentage of the maximum effect of each agonist from computer-constructed sigmoid logistic dose-response curves.

Results

Studies in vitro

Inhibition of nitric oxide synthase Cyclic GMP formation in endothelial cytosol was stimulated from 1.54 ± 0.16 to 35.55 ± 2.88 pmol min^{-1} mg^{-1} protein ($n = 10$) by L-arginine (30 μM: EC_{50} 18.8 ± 2.6 μM; $n = 3$). This was dependent on the presence of NADPH (100 μM; $n = 6$). Endothelial cytosol, in the presence of NADPH (100 μM), also formed [^{3}H]-citrulline (43.9 ± 7.6 fmol min^{-1} mg^{-1} protein; $n = 10$) from [^{3}H]-L-arginine (3 μM).

The formation of cyclic GMP in the presence of L-arginine (30 μM) was inhibited by EGTA (0.001–1 mM; IC_{50} = 46.0 ± 7.1 μM; $n = 3$) and by EDTA (0.001–1 mM; IC_{50} = 46.8 ± 10.4 μM; $n = 5$). The formation of [^{3}H]-citrulline in the presence of L-arginine (3 μM) was also inhibited by these concen-

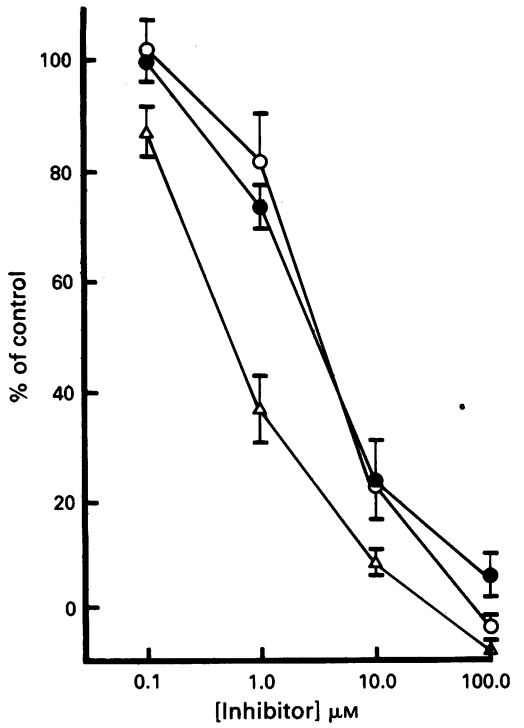

Figure 2 Effects of L-NMMA (●), L-NIO (△), and L-NAME (○) on the stimulation of soluble guanylate cyclase in endothelial cytosol in the presence of L-arginine (30 μM) and NADPH (100 μM). Data are expressed as % inhibition of control (35.55 ± 2.88 pmol min^{-1} mg^{-1} protein, $n = 10$). Each point is the mean of 4 experiments with s.e.mean shown by vertical bars.

trations of EGTA (IC_{50} = 40.8 ± 7.2 μM; $n = 5$) and EDTA (IC_{50} = 45.0 ± 12.0 μM; $n = 5$).

L-NMMA, L-NIO and L-NAME (all at 0.1–100 μM; Figure 2) in the presence of L-arginine (30 μM) and NADPH (100 μM), inhibited cyclic GMP formation by endothelial cytosol with IC_{50}s of 2.9 ± 0.2, 0.5 ± 0.1 and 3.1 ± 0.4 μM ($n = 4$ for each) respectively. The stimulation of soluble guanylate cyclase by sodium nitroprusside (100 μM; 705.6 ± 60.9 pmol min^{-1} mg^{-1} protein; $n = 4$) was not affected by L-NMMA, L-NIO or L-NAME (100 μM; $n = 3$ for each). These compounds also inhibited the formation of [^{3}H]-citrulline in the presence of L-arginine (3 μM) and NADPH (100 μM) with IC_{50}s of 0.092 ± 0.08, 0.015 ± 0.01 and 0.090 ± 0.004 μM ($n = 4$ for each) respectively.

Effects on vascular rings L-NMMA, L-NIO and L-NAME (100 μM) alone induced a small but significant endothelium-dependent contraction of rings of rat aorta of 5.5 ± 0.5, 11.8 ± 1.5 and 5.2 ± 0.6% respectively (n = 17–19) of that induced by phenylephrine (750 nM; tension approx. 3 g). In the presence of a threshold concentration of phenylephrine (10 nM), however, these compounds (0.1–300 μM) induced a greater endothelium-dependent contraction of the rings (Figure 3). The EC_{50}s for the three compounds were 12.5 ± 1.3, 2.1 ± 0.6 and 26 ± 6 μM, respectively (n = 6–8). The maximum contraction induced by L-NMMA, L-NIO and L-NAME was 40.5 ± 6.5, 92.3 ± 9.7 and 39.7 ± 7.4% respectively (n = 6–8) of that induced by phenylephrine (750 nM). The contractions induced by equieffective concentrations of L-NMMA (10 μM) and L-NIO (0.3 μM) were rapid in onset and reached a plateau in 8.3 ± 2.4 and 8.2 ± 2.3 min ($n = 3$ for each), whereas L-NAME (10 μM) produced a slower response which only reached a plateau in 15.7 ± 1.9 min ($n = 3$). Their D-enantiomers had no effect (100 μM; $n = 3$ for each).

Acetylcholine (0.01–3 μM) caused endothelium-dependent relaxation of rings of rat aorta contracted (approx. EC_{90}) with phenylephrine (750 nM). The maximum relaxation observed was 86.7 ± 1.7% ($n = 8$) of the contraction induced by phenylephrine. The relaxation induced by ACh was inhibited in a concentration-dependent manner by L-NMMA (1–1000 μM), L-NIO (0.3–30 μM) and L-NAME (0.1–10 μM) with IC_{50}s of 9.5 ± 1.1, 2.0 ± 0.3 and 0.54 ± 0.04 μM ($n = 3$ for each) respectively (Figure 4). The maximum degree of inhibition of ACh-

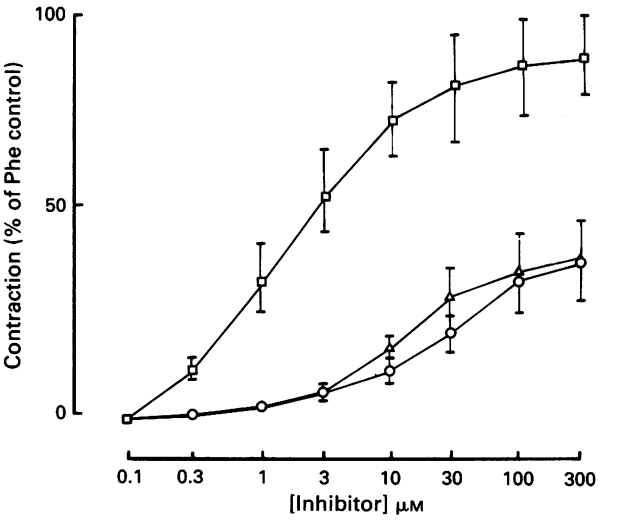

Figure 3 Contraction of rings of rat aorta induced by L-NMMA (△), L-NIO (□) and L-NAME (○) in the presence of 10 nM phenylephrine, expressed as a % of the contraction induced by phenylephrine (Phe, 750 nM). Each point is the mean of 6–8 experiments with s.e.mean shown by vertical bars.

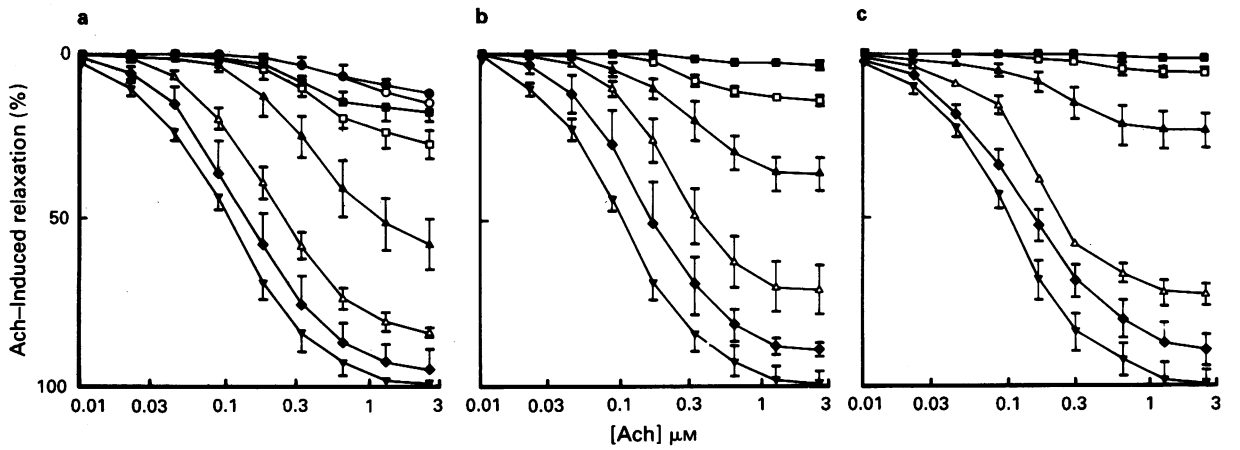

Figure 4 Inhibition by (a) L-NMMA (1–1000 μM), (b) L-NIO (0.3–30 μM) and (c) L-NAME (0.1–10 μM) of the relaxation of rat aortic rings induced by acetylcholine (ACh), expressed as a % of the maximum relaxation induced by ACh. In (a), (▼) control; (◆) 1; (△) 3; (▲) 10; (□) 30; (■) 100; (○) 300 and (●) 1000 μM L-NMMA. In (b), (▼) control; (◆) 0.3; (△) 1; (▲) 3; (□) 10 and (■) 30 μM L-NIO. In (c), (▼) control; (◆) 0.1; (△) 0.3; (▲) 1; (□) 3 and (■) 10 μM L-NAME. Each point is the mean of 3 experiments with s.e.mean shown by vertical bars.

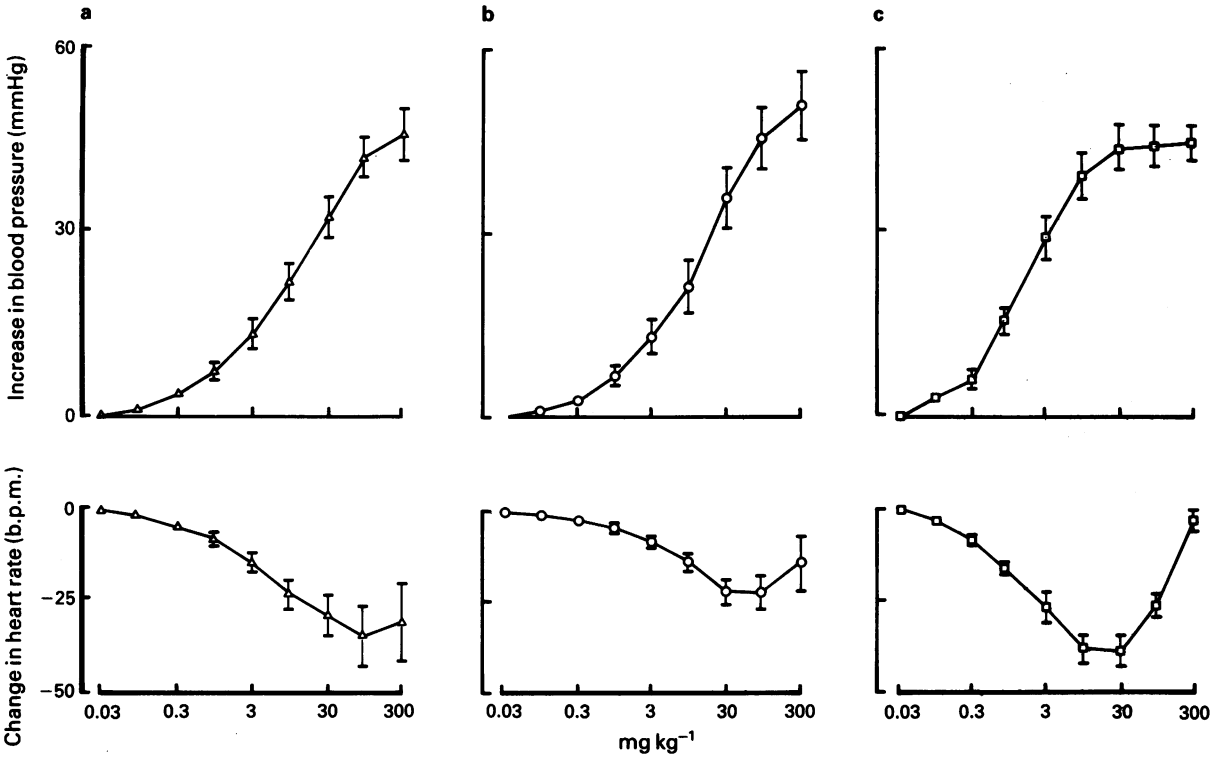

Figure 5 Effect of (a) L-NMMA, (b) L-NIO and (c) L-NAME (0.03–300 mg kg^{-1}, i.v. for each) on mean arterial blood pressure and heart rate in anaesthetized rats. The resting blood pressures were 104.6 ± 8.2 (n = 7), 102.9 ± 2.6 (n = 8) and 111.3 ± 2.6 mmHg (n = 6) for L-NMMA-, L-NIO- and L-NAME-treated rats respectively. The resting heart rates were 366 ± 16 (n = 7), 362 ± 11 (n = 8) and 372 ± 6 (n = 6) b.p.m. respectively. Each point is the mean of 6–8 experiments with s.e.mean shown by vertical bars.

induced relaxation by L-NMMA (1000 μM), L-NIO (30 μM) and L-NAME (10 μM) was 89.4 ± 1.1, 96.6 ± 1.3 and 98.3 ± 0.2% (n = 3 for each) respectively. Their D-enantiomers were without effect (100 μM; n = 3).

In vivo

L-NMMA, L-NIO and L-NAME (0.03–300 mg kg^{-1}, i.v.), but not their D-enantiomers (100 mg kg^{-1}, i.v.), caused a dose-dependent increase in mean arterial blood pressure (Figure 5) with EC_{50}s of 16.7 ± 4.4, 19.5 ± 5.8 and 2.4 ± 0.4 mg kg^{-1} (n = 6–8) respectively. The maximum increases in blood pressure produced by L-NMMA, L-NIO and L-NAME (300 mg kg^{-1}, i.v. for each) were 45.6 ± 4.4 (n = 7), 50.7 ± 5.5 (n = 8) and 44.1 ± 3.1 mmHg (n = 6) and were not significantly different from each other. The increase in blood pressure was rapid in onset for L-NMMA and L-NIO (10 mg kg^{-1}, i.v. for both) and reached a plateau in 4.2 ± 1.1 and 3.5 ± 0.8 min (n = 4) respectively, whereas L-NAME (1 mg kg^{-1}, i.v.) produced a slower increase in blood pressure and reached a plateau in 6.0 ± 0.4 min (n = 6). The dose-dependent increase in blood pressure induced by L-NMMA,

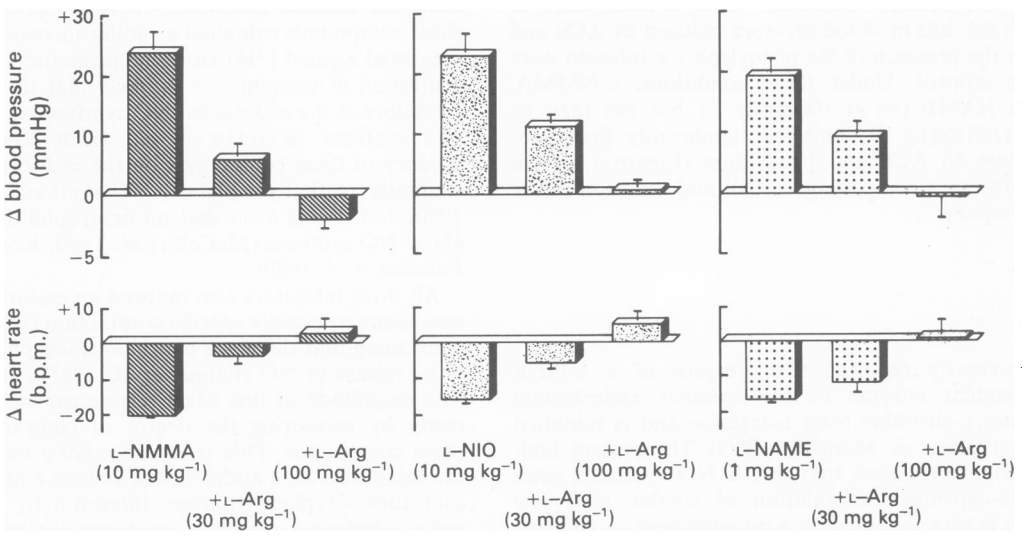

Figure 6 Reversal by L-arginine (L-Arg, 30 and 100 mg kg^{-1}, i.v.) of the effects of L-NMMA (10 mg kg^{-1}, i.v.), L-NIO (10 mg kg^{-1}, i.v.) and L-NAME (1 mg kg^{-1}, i.v.) on blood pressure and heart rate in the anaesthetized rat. The resting blood pressures were 98.6 ± 5.3 (n = 4), 105 ± 5.3 (n = 4) and 108.4 ± 4.4 (n = 6) mmHg respectively and the corresponding resting heart rates were 381 ± 15 (n = 4), 385 ± 13 (n = 4) and 377 ± 14 (n = 6) b.p.m. Each column is the mean of 4–6 experiments with s.e.mean shown by vertical bars.

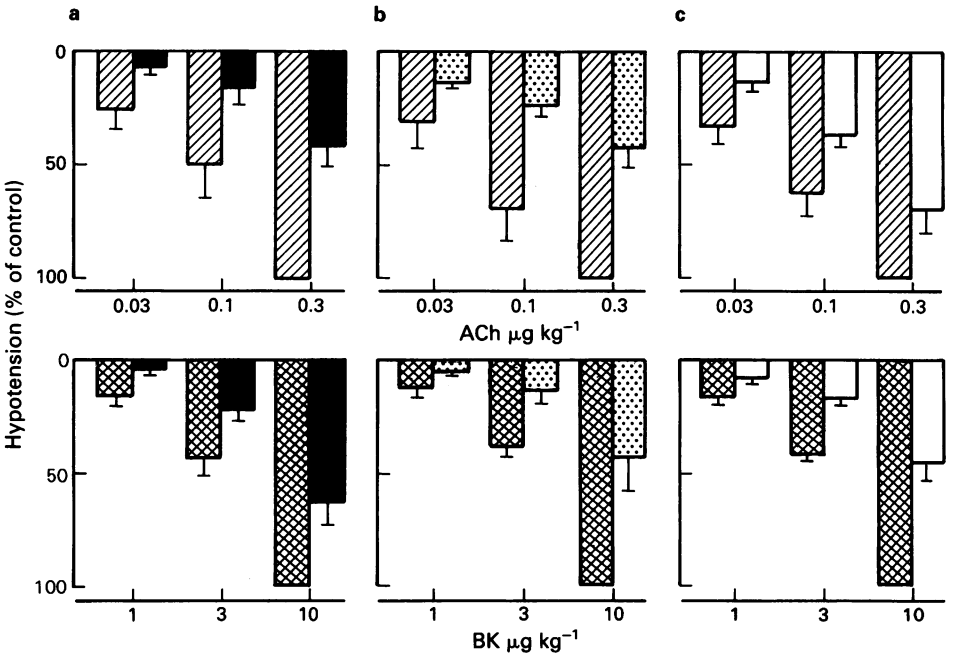

Figure 7 Inhibition by (a) L-NMMA (solid columns), (b) L-NIO (stippled columns) and (c) L-NAME (open columns) (100 mg kg^{-1} for each) of the hypotensive response to acetylcholine (ACh, hatched columns 0.03–0.3 μg kg^{-1}, i.v.) and bradykinin (BK, cross-hatched columns 1–10 μg kg^{-1}, i.v.) in anaesthetized rats. The hypotension was measured as the area comprising the fall and the duration of the response and was expressed as a % of the maximum control response. Each column is the mean of 4–5 experiments with s.e.mean shown by vertical bars.

L-NIO and L-NAME was accompanied by a dose-dependent bradycardia which tended to reverse at higher doses (Figure 5).

Administration of L-arginine (30–100 mg kg^{-1}, i.v.), which had no direct effect either on mean arterial blood pressure or on heart rate, reversed in a dose-dependent manner the increases in blood pressure and bradycardia produced by L-NMMA (10 mg kg^{-1}, i.v., $n = 4$), L-NIO (10 mg kg^{-1}, i.v., $n = 4$) and L-NAME (1 mg kg^{-1}, i.v., $n = 6$) (Figure 6).

Acetylcholine (0.03–0.3 μg kg^{-1}) and bradykinin (1–10 μg kg^{-1}) caused a dose-dependent fall in mean arterial blood pressure. The absolute falls in mean arterial blood pressure induced by ACh (0.3 μg kg^{-1}) and bradykinin (10 μg kg^{-1}) were 47.9 ± 3.4 ($n = 15$) and 51.2 ± 3.1 mmHg ($n = 12$) respectively. Phenylephrine (150–300 μg kg^{-1} h^{-1}) was used to raise the blood pressure to a level similar to that induced by L-NMMA, L-NAME and L-NIO (all at 100 mg kg^{-1}); the falls in blood pressure induced by ACh and bradykinin in the presence of the phenylephrine infusion were taken as the control. Under these conditions, L-NMMA, L-NIO and L-NAME (all at 100 mg kg^{-1}), but not their D-enantiomers (100 mg kg^{-1}), inhibited significantly the hypotensive response to ACh and bradykinin (Figure 7), when measured as the area comprising the fall and duration of the hypotensive response.

Discussion

We have previously reported the presence of a soluble, NADPH-dependent enzyme in the vascular endothelium which generates L-citrulline from L-arginine and is inhibited by L-NMMA (Palmer & Moncada, 1989). The present findings support our conclusion that this is NO synthase, since the L-arginine-dependent stimulation of soluble guanylate cyclase, which is now accepted as a measurement of NO formation (Knowles *et al.*, 1989; Mülsch *et al.*, 1989), is also dependent on NADPH and is inhibited by L-NMMA.

Furthermore, the elevation of cyclic GMP and the formation of [^{3}H]-citrulline are inhibited by EGTA and EDTA over the same range of concentrations, indicating that endothelial NO synthase is Ca^{2+}-dependent, since this is the only divalent cation for which both chelating agents have similar affinities (Dawson *et al.*, 1986). These data are in agreement with a recent report (Meyer *et al.*, 1989) and show that this NO synthase is similar to that in the brain (Knowles *et al.*, 1989) and in the adrenal gland (Palacios *et al.*, 1989) in terms of their dependence on this divalent cation.

The endothelial NO synthase is also inhibited in an enantiometrically specific manner by L-NIO and L-NAME which have recently been characterized as inhibitors of NO synthase in the adrenal gland (Palacios *et al.*, 1989) and brain (Knowles *et al.*, 1990). L-NIO was approximately 5 times more potent as an inhibitor of endothelial NO synthase than the other arginine analogues. Since none of these compounds affected the rise in cyclic GMP induced by sodium nitroprusside, the data indicate that these compounds inhibit NO synthase rather than the soluble guanylate cyclase. Furthermore, since all these compounds exhibited a similar increase in potency when measured against [^{3}H]-citrulline formation from a lower concentration of L-arginine, it is likely that they are competitive inhibitors of the endothelial NO synthase as they are for brain NO synthase (Knowles *et al.*, 1990). The rank order of potency of these compounds on the endothelial NO synthase is similar to that on platelet NO synthase (Radomski *et al.*, 1990), but differs from that on neutrophil, brain and adrenal gland NO synthase (McCall *et al.*, 1990; Knowles *et al.*, 1990; Palacios *et al.*, 1989).

All three inhibitors also induced an endothelium-dependent and enantiomerically specific contraction of the vascular rings, confirming that there is a continuous use of L-arginine for the basal release of NO (Palmer *et al.*, 1988b; Rees *et al.*, 1989a). The magnitude of this basal release can be determined indirectly by measuring the degree of contraction induced by these compounds. This could be readily demonstrated when the inhibitors were added in the presence of a threshold concentration of phenylephrine. Interestingly, L-NIO, which is not a substituted guanidino analogue of L-arginine, was again approximately 5–10 times more potent than L-NMMA and L-NAME and the contraction induced by L-NIO reached a significantly greater maximum. Since this effect was enantiomerically specific it is likely that it is due solely to an effect on NO synthesis.

The endothelium-dependent relaxations induced by ACh were inhibited by all three compounds by at least 90%. Previous studies have shown that in rings of rabbit aorta (Rees *et al.*, 1989a) and guinea-pig pulmonary artery (Sakuma *et al.*, 1988) L-NMMA only inhibits ACh-induced relaxation by approximately 65%. However, N^G-nitro-L-arginine inhibits the ACh-induced relaxation in the rabbit aorta by more than 90% (Kobayashi & Hattori, 1990) and in the rabbit femoral artery by about 80% (Mülsch & Busse, 1990). These data suggest that the difference in the maximum effect in different preparations is probably due to variations in the uptake and metabolism of these compounds by endothelial cells, rather than to the involvement of another mediator in endothelium-dependent relaxation.

All three compounds inhibited endothelium-dependent relaxation induced by ACh and caused an increase in blood pressure, with L-NAME being more potent than L-NIO and L-NMMA in both systems. These findings are consistent with previous reports that N^G-nitro-L-arginine is more potent than L-NMMA in other vascular preparations *in vitro* (Mülsch & Busse, 1990; Moore *et al.*, 1990). This rank order of potency differs from that for inhibition of NO synthase and for their contractile activity on rings of rat aorta. This difference is largely attributable to the substantial increase in potency of L-NAME relative to the other two analogues, the reasons for which are not clear. An interesting possibility suggested by Mülsch *et al.* (1989) is that there are two NO synthases in the vascular endothelium, one Ca^{2+}-dependent and one Ca^{2+}-independent. If one is agonist-activated and is more sensitive to L-NAME, then this would explain the increased potency of this compound against ACh-induced relaxation. Further work is required to clarify these differences.

The rate of onset of the hypertensive response to all three compounds was similar to that of the increase in basal tone of rings *in vitro*, further indicating that the hypertensive response is mediated by inhibition of basal endothelial NO synthesis. The maximum increase in blood pressure induced by all three compounds was similar. Therefore, the level of increase in blood pressure achieved probably represents the total vasodilator tone produced by NO.

The bradycardia which accompanies the elevation in blood pressure was reversed at higher doses of the compounds, particularly with L-NAME. An L-arginine:NO pathway has been implicated in neurotransmission in non-adrenergic, non-cholinergic preparations (Gillespie *et al.*, 1989; Gibson *et al.*, 1990) and in the brain (Knowles *et al.*, 1989; Garthwaite *et al.*, 1989). The accompanying bradycardia may therefore involve a neuronal L-arginine:NO pathway.

L-Arginine has no direct effect on blood pressure or heart rate, indicating that its availability is not rate-limiting for basal NO synthesis in the rat and confirming previous reports for other species (Rees *et al.*, 1989b; Aisaka *et al.*, 1989a). The increase in blood pressure and the accompanying bradycardia induced by equi-effective doses of L-NMMA, L-NIO and L-NAME were fully reversed following administration of L-arginine. Since L-NAME is about 10 fold more potent than L-NMMA and L-NIO in increasing blood pressure, a 10 fold greater molar excess of L-arginine is required to reverse its effects fully. This is consistent with these compounds inhibiting NO synthesis by competing with the substrate.

In contrast to the effect of L-NMMA on hypotension induced by ACh in the rabbit (Rees *et al.*, 1989b), the inhibitory effect of L-NMMA, L-NIO and L-NAME on ACh- and bradykinin-induced hypotension in the rat was only observed when measured in terms of fall and duration, rather than fall alone, confirming similar findings in the guinea-pig (Aisaka *et al.*, 1989b). These authors proposed that the ACh-induced hypotension in the guinea-pig is the resultant of two phases, an immediate vasodilatation, which is resistant to blockade by L-NMMA, and a longer-lasting vasodilatation, which is susceptible to blockade by L-NMMA. They therefore suggest that only the latter requires the synthesis of NO from L-arginine. However, *in vitro* studies (Giles *et al.*, 1990) have recently shown that resistance to blockade by L-NAME is more apparent in vascular preparations with a highly efficient receptor-effector coupling, such as when ACh is a potent full agonist. Indeed, the finding that L-NMMA inhibits the initial fall in blood pressure induced by ACh in the rabbit (Rees *et al.*, 1989b), where the potency of ACh is 10 fold lower than in the rat, is consistent with this explanation.

In summary, L-NMMA, L-NIO and L-NAME are inhibitors of the formation of NO from L-arginine by the vascular endothelium, which behave in a qualitatively similar pharmacological manner *in vitro* and *in vivo*. Although all their effects are consistent with inhibition of NO synthase, there are differences in potency of these compounds in different systems which require further investigation. Furthermore, the activity of these compounds *in vitro* and *in vivo* confirms the important role of vascular endothelial NO synthesis in regulating blood pressure and flow. Pharmacokinetic and metabolic studies on these compounds and characterization of the distribution and activity of the L-arginine:NO pathway at the different levels of the vascular tree are now required.

The authors are indebted to Mr N.A. Foxwell, Mrs L.J. Bridge and Miss T. Andrews for excellent technical assistance and to Mrs G. Henderson and Mrs E.A. Higgs for assistance with the preparation of the manuscript.

R. S. is a fellow of the Medical Research Council of Canada and the Alberta Heritage Foundation for Medical Research.

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(Received June 5, 1990
Revised July 2, 1990
Accepted July 3, 1990)

Br. J. Pharmacol. (1990), **101**, 753–761

The pharmacological properties of the imidazobenzodiazepine, FG 8205, a novel partial agonist at the benzodiazepine receptor

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1 The pharmacological properties of the benzodiazepine receptor ligand, FG 8205 (7-chloro-5,6-dihydro-5-methyl-6-oxo-3-(5-isopropyl-1,2,4-oxadiazol-3-yl)-4H-imidazol[1,5a][1,4]benzodiazepine) have been examined.

2 FG 8205 potently displaced [^{3}H]-flumazenil binding in rat cortical membranes with a K_i of 3.3 nM, but was inactive at 13 neurotransmitter recognition sites.

3 Consistent with a partial agonist profile, the affinity of FG 8205 for the benzodiazepine recognition site was increased in the presence of γ-aminobutyric acid (GABA, 300 μM) by a degree ($-\log [IC_{50}$ in the presence of GABA/IC_{50} alone] = 0.34) significantly less than found for diazepam (0.46). FG 8205 also potentiated the inhibitory potency of the $GABA_A$-receptor agonist, isoguvacine, on the hippocampal CA1 population spike and, again, the maximum shift ($-\log$ dose-ratio = 0.2) was significantly less than that seen with diazepam (0.4).

4 In anticonvulsant studies, the ED_{50} doses of FG 8205 and diazepam needed to antagonize seizures induced by pentylenetetrazol (PTZ) or by sound in audiogenic seizure prone mice were similar with values of 0.2–0.3 mg kg^{-1}, i.p. However, even high doses of FG 8205 (50 mg kg^{-1}) did not protect against seizures induced by electroshock.

5 FG 8205 released responding suppressed by footshock in a rat operant conditioned emotional response task over the dose range 0.5–50 mg kg^{-1} (i.p.). Similar doses of FG 8205 had a marked taming effect in cynomolgus monkeys. However, measures of sedation and ataxia (as measured by rotarod in the mouse, climbing behaviour in the rat, and by scoring arousal and co-ordination in primates) were slight and only transiently affected by FG 8205, and FG 8205 significantly antagonized the rotarod performance deficit induced by diazepam in the mouse.

6 While the potentiation by FG 8205 of the response to isoguvacine in the rat hippocampal slice and the anxiolytic-like effects of the compound in both rats and primates were reversed by the benzodiazepine receptor antagonist, flumazenil, high doses of the antagonist were able only marginally to block the protective effects of FG 8205 against seizures induced by PTZ in the mouse.

7 Thus, FG 8205 does not show the marked motor impairment characteristic of full agonists at the benzodiazepine receptor, consistent with its partial agonist profile in *in vitro* assay systems. Nevertheless, the compound has sufficient intrinsic activity to maintain high efficacy in anticonvulsant and anxiolytic tests.

Introduction

Recognition sites for benzodiazepines are known to be linked to the γ-aminobutyric $acid_A$ ($GABA_A$)-receptor associated with a chloride channel within the same macromolecular complex (Olsen, 1982; Ehlert *et al.*, 1982; Wong, 1989). Occupation of the site by an agonist, such as diazepam, enhances $GABA_A$-mediated responses by increasing the amount of Cl^- current gated by the binding of a GABA receptor agonist (Study & Barker, 1981).

Benzodiazepines and other compounds capable of interacting with the benzodiazepine recognition site possess a wide range of pharmacological actions: the prototypical full agonist, diazepam, is anxiolytic, sedative, muscle relaxant and anticonvulsant over a narrow dose range (Braestrup *et al.*, 1984; Schneider *et al.*, 1989). A second group of compounds produce effects that are diametrically opposed to those of full agonists; that is they reduce the ability of GABA to increase chloride ion permeability and cause a rightward shift in concentration-effect curves of $GABA_A$-receptor agonists: these are known as inverse agonists (Polc *et al.*, 1982; Braestrup *et al.*, 1982; Haefely *et al.*, 1985; Kemp *et al.*, 1987). Behaviourally, they are convulsant, anxiogenic and heighten arousal. A third class, the benzodiazepine receptor antagonists, such as flumazenil and ZK 93426, are themselves essentially neutral and block the actions of both agonists and inverse agonists (Hunkeler *et al.*, 1981; Bernard *et al.*, 1981; Haefely *et al.*, 1985; Schneider *et al.*, 1989).

The continuum from full agonist to full inverse agonist clearly covers a wide range of possible functional effects *in vivo*: compounds that are partial agonists should be capable of inducing the effects of full agonists which are mediated at low levels of receptor occupancy, but incapable of mimicking the effects of full agonists which require high levels of receptor occupancy (Haefely & Polc, 1986).

There is some evidence that the anticonvulsant effects of the benzodiazepines occur at low benzodiazepine receptor occupation, whilst the myorelaxant and sedative effects require almost 100% occupancy (Jensen & Petersen, 1983). In the present study, evidence is presented indicating that the imidazobenzodiazepine, FG 8205 (7-chloro-5,6-dihydro-5-methyl-6-oxo-3(5-isopropyl-1,2,4-oxadiazol-3-yl)-4H-imidazo-[1,5a][1,4]benzodiazepine) is a partial agonist at the benzodiazepine receptor. In rodents and primates the compound does not induce the marked motor impairment characteristic of full agonists, but nevertheless has high efficacy in tests of anticonvulsant and anxiolytic activity.

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Methods

Animals

Male Sprague-Dawley rats (250–300 g), Swiss Webster (18–20 g) and DBA/2 mice (8–9 g, 21–23 days of age) were obtained from Bantin and Kingman (Hull, U.K.) and caged in groups of 5. Male cynomolgus monkeys (Macaca fascicularis, circa 6 kg, Shamrock Farms, U.K.) were individually housed. All animals were kept under standard laboratory conditions of temperature and humidity and a 12 h light-dark cycle (lights on at 07 h 00 min) with food and water freely available.

Radioligand binding studies

[^{3}H]-flumazenil binding, in vitro Affinity for the benzodiazepine recognition site was determined essentially as described by Wong & Iversen (1985) in membranes derived from rat cerebral cortex. Animals were killed by stunning and decapitation and the whole cortex removed and homogenised in 9 vol of ice-cold 0.32 M sucrose by 10 strokes in a glass teflon homogeniser at 500 r.p.m. All further procedures were carried out at 4°C.

Homogenates were centrifuged at 1000 *g* for 10 min and the supernatant was recentrifuged at 10,000 *g* for 20 min. The P_2 pellet was resuspended in 30 volumes of ice-cold distilled water and centrifuged at 8000 *g* for 20 min. The upper 'buffy coat' and the loosely sedimented pellet were suspended in 20 volumes of ice-cold water and centrifuged at 50,000 *g* for 20 min. The water washing step was repeated 3 times and the resultant pellet stored at −20°C for at least 18 h. On the day of the experiment, the membranes were thawed at room temperature for 30 min, mixed with 20 volumes of 5 mM Tris HCl (pH 7.4) and left at room temperature for a further 30 min, then centrifuged at 50,000 *g* for 10 min. The pellet was resuspended in 20 volumes of the Tris buffer and incubated at room temperature for 15 min before centrifugation. This washing step was repeated three times, prior to resuspension for the binding assay in Krebs buffer (pH 7.4) of the following composition (mM): NaCl 118, KCl 4.7, $MgSO_4$ 1.2, $NaHCO_3$ 5, HEPES 20, KH_2PO_4 1.2, $CaCl_2$ 2.5, glucose 11.

Benzodiazepine receptors were labelled with 1.0 nM [^{3}H]-flumazenil (100 μl of 10 nM), 100 μl of displacer (test compound) or 30 μM clonazepam (for defining non-specific binding), 50 μl of buffer or 6 mM GABA (for GABA-shift measurements), and 750 μl of membrane homogenate. To control for possible uptake of GABA, 0.1 mM nipecotic acid (final concentration) was added to the assay buffer in all experiments. Duplicate samples containing 0.2 mg protein were incubated for 60 min at 30°C. The binding reaction was terminated by filtration through Whatman GF/B glass fibre filters in a Brandel M24 cell harvester followed by 2 × 5 ml washes with ice-cold 0.9% NaCl. The filters were soaked in 10 ml of Hydrofluor (National Diagnostics) overnight before liquid scintillation counting. Potencies for displacement (IC_{50} or K_i) in the absence or presence of GABA were determined by including at least 5 concentrations of the test compound, and the data analysed by a computer curve fitting programme.

Affinity of FG 8205 for other neurotransmitter recognition sites The affinity of FG 8205 for neurotransmitter recognition sites other than the benzodiazepine receptor was examined in a similar manner to that described for [^{3}H]-flumazenil binding. Details of the radioligands used and the methods for determining non-specific binding are shown in Table 1.

Electrophysiological studies

The ability of benzodiazepine receptor agonists, antagonists and inverse agonists to modulate the inhibitory potency of the $GABA_A$-receptor agonist, isoguvacine, on the CA1 population spike recorded from slices of rat hippocampus was determined.

Male Sprague-Dawley rats (approximately 100 g) were killed by decapitation and their brains rapidly removed. Slices 350 μm thick, from the dorso-medial part of the hippocampus were cut in artificial CSF (aCSF), at room temperature (20°C), with an Oxford vibratome. Slices were placed on a nylon mesh and completely submerged in a small superfusion chamber (volume = 0.3 ml), and continuously superfused with oxygenated aCSF at a rate of approximately 1.5 ml min^{-1}, at room temperature. The aCSF had the following composition (mM): NaCl 124, KCl 5, KH_2PO_4 1.25, $MgSO_4$ 2, $CaCl_2$ 2, $NaHCO_3$ 25 and glucose 11.

The Schaffer collateral-commissural pathway was stimulated every 30 s with a metal bipolar electrode, made from two tungsten microelectrodes (TM25-5, Clark electromedical), placed in the stratum radiatum. Population spikes were recorded from the cell body layer of the CA1 pyramidal cells by use of glass micropipettes filled with 3 M NaCl and having resistances of 2–10 MΩ.

In most experiments two slices were placed in the perfusion chamber and their responses recorded in parallel. The recorded potentials were digitised by a Gould OS4040 digital oscilloscope. A BBC microcomputer based system was used to average and measure the peak height of the population spikes. The average of four submaximal control responses was taken and then the perfusing medium changed to one containing isoguvacine by means of a three-way tap. Each concentration of isoguvacine was perfused for a 5 min period, to allow for equilibration within the slice, and the last four responses at each concentration averaged. Increasing concentrations were added cumulatively and the concentration-response curve generated by plotting isoguvacine concentration against % reduction of the population spike. The benzodiazepine receptor ligands under study were perfused for 30 min before and during re-determination of the isoguvacine concentration-response curve. The dose-ratio between the control and drug-treated curves was always measured at the 50% inhibition level. Only one concentration of one ligand was tested on each slice.

Table 1 Details of ligand binding assays

Recognition site	*Radioligand*	*Tissue*	*Non-specific binding determined by:*	*Non-specific conc.* (μmol l^{-1})	*Radioligand conc.* (nmol l^{-1})	*Incub. time* (min)	*Temp.* (°C)
5-HT_{1A}	[^{3}H]-8-OH-DPAT	Cortex	5-HT	10	1	5	37
5-HT_2	[^{3}H]-ketanserin	Cortex	Methysergide	1	2	15	37
5-HT_3	[^{3}H]-quat-ICS 205-930	Cortex	MDL72222	10	0.5	15	4
α_1	[^{3}H]-prazosin	Whole brain	Phentolamine	10	1	30	23
β	[^{125}I]-cyanopindolol	Cortex	(−)-Isoprenaline	200	0.15	20	37
D_2	[^{3}H]-ADTN	Striatum	Dopamine	10	16	20	23
CCK_A	[^{125}I]-BH-CCK	Pancreas	CCK	1	0.05	120	23
CCK_B	[^{125}I]-BH-CCK	Cortex	CCK	1	0.05	120	23
NMDA	[^{3}H]-MK-801	Cortex	TCP	10	2	45	23
Glycine	[^{3}H]-glycine	Cortex/hippo	Glycine	1000	50	30	4
Sigma	[^{3}H]-DTG	G P whole brain	Haloperidol	1	5	90	23
Muscarinic	[^{3}H]-oxotremorine	Cortex	Atropine	100	3	40	30
	[^{3}H]-N-methylscopolamine	Cortex	Atropine	100	0.1	40	30

Anticonvulsant activity

Seizures induced by pentylenetetrazol Mice were injected subcutaneously with pentylenetetrazol (PTZ, 120 mg kg^{-1}, s.c.) and observed for the following 30 min. FG 8205 and diazepam were administered i.p., 30 min before injection of PTZ. Animals not exhibiting tonic seizures during the following 30 min observation period were considered protected. The dose of antagonist giving 50% protection (ED_{50}) was calculated by probit analysis.

Seizures induced by electroshock Tonic seizures were induced in mice by application of electroshock (0.5 mA, 0.2 s) via corneal electrodes. FG 8205 and diazepam were given i.p., 30 min before application of shock and the dose giving 50% protection calculated by probit analysis.

Audiogenic seizures Seizures were induced in 21–23 day-old male DBA/2 mice by exposure for 30 s to a 120 dB, 1.4 kHz bell. FG 8205 and diazepam were given i.p., 30 min before exposure to the sound. Animals not exhibiting a tonic seizure within the 30 s of sound exposure were considered protected. The dose of compound giving 50% protection against each convulsant was calculated by probit analysis.

Anxiolytic studies

Rat conditioned emotional response Rats (250–300 g) were trained to lever-press on a variable interval, VI 60 s schedule for food reinforcement in a standard conditioning chamber (Gerbrand's Instruments) over weekly (five days per week) training sessions. All animals then received daily 20 min conditioning sessions, each session being partitioned into alternating 5 min light (L) and 2.5 min dark (D) periods in a fixed LDLDL sequence. During both types of period (L,D), lever-presses delivered food pellets on a V1 60 s schedule; in the dark periods (D), lever-presses also elicited mild footshock (0.8 mA, 0.5 s) on an independent shock presentation schedule of V1 20 s.

Lever-pressing was suppressed during the dark period and reflects the formation of a conditioned emotional response (CER) as the animals learn to discriminate between light and dark. Rats were tested on extinction days (shock off) separated by 1 or 2 days of baseline re-training (shock on, identical to training sessions). Subjects were injected with carboxymethylcellulose vehicle (0.5% w/v in distilled water) before every training session to eliminate any cues deriving from the injection. Unless stated otherwise, compounds were administered i.p. 40 min before testing. All drug testing was carried out in separate groups of rats (minimum of 8 per group) for each drug or dose tested. Test treatments were given according to a randomised sequence to minimize order effects. Results are expressed as response rates in the light and dark periods.

Taming effect in primates Procedures similar to those first described by Heise & Boff (1961) and Randall *et al.* (1961) were used to detect the taming effect of benzodiazepines in aggressive monkeys. Six individually-housed, adult male cynomolgus monkeys were used; attack or avoidance behaviour directed towards the observer (following (i) sudden approach and (ii) hand clapping nearby), a broom handle and a live garter snake were each rated on a scale of 0–5 to give a maximum total aggression score of 20. Animals were scored by an observer blind to the drug treatment immediately before the i.p. administration of diazepam or FG 8205 and at 30 min intervals thereafter for a 5 h observation period. Animals were subjected to these and other drug treatments usually at one week intervals.

For calculation of the ED_{50} for taming, scores were summed over the period of peak effect and plotted against drug dose on a logarithmic scale. The point at which the dose-response curve was intersected by a line representing a 50% reduction in aggression score was taken as the ED_{50}. A 50% reduction in aggression was defined as a score mid-way between the maximum observed following vehicle treatment and the minimum observed after any drug treatment.

Duration of action was estimated by examination of the time course of taming activity of the dose closest to the calculated ED_{50} value. Duration was defined as the time taken for scores to increase from 50 to 75% of control values.

Sedation and motor co-ordination

Mouse rotarod Groups of male Swiss Webster mice were trained on a rotarod apparatus (diameter of rod = 3 cm) until they were able to remain on the rotating rod (15 rev min^{-1}) for at least 120 s, at which point they moved continuously with the rod, maintaining their position on the top. Animals who fell several times were discarded. Time spent on the rotating rod was then determined at 15, 30, 45 and 60 min after the i.p. injection of FG 8205 or, for comparison, diazepam. The duration of each trial was 2 min.

Rat swimming and climbing performance A platform was positioned 1 m from the start point of a circular swimming pool, 1.32 m in diameter. When plunged into the water at room temperature, rats swam spontaneously and searched for a means of escape. The time taken to locate the platform and then to mount the platform once contact was established, was noted. FG 8205 and diazepam were injected i.p., 30 min before test.

Sedation and ataxia in primates Sedation and ataxia were each scored in male cynomolgus monkeys on a scale of 0–5, according to the ease with which the animals could be aroused and the degree of motor incoordination (for example, when picking up a peanut) and postural instability. The scores were summed to give a combined sedation + ataxia score. Behaviours were scored immediately before i.p. drug administration and every 30 min throughout the subsequent 5 h period by an observer blind to drug and dose conditions.

Drugs

The following compounds were used: FG 8205 (7-chloro-5,6-dihydro-5-methyl-6-oxo-3-(5-isopropyl-1,2,4-oxidiazol-3-yl)-4H-imidazo [1,5-a][1,4]benzodiazepine, Merck Sharp & Dohme Research Laboratories), diazepam (Roche), alprazolam (Upjohn), methyl-6,7-dimethoxy-4-ethyl-β-carboline carboxylate (DMCM, Research Biochemicals Inc.), flunitrazepam (Roche) and flumazenil (ethyl-8-fluoro-5,6-dihydro-5-methyl-6-oxo-4H-imidazo(1,5a)(1,4)benzodiazepine-3-carboxylate, Roche). Bretazenil (t-butyl(S)-8-bromo-11,12,13,13a-tetrahydro-9-oxo-9H-imadazo[1,5-a]-pyrrolo-[2,1-c][1,4] benzodiazepine-1-carboxylate) and Ro 17-1812(cyclopropylmethyl (5)-8-chloro-12,12a-dihydro-9-oxo-9H,11H-aceto[2,1-C]-imidazo-[1,5-a][1,4]benzodiazepine-1-carboxylate) were synthesized at Merck Sharp & Dohme Research Laboratories.

When administered to rodents, compounds were suspended in 0.5% w/v carboxymethylcellulose in distilled water with the aid of ultrasonification. In the cynomolgus monkey, 5% (w/v) cremophore in 0.9% NaCl was used as the drug vehicle.

Results

Affinities and GABA-shift values for ligands at rat brain benzodiazepine receptors

FG 8205 was about 10 to 20 fold more potent than alprazolam or diazepam and approximately equipotent with flumazenil in displacing [^{3}H]-flumazenil from the benzodiazepine recognition site in rat cortical membranes. Bretazenil was the most potent compound examined with a K_i 86 fold lower than that of diazepam (Table 2).

Table 2 Potencies and γ-aminobutyric acid (GABA)-shift values for ligands at benzodiazepine receptors in rat cerebral cortical membranes

	K_i (nM) *vs* [^3H]-*flumazenil*	*GABA-shift*	*log GABA shift*
FG 8205 (6)	3.3 ± 0.4	2.2 ± 0.10*	0.34
Diazepam (8)	62.0 ± 9.0	2.9 ± 0.06	0.46
Alprazolam (4)	34.0 ± 6.7	2.9 ± 0.18	0.46
Bretazenil (5)	0.7 ± 0.2	1.4 ± 0.12*	0.15
Ro 17-1812 (5)	5.2 ± 0.8	1.7 ± 0.08*	0.23
Flumazenil (5)	5.7 ± 1.1	1.0 ± 0.04*	0
DMCM (3)	3.4 ± 0.5	0.4 ± 0.03*	−0.40

Values are mean ± s.e.mean from (*n*) number of experiments. The GABA-shift is the control IC_{50}/IC_{50} in the presence of 3×10^{-4} M GABA. For direct comparison with the electrophysiological data, the log GABA shift is also given. *Significantly different from value for diazepam, $P < 0.05$ (unpaired *t* test).

The affinity of both diazepam and alprazolam for the benzodiazepine recognition site was increased in the presence of GABA, giving a GABA-shift (−log ratio of IC_{50} values obtained in the presence and absence of GABA) of 0.46. The GABA-shift for FG 8205 (0.34) was significantly less than that of either diazepam or alprazolam ($P < 0.05$, unpaired *t* test), but not as low as that of bretazenil (0.15), flumazenil (0) or DMCM (−0.4) (Table 2). Similar affinity and GABA-shift values were observed for FG 8205 when [^{3}H]-flumazenil binding was carried out in human cortical membranes (results not shown).

Affinity of FG 8205 at other neurotransmitter recognition sites

At concentrations of 10 to 100 μM, FG 8205 did not displace binding by more than 6% at recognition sites for 5-hydroxytryptamine (5-HT) (5-HT_{1A}, 5-HT_2, 5-HT_3), dopamine (D_2), acetylcholine (muscarinic), noradrenaline (α_1 and β-adrenoceptors), glutamate N-methyl-D-aspartate (NMDA), substance P and cholecystokinin (CCK_A and CCK_B), at the σ-recognition site or the strychnine-insensitive glycine modulatory site on the NMDA receptor (results not shown).

Effects of FG 8205 in the rat hippocampal slice

FG 8205 produced a leftward shift of the isoguvacine concentration-response curve (Figure 1) with a threshold concentration of 10 nM. The maximum shift was achieved at 30 nM (−log concentration-ratio = 0.2) and this was maintained up to the highest concentration tested (300 nM). This compares to maximum shifts produced by diazepam and flunitrazepam of 0.41 and 0.38 respectively (Kemp *et al.*, 1987). The partial agonist, bretazenil, also produced a maximum shift (0.2 at 30 nM) which was also less than that of diazepam, whereas the inverse agonist, DMCM, shifted the isoguvacine concentration-response curve to the right, maximally at 1 μM giving a log concentration-ratio of 0.38.

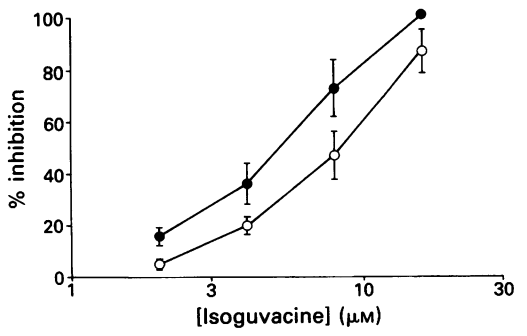

Figure 1 Concentration-response curve for the inhibition of the CA1 population spike in slices of rat hippocampus by isoguvacine before (○) and after (●) 30 min perfusion with FG 8205 (30 nM). Data are shown as the mean percentage inhibition of 4 separate slices; vertical lines indicate s.e.mean.

To confirm that the potentiating effect of FG 8205 was mediated by an action at the benzodiazepine receptor, the effect of the benzodiazepine receptor antagonist, flumazenil was investigated. The increase in the potency of isoguvacine induced by FG 8205 (30 nM) was first determined and then flumazenil (300 nM) added together with FG 8205 for a further 30 min, and the isoguvacine dose-response curve re-determined. The mean −log concentration-ratio produced by FG 8205 was 0.197 ± 0.018 (±s.e.mean, $n = 6$). This was reduced significantly to 0.010 ± 0.017 by flumazenil ($P < 0.001$, paired *t* test).

Anticonvulsant studies

Under the present conditions, PTZ (30–120 mg kg^{-1}) dose-dependently induced tonic seizures in the mouse: the dose calculated by probit analysis to induce seizures in 50% of the animals tested was 94 mg kg^{-1} (Singh *et al.*, 1990). For antagonist studies, a dose of 120 mg kg^{-1} PTZ was chosen since this was the minimum dose that reliably induced seizures in 100% of the control animals.

The ED_{50} doses of diazepam and FG 8205 given 30 min before testing against tonic seizures induced in the mouse by pentylenetetrazol or by sound in audiogenic seizure prone DBA/2 mice were similar (Table 3). Diazepam was about 10 fold weaker against seizures induced by electroshock than against the convulsant action of PTZ or sound. However, FG 8205 did not protect against electroshock even at the high dose of 50 mg kg^{-1}.

When co-administered with diazepam, flumazenil (1–40 mg kg^{-1}) dose-dependently antagonized the protection against PTZ (120 mg kg^{-1}), such that the ED_{50} for diazepam increased from 0.39 ± 0.035 mg kg^{-1} (mean of 4 separate determinations ± s.e.mean) in vehicle-treated controls to 1.8 mg kg^{-1} ($n = 2$) and 4.7 ± 1.25 mg kg^{-1} ($n = 4$, $P < 0.01$, 2-tailed *t* test) in the presence of doses of 20 mg kg^{-1} and 40 mg kg^{-1} flumazenil, respectively. In contrast, 40 mg kg^{-1} flumazenil had little effect on the protective effect of FG 8205 against PTZ, the ED_{50} dose increasing by only 1.3 fold from 0.34 ± 0.087 mg kg^{-1} ($n = 3$) to 0.45 ± 0.173 mg kg^{-1} ($n = 3$, $P > 0.05$, two-tailed *t* test). Flumazenil (1–40 mg kg^{-1}) alone was without significant effect on PTZ-induced seizures (results not shown).

Effects of benzodiazepine ligands in the rat conditioned emotional response test

After approximately six weeks of training, lever pressing was completely suppressed by footshock during the dark phase of the conditioned emotional response (CER) test. Response suppression was subsequently maintained in the absence of footshock when extinction sessions were interspersed on a twice

Table 3 Anticonvulsant potency of FG 8205 and diazepam

	Anticonvulsant ED_{50} (mg kg^{-1}) vs		
	PTZ	*Audiogenic seizures*	*Electroshock*
FG 8205	0.26 (0.18–0.34)	0.18 (0.13–0.24)	>50
Diazepam	0.39 (0.31–0.47)	0.18 (0.02–0.04)	3.0 (1.7–5.2)

Groups of 8 mice were given FG 8205 or diazepam 30 min before injection of pentylenetetrazol (PTZ), exposure to sound or electroshock and the number of animals convulsing noted. ED_{50} values were determined by probit analysis. 95% confidence limits are given in parentheses.

weekly basis with three days of training in the presence of shock.

At a dose of 10 mg kg^{-1}, diazepam significantly increased the response rate in the dark to a level not significantly different from the rate in the light phase. However, the increase in dark phase responding occurred over a narrow dose range. Response rate decreased in both light and dark phases following a dose of 20 mg kg^{-1} diazepam (Figure 2a). A very similar pattern of responding was seen with the benzodiazepine receptor agonist, alprazolam (Figure 2b). In contrast, FG 8205 (0.2–50 mg kg^{-1}) dose-dependently increased the dark phase response rate over a wide dose range and without suppression of responding in the light (Figure 2c). Indeed, FG 8205 tended to increase the light phase response rate, although the effect was not statistically significant. The estimated ED_{50} dose of FG 8205 (the dose increasing the dark phase response rate by 50% of the maximum increase seen) when given intraperitoneally was 1.3 mg kg^{-1}. At the ED_{50} dose, the dark phase response rate was highest in animals injected immediately before the start of the test and declined steadily with increasing pretreatment time (Figure 3). However, a significant increase in response rate was still present when the compound was given 180 min before testing. Given orally 40 min before testing, the ED_{50} for FG 8205 was 5 mg kg^{-1} (Figure 4).

The ability of the benzodiazepine receptor antagonist, flumazenil to antagonize the effects of FG 8205 and diazepam in the CER test was also examined. When co-administered with 5 mg kg^{-1} FG 8205 or diazepam, 40 min before test, flumazenil (10 and 20 mg kg^{-1}) dose-dependently antagonized the increase in dark phase response rate induced by both compounds (Figures 5 and 6).

In contrast to the marked release of suppressed responding by FG 8205, the benzodiazepine partial agonist, bretazenil (0.2–50 mg kg^{-1}), induced only a very moderate increase in dark phase response rate at all doses tested (Figure 2d). When co-administered with 5 mg kg^{-1} diazepam, 40 min before test, bretazenil (0.2–5 mg kg^{-1}) antagonized the diazepam-induced increase in dark phase response rate (Figure 7). FG 8205 (0.2–25 mg kg^{-1}) did not alter the increase in dark phase response rate induced by 5 mg kg^{-1} diazepam (results not shown).

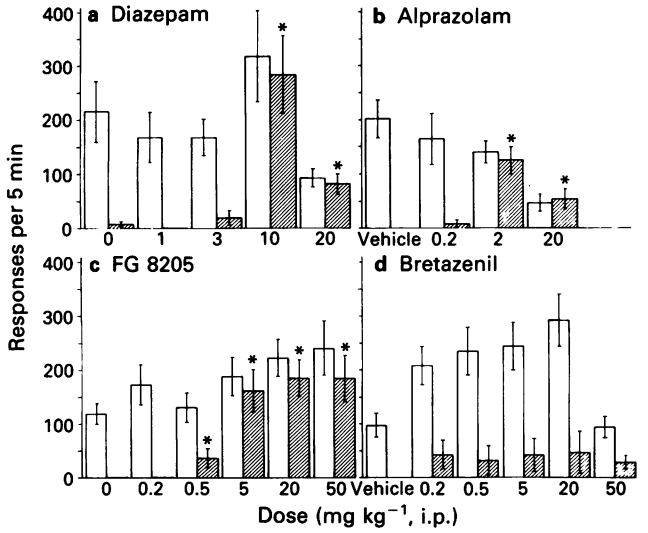

Figure 2 The effects of benzodiazepine receptor agonists in the rat conditioned emotional response test. Columns indicate the mean lever pressing response rate and bars s.e.mean of 8 or more animals per dose group during the light (open columns) and dark (hatched columns) periods of the test. The dark period was associated with punishment (footshock) during training. Drugs were administered i.p. 40 min before testing: (a) diazepam; (b) alprazolam; (c) FG 8205; (d) bretazenil. * Significantly different from vehicle-treated control dark response rate, $P < 0.05$ (ANOVA followed by Dunnett's t test).

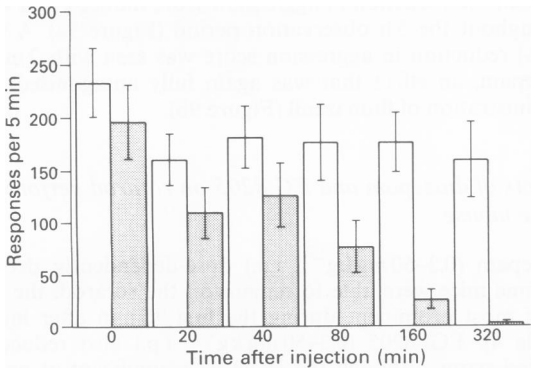

Figure 3 The duration of action of FG 8205 in the rat conditioned emotional response test. Columns indicate the mean lever pressing response rate and bars s.e.mean of 8 or more animals per dose group during the light (open columns) and dark (stippled columns) periods of the test. The dark period was associated with punishment (footshock) during training. FG 8205 (1.3 mg kg^{-1}) was administered i.p. immediately before (0) or at 20, 40, 80, 160, or 320 min before testing.

Taming effect of benzodiazepines in primates

Administration of FG 8205 (0.3–5 mg kg^{-1}, i.p.) induced a marked and dose-dependent reduction in aggression scores by

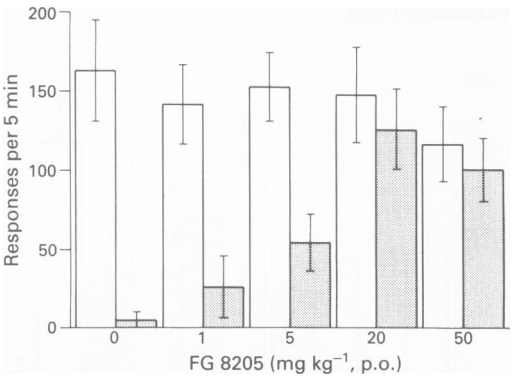

Figure 4 The oral activity of FG 8205 in the rat conditioned emotional response test. Columns indicate the mean lever pressing response rate and bars s.e.mean of 8 or more animals per dose group during the light (open columns) and dark (stippled columns) periods of the test. The dark period was associated with punishment (footshock) during training. FG 8205 was administered orally, 40 min before testing.

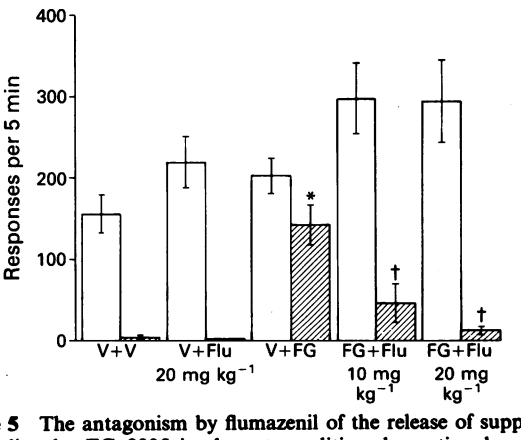

Figure 5 The antagonism by flumazenil of the release of suppressed responding by FG 8205 in the rat conditioned emotional response test. Columns indicate the mean lever pressing response rate and bars s.e.mean of 8 or more animals per group during the light (open columns) and dark (hatched columns) periods of the test. The dark period was associated with punishment (footshock) during training. V = drug vehicle; FG = FG 8205 (5 mg kg^{-1}); Flu = flumazenil, 10 or 20 mg kg^{-1}. Drugs were administered i.p. 40 min before testing. * Significantly different from vehicle-treated control dark phase response rate, $P < 0.05$; † significantly different from dark phase response rate of animals given only FG 8205, $P < 0.05$ (ANOVA followed by Dunnett's t test).

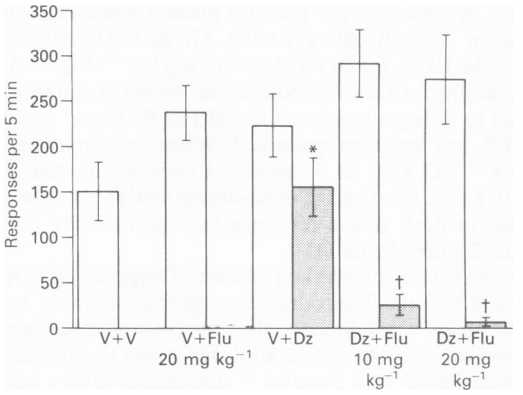

Figure 6 The antagonism by flumazenil of the release of suppressed responding by diazepam in the rat conditioned emotional response test. Columns indicate the mean lever pressing response rate and bars s.e.mean of 8 or more animals per group during the light (open columns) and dark (stippled columns) periods of the test. The dark period was associated with punishment (footshock) during training. V = drug vehicle; Dz = diazepam (5 mg kg^{-1}); Flu = flumazenil, 10 or 20 mg kg^{-1}. Drugs were administered i.p. 40 min before testing. * Significantly different from vehicle-treated control dark phase response rate, $P < 0.05$; † significantly different from dark phase response rate of animals given only diazepam, $P < 0.05$ (ANOVA followed by Dunnett's t test).

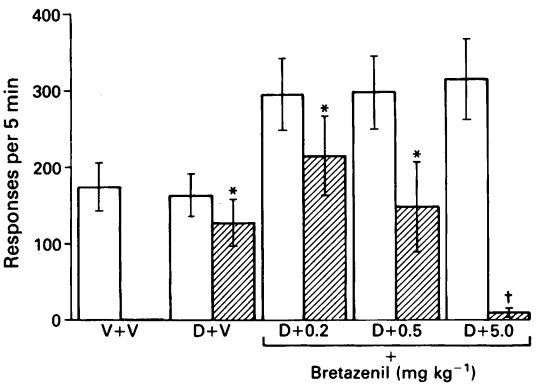

Figure 7 The antagonism by bretazenil of the release of suppressed responding by diazepam in the rat conditioned emotional response test. Columns indicate the mean lever pressing response rate and bars s.e.mean of 8 or more animals per group during the light (open columns) and dark (hatched columns) periods of the test. The dark period was associated with punishment (footshock) during training. V = drug vehicle; D = diazepam (5 mg kg^{-1}). Drugs were administered i.p. 40 min before testing. * Significantly different from vehicle-treated control dark response rate, $P < 0.05$; † significantly different from dark response rate of animals given only diazepam, $P < 0.05$ (ANOVA followed by Dunnett's t test).

comparison to vehicle treatment throughout the 5 h observation period. The taming effect induced by the lowest dose of FG 8205 (0.1 mg kg^{-1}) was the least pronounced, but still significant for up to 2 h after administration (Figure 8a). In contrast, taming was not induced by low doses of diazepam (0.1–0.3 mg kg^{-1}). A dose of 1 mg kg^{-1} diazepam transiently reduced aggression scores between 0.5 and 1.5 h, but only the highest dose (5 mg kg^{-1}) produced marked and sustained taming (Figure 8b).

Estimation of the ED_{50} doses for both FG 8205 and diazepam as the dose reducing the maximum difference between vehicle and drug-treated animals by 50% gave values of 0.1 and 1 mg kg^{-1}, respectively. At the ED_{50} doses, the time taken for the taming score to drop from 50% to 25% of the maximum effect was about 3 h for FG 8205 and 5 h for diazepam.

In a separate experiment, the ability of flumazenil to block the taming effect of FG 8205 and diazepam was examined.

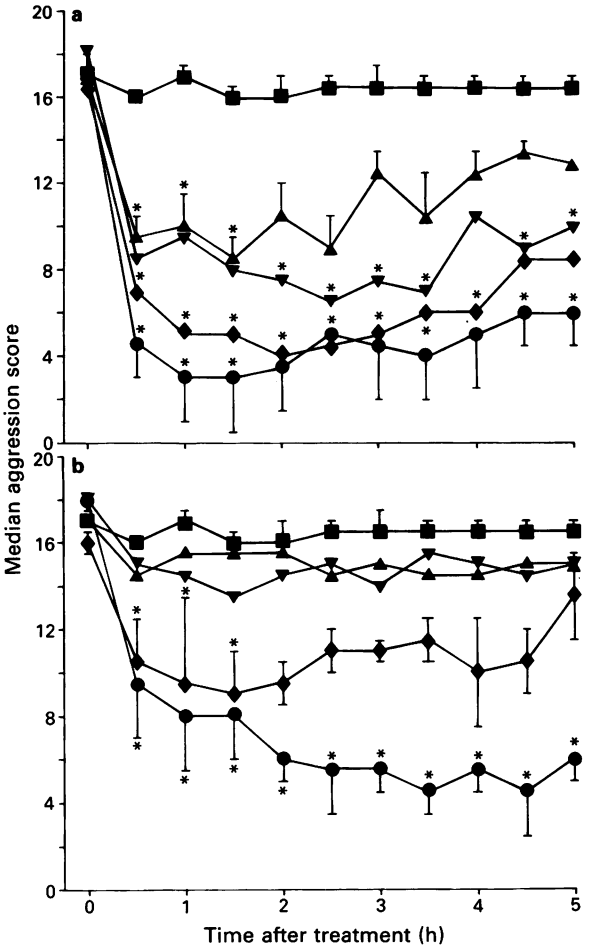

Figure 8 The effect of FG 8205 on aggressive behaviour in cynomolgus monkeys. Attack and avoidance behaviour to the observer (following sudden approach or clapping of hands), a broom handle or a live garter snake was rated on a ranked intensity scale, allowing calculation of the median aggression score of the six animals used. Observations were made immediately before and at 30 min intervals after i.p. injection of (a) FG 8205 or (b) diazepam over a five hour period. (■) Vehicle; (▼) 0.1 mg kg^{-1}; (▲) 0.3 mg kg^{-1}; (◆) 1 mg kg^{-1}; (●) 5 mg kg^{-1} FG 8205 (a) or diazepam (b). Vertical lines indicate the semi-interquartile range. * Significantly different from vehicle-treated control scores, $P < 0.05$ (Mann-Whitney U test).

Treatment with 0.2 mg kg^{-1} FG 8205 reduced aggression scores to about 41% of the pretreatment baseline for up to 3.5 h. Co-administration of flumazenil (5 mg kg^{-1}, i.p.) fully blocked the reduction in aggression score induced by FG 8205 throughout the 5 h observation period (Figure 9a). A similar (53%) reduction in aggression score was seen with 2 mg kg^{-1} diazepam, an effect that was again fully antagonised by co-administration of flumazenil (Figure 9b).

Effects of diazepam and FG 8205 on rotarod performance in the mouse

Diazepam (0.3–50 mg kg^{-1}, i.p.) dose-dependently decreased the time mice were able to remain on the rotarod, the effects being most prominent during the first 15 min after injection (Table 4). FG 8205 (0.3–50 mg kg^{-1}, i.p.) also reduced the rotarod score, although the effect was significant at only one dose level (30 mg kg^{-1}) and one time point (15 min after injection). Indeed, when animals were co-administered FG 8205 (0.2–25 mg kg^{-1}) with diazepam (20 mg kg^{-1}), the impairment in rotarod performance determined 30 min after injection was dose-dependently reduced, although even after 25 mg kg^{-1} FG 8205 complete reversal of the diazepam-induced deficit was not achieved (Figure 10).

Table 4 Effect of FG 8205 and diazepam on rotarod performance in the mouse

Compound	Dose ($mg\,kg^{-1}$)	Time time on rotarod (s) 15	30	45	60 min
Vehicle	—	109 ± 7	115 ± 5	120	120
FG 8205	0.3	92 ± 13	115 ± 5	120	109 ± 8
	1	91 ± 10	116 ± 3	120	120
	3	115 ± 4	116 ± 4	107 ± 9	120
	10	79 ± 12	95 ± 13	100 ± 11	116 ± 4
	30	71 ± 9*	107 ± 7	109 ± 8	113 ± 7
	50	89 ± 12	99 ± 9	108 ± 8	112 ± 8
Vehicle	—	120	112 ± 8	120	120
Diazepam	0.3	120	120 ± 3	107 ± 9	116 ± 4
	1	113 ± 5	120	114 ± 6	120
	3	98 ± 11	105 ± 11	115 ± 5	116 ± 3
	10	64 ± 11*	71 ± 13	94 ± 11	103 ± 10
	30	15 ± 6**	45 ± 16	70 ± 19	74 ± 18
	50	40 ± 14**	33 ± 14**	61 ± 19*	69 ± 20*

Mice were tested for their ability to remain on the rotarod at 15, 30, 45 and 60 min after drug injection. Values are means ± s.e.mean of 8–10 animals per group.
* Significantly different from vehicle-treated controls, $P < 0.05$ (Kruskal Wallis ANOVA followed by Mann-Whitney U test).

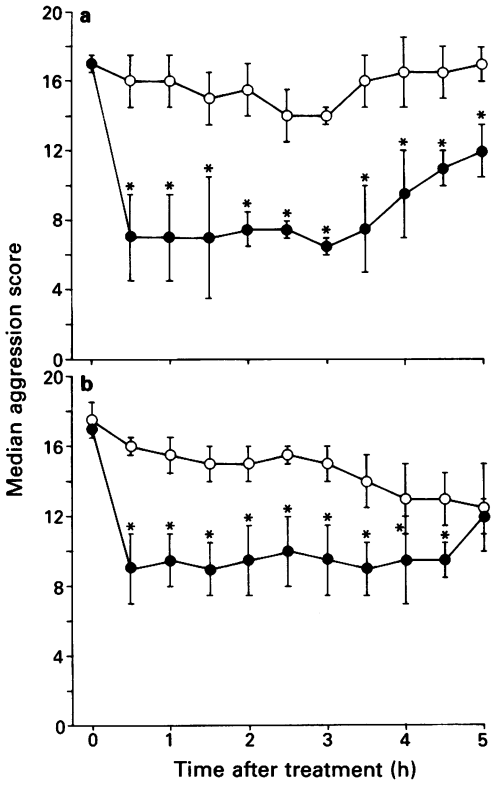

Figure 9 The antagonism by flumazenil of the anti-aggressive effect of (a) FG 8205 and (b) diazepam in cynomolgus monkeys. Flumazenil ($5\,mg\,kg^{-1}$) was co-administered i.p. with either (a) FG 8205 ($0.2\,mg\,kg^{-1}$) or (b) diazepam ($2\,mg\,kg^{-1}$) and aggressive behaviour rated as described in the legend to Figure 8. (○) FG 8205 or diazepam; (●) FG 8205 or diazepam + flumazenil. * Significantly different from animals given only FG 8205 or diazepam (Mann-Whitney U test).

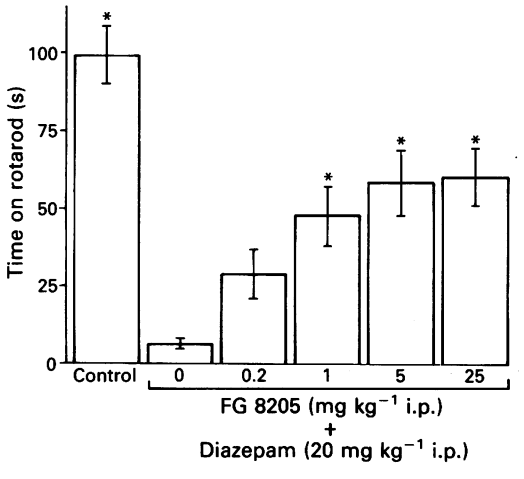

Figure 10 Antagonism by FG 8205 of the rotarod deficit induced in the mouse by diazepam. Animals were trained to remain on a rotarod revolving at 15 r.p.m. for 120 s and then co-administered various doses of FG 8205 with diazepam ($20\,mg\,kg^{-1}$) i.p. Thirty minutes later, they were returned to the rotarod and the latency to fall noted. Columns represent the mean time spent on the rotarod and bars s.e.mean of at least 8 animals per group. * Significantly different from animals given only diazepam, $P < 0.05$ (Kruskal Wallis ANOVA followed by Mann-Whitney U test).

Effects of diazepam and FG 8205 on swimming and climbing performance in the rat

Rats in their home cage were calm and inactive following the acute administration of diazepam ($1–50\,mg\,kg^{-1}$) or FG 8205 ($1–50\,mg\,kg^{-1}$). However, neither FG 8205 ($1–50\,mg\,kg^{-1}$) nor diazepam ($1–50\,mg\,kg^{-1}$) significantly altered the time taken to locate the platform of a swim maze (Table 5). On the other hand, diazepam markedly increased the time taken to mount the platform: animals given a dose of $30\,mg\,kg^{-1}$ took more than 20 s to escape the water. In contrast, FG 8205 increased the time taken to mount the platform by a maximum of only 2.4 s (Table 5).

Sedation and ataxia in primates

Scores for sedation and ataxia in cynomolgus monkeys were obtained at each 30 min observation period following drug administration. Cumulative scores are shown in Table 6. A slight, but significant increase in the score was seen only after a dose of FG 8205 ($5\,mg\,kg^{-1}$), 50 fold higher than the minimum effective dose to induce taming. In contrast, a high sedation/ataxia score was seen at the lowest dose of diazepam that produced a marked and sustained taming effect.

Discussion

The imidazobenzodiazepine, FG 8205 potently inhibited [^{3}H]-flumazenil binding in rat cortical membranes, but was without significant affinity for thirteen neurotransmitter

Table 5 Effects of diazepam and FG 8205 on swimming performance in the rat

	Dose ($mg\,kg^{-1}$)	Latency to reach platform (s)	Latency to mount platform (s)
Vehicle	—	33.9 ± 6.2	1.8 ± 0.3
Diazepam	1	32.8 ± 7.8	2.9 ± 0.3
	3	42.7 ± 6.9	2.9 ± 0.3
	10	47.1 ± 6.8	6.8 ± 1.9*
	30	47.6 ± 8.3	21.4 ± 2.7*
	50	49.1 ± 6.2	27.6 ± 1.8*
Vehicle	—	32.7 ± 5.2	1.7 ± 0.2
FG 8205	1	34.5 ± 4.4	2.9 ± 0.3*
	3	37.4 ± 3.6	3.9 ± 0.5*
	10	43.8 ± 6.6	4.0 ± 0.4*
	30	42.7 ± 5.3	4.1 ± 0.6*
	50	35.4 ± 6.7	3.9 ± 0.5*

Rats were plunged into a swimming pool 1 m from a visible platform and the time taken to (a) locate the platform and (b) mount the platform was noted. Rats were tested 30 min after i.p. injection of either diazepam or FG 8205. Values are means ± s.e.mean of 8–10 animals per group. * Significantly different from vehicle-treated controls, $P < 0.05$ (ANOVA followed by Dunnett's t test).

Table 6 Sedation and ataxia induced by FG 8205 or diazepam in primates

Dose ($mg\,kg^{-1}$)	Summed sedation and ataxia score/30–210 min FG 8205	Diazepam
0.1	0	0
0.3	1.0 ± 2	0
1.0	3.5 ± 4.5	2.5 ± 2
5.0	5.5 ± 4*	18.5 ± 6*

Animals were scored for sedation and ataxia on a scale of 0–5 according to the ease with which animals could be aroused and their motor co-ordination. Summed scores were compared with those of vehicle-treated animals by use of Kruskal Wallis analysis of variance of ranks followed by Mann-Whitney U-tests. The summed score of vehicle-treated animals was 0.
* $P < 0.05$ compared with control treatment.

recognition sites, indicating its very high selectivity for the benzodiazepine receptor. As with diazepam and alprazolam, compounds considered to be full benzodiazepine receptor agonists, the affinity of FG 8205 for the [^{3}H]-flumazenil recognition site was increased by the presence of GABA. However, the magnitude of the 'GABA shift' suggests that FG 8205 has a level of efficacy less than that of diazepam, but greater than that of the benzodiazepine partial agonists, bretazenil and Ro 17-1812 (Kemp *et al.*, 1987). The GABA shifts of the latter two compounds were, in turn, greater than that of the neutral antagonist, flumazenil, which had no GABA shift, and opposite to that of the beta-carboline inverse agonist, DMCM.

In the rat hippocampal slice, benzodiazepine receptor agonists dose-dependently potentiate the inhibitory potency of the selective $GABA_A$-receptor agonist, isoguvacine, on the CA1 population spike. Previous studies (Kemp *et al.*, 1987) have shown that this potentiation closely parallels the degree of benzodiazepine receptor occupation and thus, partial agonists are unable to produce the same maximum potentiation as full agonists. Consistent with its low GABA shift, FG 8205 gave a maximum potentiation of the isoguvacine-induced inhibition which was significantly less than that produced by diazepam or alprazolam. The shift in potency induced by FG 8205 was, in this case, similar to that seen with bretazenil and Ro 17-1812, but again opposite to that produced by DMCM.

Whilst the intrinsic activity of FG 8205 is low compared to diazepam and alprazolam, sufficient efficacy remains for the genesis of both anticonvulsant and anxiolytic effects in a number of test situations. Thus, the compound was equipotent with diazepam against seizures induced by PTZ or by sound in audiogenic seizure prone mice, two models that are highly sensitive to benzodiazepines. Diazepam was itself a much weaker antagonist of seizures induced by electroshock and, perhaps consistent with the lower intrinsic activity and inactivity of other partial benzodiazepine receptor agonists (Petersen *et al.*, 1984; Haefely, 1984; Schneider *et al.*, 1989), FG 8205 was inactive in this test even at a dose 200 fold greater than that required to block seizures induced by sound or PTZ.

In the conditioned emotional response task, the full benzodiazepine receptor agonists, diazepam and alprazolam, were able to increase lever pressing in the dark phase of the test associated with punishment (footshock) during training. However, the response to both compounds was biphasic, the higher doses used giving lower response rates in both dark and light, probably reflecting the onset of sedation. FG 8205 dose-dependently increased the dark phase response rate over a wide dose range and without suppression of responding in the light. In contrast to FG 8205, the somewhat lower efficacy benzodiazepine receptor agonist, bretazenil (at least in terms of its lower GABA shift) induced only a very moderate increase in dark phase response rate in the CER test. In addition, bretazenil was able to antagonize the increase in dark phase response rate induced by diazepam, while FG 8205 had no such effect. In view of their seemingly modest differences in *in vitro* measures of efficacy, the differences in the behavioural profiles of FG 8205 and bretazenil are perhaps surprising. They are, nevertheless, not inconsistent with the benzodiazepine 'efficacy' hypothesis.

Compounds that are anxiolytic in man are often able to release suppressed responding in rodent behavioural models similar to the CER test described here: it is likely, therefore, that FG 8205 would also be anxiolytic in man. This is further supported by the taming effects of the compound in aggressive cynomolgus monkeys. Like diazepam, FG 8205 reduced attack and avoidance behaviour to aversive stimuli. Although FG 8205 was approximately 10 fold more potent than diazepam, the duration of action of the compound at its ED_{50} dose was shorter than that of diazepam (3 h cf 5 h for diazepam).

Sedation and ataxia are marked features of the behavioural and neurological response to benzodiazepine receptor agonists in both rodents and primates. In the mouse, this was clearly reflected in the disruption of performance on the rotarod following treatment with diazepam. In contrast to diazepam, FG 8205 induced only a very mild and transient performance deficit at high doses. In the rat, FG 8205 decreased activity in the home cage, but only slightly impaired the ability of animals to climb from a swimming pool, a task that was again markedly disrupted by diazepam. Similarly, in primates sedation and ataxia induced by FG 8205 was slight and transient after doses ten fold higher than the minimum required for a significant taming effect.

It has been hypothesized that benzodiazepine-mediated sedation reflects the occupation of the receptor by high efficacy agonists in regions of low receptor reserve (Jensen & Petersen, 1983; Haefely & Polc, 1986). For example, with *in vivo* radioligand binding techniques, the anticonvulsant effects of diazepam and the benzodiazepine partial agonist, bretazenil have been found to occur at receptor occupancies of 5% and 40%, respectively (Potier *et al.*, 1988). In contrast, diazepam did not induce marked motor deficits until 35% occupancy had been achieved, whilst even at an occupancy of 90–100%, bretazenil was without myorelaxant effects. Hence, the low liability of FG 8205 to induce sedation and ataxia in rodents and primates is entirely predictable from the low intrinsic activity of the compound in *in vitro* biochemical and electrophysiological test systems. The 'partial agonist' or 'efficacy' theory also predicts that compounds of low intrinsic efficacy

will antagonize the actions of full agonists. Consistent with this hypothesis, FG 8205 clearly attenuated the rotarod performance deficit induced in the mouse by diazepam.

However, the varied pharmacological profile of partial benzodiazepine receptor agonists invites alternative explanations: the different behavioural effects of these compounds could equally reflect different degrees of efficacy at more than a single benzodiazepine recognition site. The anticonvulsant profile of FG 8205 suggests the existence of one subtype that is insensitive to the benzodiazepine receptor antagonist, flumazenil. Thus, while the potentiation by FG 8205 of the response to isoguvacine in the rat hippocampal slice and the anxiolytic-like effects of the compound in both rats and primates were reversed by flumazenil, high doses of the antagonist were able to block only marginally the protective effects of FG 8205 against seizures induced by PTZ in the mouse. Preliminary studies also suggest this to be the case in the rat (Tricklebank, unpublished observations). Since the anticonvulsant effects of diazepam were reversed by flumazenil under an identical dosing regime, it seems unlikely that the insensitivity of FG 8205 can be explained by insufficient occupation of the receptor by the antagonist. On the other hand, although cloning experiments have strongly supported the existence of multiple benzodiazepine receptors (Pritchett *et al.*, 1989a,b; Khrestchatisky *et al.*, 1989), none have yet been shown to be flumazenil-insensitive. It remains to be seen whether FG 8205 will play a useful role in providing the functional evidence necessary to substantiate the pharmacological relevance of putative subtypes of the $GABA_A$-receptor.

In conclusion, *in vitro* test systems have shown FG 8205 to have many of the characteristics of a partial agonist at the benzodiazepine receptor. In rodents and primates, the compound lacks the sedation/ataxia profile of full agonists. Intrinsic activity is nevertheless sufficient to endow FG 8205 with good activity in anxiolytic paradigms, although it is not clear whether its anticonvulsant effects reflect partial agonist properties at the benzodiazepine receptor; at a subtype of the benzodiazepine receptor or (despite its lack of activity in many other neurotransmitter binding assays) an interaction at non-benzodiazepine sites involved in the generation or expression of seizures. Other studies have indicated that mice are less likely to become tolerant to the anticonvulsant effects of FG 8205 than they are to diazepam and show little evidence of a dependence syndrome on withdrawal from chronic treatment (Tricklebank *et al.*, unpublished observations). As such, FG 8205 should have a better side-effect profile in man than many of the currently available full benzodiazepine receptor agonists.

We thank Hoffmann-La Roche and Upjohn for the gift of drugs.

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(Received March 1, 1990
Revised July 2, 1990
Accepted July 7, 1990)

Br. J. Pharmacol. (1990), **101**, 762–768

Effect of blockade of noradrenaline re-uptake on evoked tritium overflow from mouse vasa deferentia and rat cortex slices

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1 In tissues previously incubated with [^{3}H]-noradrenaline exposure to cocaine (0.1 to 10 μM) or desmethylimipramine (0.01 to 1 μM) produced a concentration-dependent increase (up to 2 fold) in electrically evoked (3 Hz, 2 ms, 20 mA, 120 s every 20 min) fractional overflow of tritium from rat brain cortex slices but not from mouse vas deferens (2.5 Hz, 2 ms, 400 mA, for 90 s every 14 min).

2 Yohimbine and idazoxan (0.01 to 1 μM) increased fractional evoked overflow of tritium by up to 2 fold; in the presence of these drugs, cocaine (10 μM) produced an increase in both tissues (up to 3.5 fold over control).

3 In brain slice an increase in stimulation frequency (0.1, 0.5, 1, 3 and 6 Hz) decreased fractional evoked overflow of tritium per pulse but cocaine (10 μM) produced a significant enhancement at each frequency except 6 Hz. In vas deferens fractional tritium overflow per pulse changed little with increasing frequency and cocaine produced no effect.

4 In both tissues fractional evoked overflow of tritium was dependent on the stimulation current; cocaine (10 μM) increased fractional evoked overflow from brain slice at every current tested but was without effect in vas deferens.

5 Chromatographic separation of the released tritium showed there was little difference in the proportions of [^{3}H]-noradrenaline and ^{3}H-metabolites overflowing from the tissues. Cocaine increased the proportion of [^{3}H]-noradrenaline and decreased the proportion of [^{3}H]-DOPEG overflowing both at rest and during stimulation.

6 In brain slice an increase in electrically evoked overflow was produced by cocaine (10 μM) whether total tritium overflow (1.8 fold), overflow of [^{3}H]-noradrenaline (1.8 fold) or overflow of unlabelled noradrenaline (1.8 fold) was measured. Evoked overflow from vas deferens was unaffected when assessed by any of these three methods.

7 The mechanism responsible for this differential effect of cocaine is unclear but may involve differences in the physical relationship between release sites, reuptake sites and presynaptic autoreceptors.

Introduction

A variety of drugs block neuronal re-uptake of noradrenaline and increase the amount of transmitter which overflows after stimulation of noradrenergic nerves (ear artery, Auch-Schwelk *et al.*, 1983; mesenteric artery, Mishima *et al.*, 1984; hypothalamic (Irie & Wurtman, 1987) and cortical (Hagan & Hughes, 1981) brain slices; atria, Rand *et al.*, 1982; heart, Fuder & Muscholl, 1978; saphenous vein, Verbeuren & Vanhoutte, 1982; iris, Farnebo & Hamberger, 1971; superior cervical ganglion, Noon & Roth, 1974; rabbit vas deferens, Hughes, 1972). In other tissues blockade of re-uptake appears to have little or no effect on evoked noradrenaline overflow (spleen, Langer & Dubocovich, 1981; mouse vas deferens, Hagan & Hughes, 1981; portal vein, Haggendal *et al.*, 1970; nictitating membrane, Nigro & Enero, 1981; aorta, Schrold & Nedergaard, 1981; colon, Boullin & Brodie, 1967). In these latter tissues presynaptic α_2-adrenoceptors may function to compensate, by reducing noradrenaline release, for the increased synaptic noradrenaline levels resulting from uptake blockade (Farnebo & Hamberger, 1971; Langer, 1977). However, such receptors function in both groups of tissues and we now describe an investigation of why compensation, if it takes place, does so in one group only. Some of these results have already been communicated to the Society (El-Mas & Hughes, 1989).

Methods

Mouse vas deferens

Mice (Tuck No. 1; 28–35 g) were killed by a blow to the head. The vas deferens was removed, stripped of mesentery and incubated for 40 min with 0.66 μM [^{3}H]-(−)-noradrenaline. Tissues were then mounted in a bath which was drained and refilled every 2 min with 1.5 ml of modified Krebs bicarbonate saline (magnesium-free) of the following composition (mM): NaCl 118, KCl 4.75, $CaCl_2$ 2.54, KH_2PO_4 0.93, $NaHCO_3$ 25 and glucose 11.1. The solution also contained (μM) disodium edetate (EDTA) 27, ascorbic acid 57 and 17-β-oestradiol 3.7, was maintained at 37°C and was aerated with a gas mixture containing 95% O_2 and 5% CO_2. After washing for 45 min, up to seven periods (S1 to S7) of electrical stimulation (2.5 Hz, 2 ms pulse width, 400 mA for 90 s unless otherwise stated) were applied transmurally through parallel platinum wire electrodes (1 mm thick, approximately 35 mm long and held 6 mm apart) at 14 min intervals. The tritium content of the two samples collected immediately before a period of stimulation was averaged to give a resting overflow. Any tritium in excess of that expected from the resting overflow which was found in the sample in which stimulation was applied and in the 3 following samples was taken as evoked overflow. Resting and evoked overflows were expressed as fractional overflows by dividing by the tissue content of tritium at the start of the stimulation period. S1 was often not representative of later stimulation periods and was discarded; S2 was taken as a control period for each tissue. Drugs were added in the bulk of the bathing solution, were in contact with the tissue 10 min before a period of stimulation and then usually remained in contact for the rest of the experiment; their effect was sometimes quantified by calculation of the ratio of fractional resting or evoked overflow at S2 to that in later periods (Sx/S2; where x = 3 to 7). At the end of the experiment the tissue was placed in a vial, overnight, with 0.5 ml OptiSolv (Packard); acetic acid (0.25 ml 4M) was then added and the sample counted for tritium as below.

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Rat cortex slices

Male Wistar rats (180–220 g) were killed by cervical dislocation and decapitated. The brains were rapidly removed and placed in ice-cold modified Krebs bicarbonate saline of the following composition (mM): NaCl 118, KCl 4.75, $CaCl_2$ 1.3, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2 and glucose 10. The solution was aerated with a gas mixture containing 95% O_2 and 5% CO_2 and also contained (μM) EDTA 30, ascorbic acid 60 and 17-β-oestradiol 4. Slices of frontal cortex were prepared with a McIlwain tissue chopper (set to 0.4 mm). Up to 8 slices (5 × 2 × 0.4 mm) were incubated for 45 min at 37°C in 2.0 ml of modified Krebs bicarbonate saline containing 0.1 μM [^{3}H]-(−)-noradrenaline. After incubation the slice was briefly washed in 2 × 25 ml ice-cold modified Krebs bicarbonate saline, placed on a nylon mesh disc and inserted into a superfusion chamber (volume 0.35 ml) between two electrodes (platinum wire (0.5 mm diameter) rings each approximately 5 mm in diameter and held 3 mm apart). The chamber was maintained at 37°C and perfused with modified Krebs bicarbonate saline at 0.5 ml min^{-1}. After 40 min washing sequential 4 min superfusate samples were collected until the end of the experiment; two periods (S1 and S2) of electrical stimulation (3 Hz, 2 ms pulse width, 20 mA for 2 min unless otherwise stated) were applied at 20 min intervals. Drugs were added to the superfusion medium 16 min before S2 and remained in contact with the slice until the end of the experiment when the amount of tritium in the tissue was determined as described above. The tritium content of the sample collected immediately before a period of stimulation was taken as the resting overflow. Any tritium in excess of that expected from the resting overflow found in the sample in which stimulation was applied and in the following 2 samples was taken as evoked overflow. Fractional resting and evoked overflow were calculated as above: S1 was taken as a control period for each tissue and the effect of drugs was sometimes determined by calculation of the ratio S2/S1.

Separation of [^{3}H]-noradrenaline and ^{3}H-metabolites

Tissues were prepared, incubated and washed as above and when appropriate the modified Krebs bicarbonate saline from the tissue baths was collected in vials containing 5 mg EDTA and 5 mg Na_2SO_3. For vas deferens and brain slice one sample immediately before a stimulation period (resting overflow) and the sample in which stimulation was applied (evoked overflow) were analysed. The treated tissues were exposed to cocaine (10 μM) 10 or 16 min before S3 or S2 respectively. Samples were analysed by a dual column method (Graefe *et al.*, 1973) involving alumina and Dowex separations which yields fractions corresponding to MOPEG + VMA (I; 3-methoxy-4-hydroxyphenylglycol + 3-methoxy-4-hydroxymandelic acid), NMN (II; normetanephrine), DOPEG (III; 3,4-dihydroxyphenylglycol), unchanged noradrenaline (IV) and DOMA (V; 3,4-dihydroxymandelic acid).

Measurement of radioactivity

All samples were mixed with an appropriate volume of Opti-Phase Safe (Packard) and counted for tritium in a Packard 300C liquid scintillation counter. Correction for quench utilised the spectral index of the external standard.

Measurement of overflow of unlabelled noradrenaline

Tissues were prepared, incubated (with unlabelled noradrenaline), washed and samples collected as described for the separation of tritiated noradrenaline and metabolites except that for vas deferens, samples collected during periods S2, S3 and S4 were pooled before analysis as were those for S5, S6 and S7. The treated tissues were exposed to cocaine (10 μM) 10 min before S5. For brain slice, samples from 3 tissues taken from the same animal were pooled before analysis.

Dihydroxybenzylamine (3 pmol) was added to each sample as an internal standard. High performance liquid chromatography (h.p.l.c.) with electrochemical detection was used to measure unlabelled noradrenaline (Watson, 1981) modified as detailed below. Catecholamines in the samples were adsorbed onto alumina and then eluted in 75 μl of 0.2 M acetic acid. A Millipore Waters model 510 solvent delivery system with a Rheodyne injector (100 μl loop) was used for h.p.l.c. The column was an Apex 1 ODS 3 μm, 10 × 0.46 cm cartridge (Jones Chromatography). The mobile phase consisted of NaH_2PO_4 (0.1 M), EDTA (0.11 mM) and octane sulphonic acid (5 mM) modified with 15% methanol and was delivered at a flow rate of 0.7 ml min^{-1}. An amperometric detector model LC-4A was used (Bioanalytical Systems Inc.). The retention time of noradrenaline was 8 min.

Drugs

The following were used: ascorbic acid (BDH), cocaine hydrochloride (Boots), desmethylimipramine hydrochloride (Geigy), idazoxan hydrochloride (Wyeth), (−)-noradrenaline bitartrate (Sigma), [^{3}H]-(−)-noradrenaline (specific activity about 15 Ci mmol^{-1}; Amersham or New England Nuclear), 17-β-oestradiol (Sigma), yohimbine hydrochloride (Sigma).

Statistical procedures

When appropriate all results are given as mean ± standard error (s.e.mean) together with the number of observations contributing to each mean (n). Student's t test (paired when appropriate) or the Mann-Whitney rank test as specified (Snedecor & Cochran, 1980) were used to calculate statistical significance.

Results

Effect of drugs on resting and evoked tritium overflow

The tritium content of brain slices immediately before S1 averaged 126379 ± 2616 d.p.m. in a typical series of experiments (n = 32). Resting overflow averaged 1578 ± 40 d.p.m. and evoked overflow averaged 3628 ± 98 d.p.m.; in fractional terms these equate to 1.02 ± 0.04% and 2.80 ± 0.07% respectively. In control tissues fractional resting overflow of tritium fell slightly between S1 and S2 and gave an S2/S1 ratio which was consistently less than unity. Fractional evoked overflows were better maintained and the S2/S1 ratios were close to unity (Figure 1). Experiments were usually limited to two periods of stimulation since overflow evoked in a third period decreased somewhat and was reduced by at least 50% during a fourth period. Evoked overflow was abolished by cinchocaine (26 μM) and by replacement of the Krebs bicarbonate saline by a solution from which calcium had been omitted.

The tritium content of vas deferens immediately before the second stimulation period (S2) averaged 420359 ± 12551 d.p.m. in a typical series of experiments (n = 23). Resting overflow averaged 2539 ± 126 d.p.m. and evoked overflow averaged 4814 ± 225 d.p.m.: in fractional terms these equate to 0.70 ± 0.02% and 1.19 ± 0.04% respectively. Fractional resting overflow showed a tendency to fall during the course of the experiment as can be seen from changes in the Sx/S2 ratio (Figure 2). Fractional evoked overflow remained relatively unchanged for the 7 periods of stimulation and was abolished by cinchocaine (13 μM) and by replacement of the Krebs bicarbonate saline by a solution from which calcium had been omitted.

Cocaine (0.1 to 10 μM) and desmethylimipramine (0.01 to 1 μM) increased fractional evoked overflow in brain slices. Cocaine (but not desmethylimipramine) also produced a small but statistically significant increase in fractional resting over-

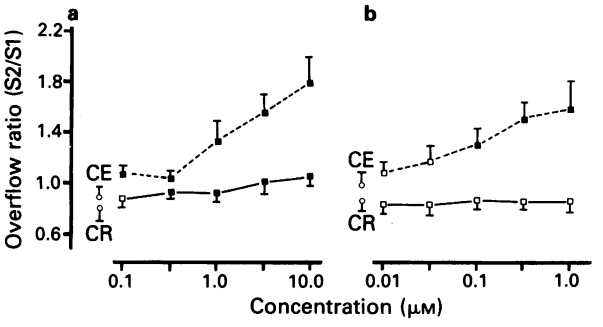

Figure 1 Showing the ratio for the fractional resting (continuous line) and the fractional evoked (broken line; 3 Hz, 2 ms pulse width, 20 mA for 120 s every 20 min) tritium overflow from rat brain cortex slices, previously incubated with [^{3}H]-noradrenaline, to the corresponding values found in the control period (S2/S1). In treated tissues (squares), but not in untreated tissues (circles), (a) cocaine (0.1–10 μM) or desmethylimipramine (0.01–1 μM) was introduced into the tissue bath 16 min before the start of the second stimulation period (S2). Each point is the mean of 5–6 experiments and the bars show standard errors. Solid symbols show statistically significant differences from values for fractional resting (CR) or evoked (CE) overflow ratios as appropriate in untreated tissues (Mann-Whitney rank test; $P < 0.05$).

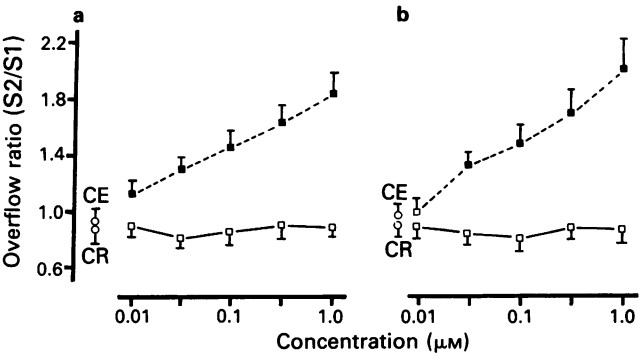

Figure 3 Showing the ratio for the fractional resting (continuous line) and the fractional evoked (broken line; 3 Hz, 2 ms pulse width, 20 mA for 120 s every 20 min) tritium overflow from rat brain cortex slices, previously incubated with [^{3}H]-noradrenaline, to the corresponding values found in the control period (S2/S1). In treated tissues (squares), but not in untreated tissues (circles), (a) yohimbine or (b) idazoxan (0.01–1 μM) was introduced into the tissue bath 16 min before the start of the second stimulation period (S2). Each point is the mean of 5–6 experiments and the bars show standard errors. Solid symbols show statistically significant differences from values for fractional resting (CR) or evoked (CE) overflow ratios as appropriate in untreated tissues (Mann-Whitney rank test; $P < 0.05$).

flow (Figure 1). In vas deferens, neither cocaine nor desmethylimipramine produced any effect on fractional evoked or on fractional resting overflow at any concentration tested and the Sx/S2 ratios were not statistically significantly different from those found in control tissues (Figure 2).

Yohimbine and idazoxan (0.01 to 1.0 μM) increased evoked tritium overflow in brain slice and vas deferens by up to 2 fold. Resting overflow was not altered (Figures 3 and 4).

The combination of cocaine (10 μM) together with either yohimbine (1 μM) or idazoxan (1 μM) produced a large increase in fractional evoked overflow in both vas deferens and in brain slice. The size of the effect (a 3 to 3.5 fold increase) was greater than any effect of cocaine alone or of yohimbine or idazoxan alone in either tissue (Figure 5).

Effect of cocaine under different experimental conditions

Cocaine was tested on vas deferens, prepared, incubated and washed as usual but then bathed, for part of the experiment,

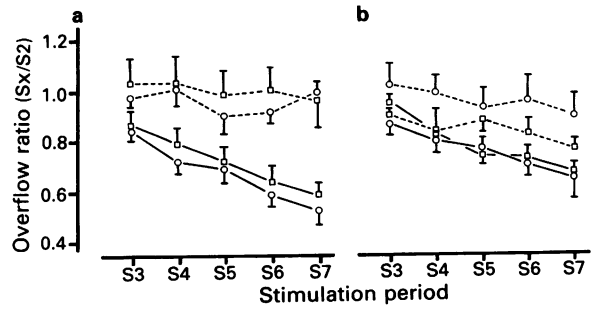

Figure 2 Showing the ratio for the fractional resting (continuous line) and the fractional evoked (broken line; 2.5 Hz, 2 ms pulse width, 400 mA for 90 s every 14 min) tritium overflow from mouse vas deferens, previously incubated with [^{3}H]-noradrenaline, to the corresponding values found in the control period (Sx/S2). In treated tissues (squares), but not in untreated tissues (circles), stimulation periods S3 to S7 were in the presence of (a) cocaine (0.1, 0.3, 1, 3 or 10 μM) or (b) desmethylimipramine (0.01, 0.03, 0.1, 0.3 or 1 μM) respectively introduced into the tissue bath 10 min before the start of the stimulation period. Each point is the mean of 6 experiments and the bars show standard errors. None of the points obtained from treated tissues are statistically significantly different (Mann-Whitney rank test; $P > 0.05$) from corresponding points from untreated tissues.

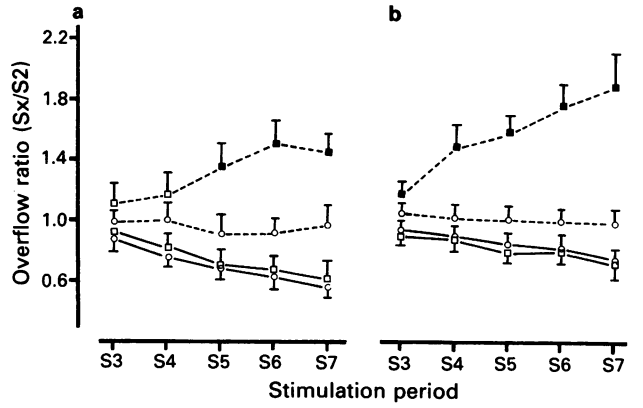

Figure 4 Showing the ratio for the fractional resting (continuous line) and the fractional evoked (broken line; 2.5 Hz, 2 ms pulse width, 400 mA for 90 s every 14 min) tritium overflow from mouse vas deferens, previously incubated with [^{3}H]-noradrenaline, to the corresponding values found in the control period (Sx/S2). In treated tissues (squares), but not in untreated tissues (circles), stimulation periods S3 to S7 were in the presence of (a) yohimbine or (b) idazoxan (0.01, 0.03, 0.1, 0.3 or 1 μM respectively) introduced into the tissue bath 10 min before the start of the stimulation period. Each point is the mean of 6 experiments and the bars show standard errors. Solid symbols show statistically significant differences from untreated tissues (Mann-Whitney rank test; $P < 0.05$).

Table 1 Effect of cocaine and of changing the composition of the bathing fluid on fractional evoked overflow of tritium from vas deferens previously incubated with [^{3}H]-noradrenaline

Stimulation period (Sx)	*Type of bathing solution used*	*Fractional evoked overflow* (Sx/S2)
S3	Vas deferens	1.07 ± 0.07
S4	Brain slice	0.46 ± 0.08*
S5	Brain slice + cocaine (10 μM)	0.48 ± 0.03*
S6	Vas deferens	0.94 ± 0.13

Values are mean ± s.e.mean, $n = 4$.
Changes in the composition (see Methods) of the bathing solution were made 10 min before each appropriate stimulation period.
* Statistically significant difference (Mann-Whitney rank test; $P < 0.05$) from S3 value.

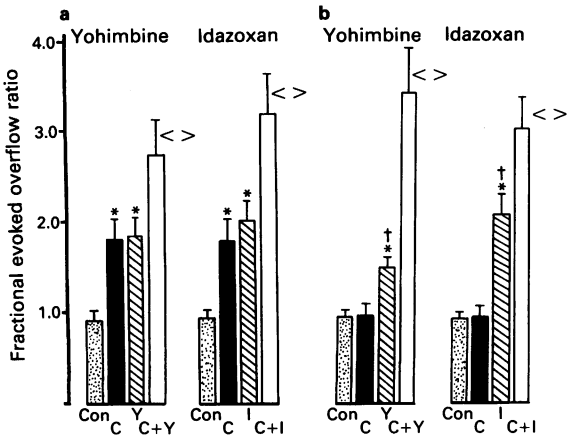

Figure 5 Showing the ratio of fractional evoked tritium overflow in an initial control period to that in a later period in the presence of no additional drugs (Con; stippled columns), cocaine alone (C; 10 μM; solid columns), yohimbine (Y; 1 μM) or idazoxan (I; 1 μM) alone (hatched columns) and for cocaine + either yohimbine or idazoxan (concentrations as above; open columns). Values obtained from (a) rat brain cortex slices and (b) mouse vas deferens are shown. The height of each column represents the mean value ($n = 6$) and the bars show standard errors. Statistically significant differences (Mann-Whitney rank test; $P < 0.05$) are shown as follows: * from control (no drugs); † from cocaine alone; <>, from other 3 associated columns. The experimental conditions for brain slice and for vas deferens are specified in Figures 3 and 4 respectively.

in the Krebs bicarbonate saline normally used for brain slice. Six periods of stimulation were applied and the S$_X$/S2 ratios for fractional evoked overflow (Table 1) show that this procedure significantly reduced the fractional evoked overflow of tritium. Under these conditions cocaine (10 μM) did not increase fractional evoked overflow. Reverting to the Krebs bicarbonate saline normally used for vas deferens increased fractional evoked overflow to a value not statistically significantly different from that obtained at the start of the experiment ($P > 0.05$; Mann-Whitney rank test). Any possible effect due to differences in the apparatus used for the two tissues was investigated in vasa deferentia prepared and incubated as described above and then mounted, superfused and stimulated electrically in the apparatus and under the conditions normally used for brain slices. After the normal wash period the tissue content of tritium averaged 478802 ± 2794 d.p.m. ($n = 8$) but stimulation at 20 mA (the current usually used for brain slices) evoked a barely detectable overflow of tritium and the strength of the stimulus was therefore increased to 200 mA. Under these conditions resting overflow in the 4 min collection period averaged 4187 ± 97 d.p.m. and evoked overflow averaged 5583 ± 140 d.p.m. ($n = 8$). In fractional terms these figures represent 0.9 ± 0.02% and 1.1 ± 0.03% respectively. Addition of cocaine (10 μM) 16 min before S2 did not alter the S2/S1 ratios for either fractional resting (1.02 ± 0.02) or fractional evoked (0.93 ± 0.08) overflows in a statistically significant manner ($P > 0.05$; Mann-Whitney rank test) when compared to control tissues without cocaine where the corresponding values were 1.06 ± 0.03 and 0.98 ± 0.08 respectively ($n = 4$ in each group of tissues).

Effect of cocaine at different stimulus currents

In both vas deferens and brain slice the ratio of the fractional evoked overflow of tritium in an initial period of stimulation (2.5 Hz, 2 ms pulse width, 400 mA for 90 s in vas deferens; 3 Hz, 2 ms pulse width, 20 mA for 2 min in brain slice) to that in a later period with a different stimulus current, increased as the current increased above the threshold value required to produce a measurable overflow of tritium (50 mA in vas deferens; 5 mA in brain slice). In vas deferens when the later periods of stimulation were carried out in the presence of cocaine (10 μM) no statistically significant difference ($P > 0.05$; Mann-Whitney rank test) was found in fractional evoked overflow ratio in comparison with experiments performed wholly in the absence of cocaine. In brain slice cocaine produced a statistically significant increase in fractional evoked overflow ratio at every stimulus current tested ($P < 0.05$; Mann-Whitney rank test) (Figure 6).

Effect of cocaine at different frequencies of stimulation

Electrical stimulation of vas deferens or of brain slices (for a fixed period of 90 or 120 s respectively) evoked a fractional overflow of tritium which increased with increasing frequency of stimulation over the range tested (0.1 to 6 Hz; Figure 7a). Since the period of stimulation was fixed, the use of different frequencies involves the application of a different number of stimuli and the data has therefore been replotted (Figure 7b) on the basis of fractional overflow per pulse which revealed a different pattern in the two tissues. In brain slices, fractional evoked overflow per pulse was high at low frequencies, decreased by about 75% at 3 Hz but showed no further decrease at 6 Hz. In vas deferens, fractional evoked overflow per pulse was generally smaller than in brain slice at low (1 Hz or less) frequencies, increased slightly over the range 0.1 to 1 Hz and then remained constant up to 6 Hz. Cocaine (10 μM) did not change this pattern of overflow and produced a statistically significant increase in fractional evoked overflow per pulse in brain slice at all frequencies except 6 Hz. In vas deferens, fractional evoked overflow per pulse was not changed in a statistically significant manner by cocaine (10 μM) at any frequency tested (Figure 7).

Effect of cocaine on the overflow of [^{3}H]-noradrenaline and ^{3}H-metabolites

The chromatographic separation yielded five fractions: MOPEG + VMA (I; 3-methoxy-4-hydroxyphenylglycol + 3-methoxy-4-hydroxymandelic acid), NMN (II; normetanephrine), DOPEG (III; 3-4,dihydroxyphenylglycol), unchanged noradrenaline (IV) and DOMA (V; 3,4-dihydroxymandelic acid). Application of the procedure to pure [^{3}H]-noradrenaline yielded 82 ± 5% recovered in fraction IV, 4.0 ± 0.5% in fraction I, 2.0 ± 0.1% in fraction V, 1.0 ± 0.1% in fraction III and less than 1% in fraction II (giving an overall recovery of radioactivity of about 90%). The percentage of the total

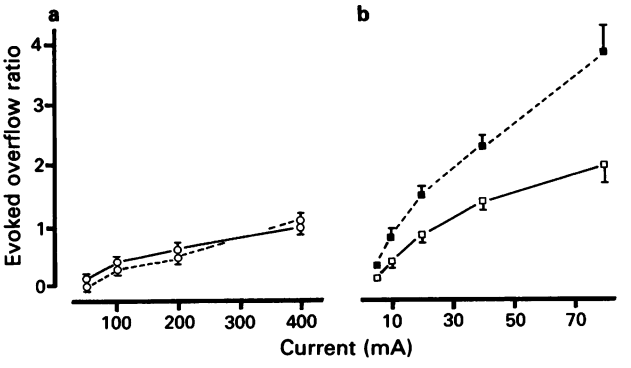

Figure 6 Ratio of the fractional evoked overflow of tritium in an initial period of stimulation (2.5 Hz, 2 ms pulse width, 400 mA for 90 s in (a) vas deferens; 3 Hz, 2 ms pulse width, 20 mA for 2 min in (b) brain slice) to that in a later period with a different stimulus current (mA). The points represent mean values and the bars show the standard error where these fall outside the area of the symbol ($n = 5$ to 6). Solid symbols show those values obtained in the presence of cocaine (10 μM; broken line) which are statistically significantly different ($P < 0.05$; Mann-Whitney rank test) from corresponding points in the absence of cocaine.

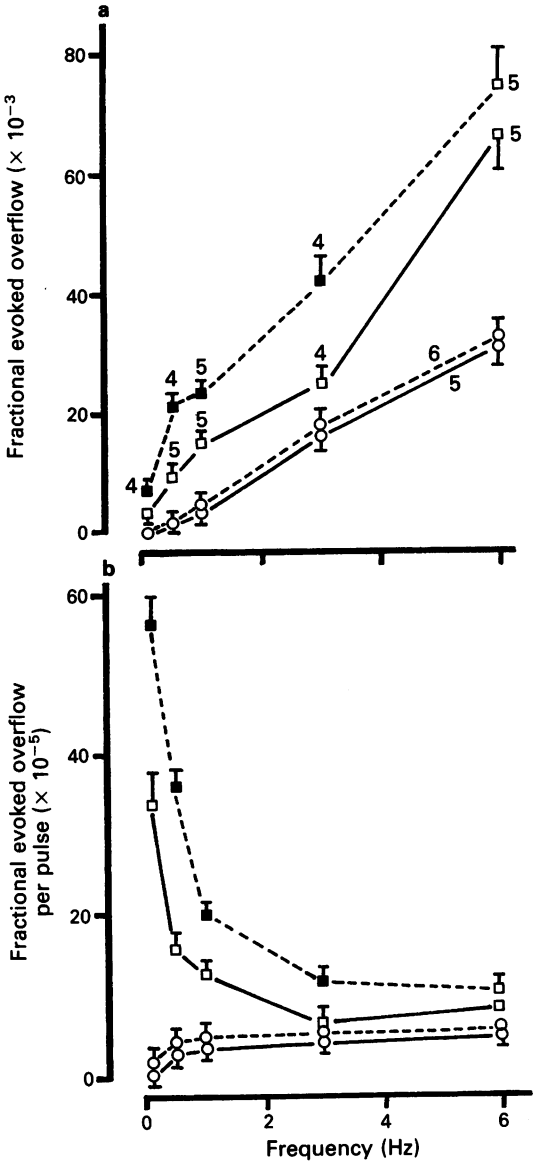

Figure 7 Effect of stimulation frequency (0.1, 0.5, 1, 3 and 6 Hz) on fractional evoked overflow of tritium (a) and fractional evoked overflow of tritium per pulse (b) in mouse vas deferens (circles) and rat brain cortex slices (squares) in the absence (continuous line) or the presence of cocaine (10 μM; broken line). The experimental conditions for brain slices and for vas deferens are specified in Figures 3 and 4 respectively. The points represent mean values and the bars show the standard error. The number of experiments contributing to each point is shown adjacent to the error bar for an individual point or adjacent to the relational line for a set of experiments as a whole. Solid symbols show those values statistically significantly different (paired t test; $P < 0.05$) from corresponding points in the absence of cocaine.

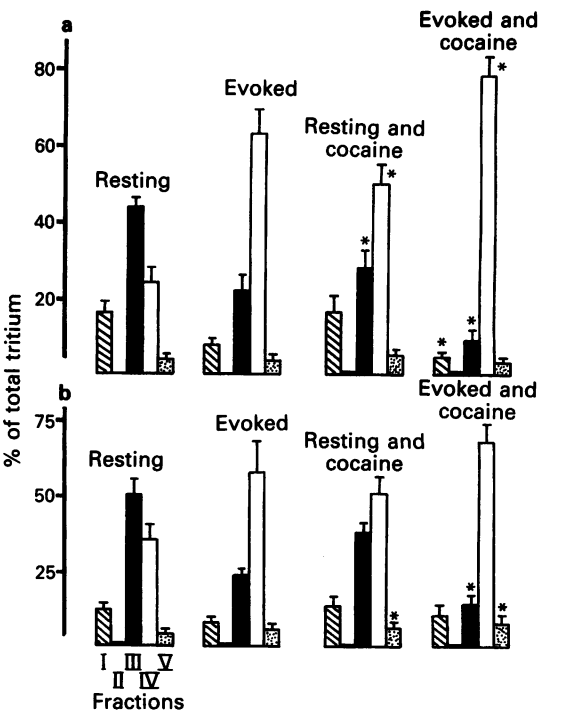

Figure 8 Effect of cocaine (10 μM) on the proportions of [^{3}H]-noradrenaline and its ^{3}H-metabolites found in the resting and evoked overflow of tritium from brain slice (a) or vas deferens (b) previously incubated with [^{3}H]-noradrenaline. The chromatographic separation yielded five fractions: MOPEG + VMA (I; 3-methoxy-4-hydroxyphenylglycol + 3-methoxy-4-hydroxymandelic acid), NMN (II; normetanephrine), DOPEG (III; 3-4,dihydroxyphenylglycol), unchanged noradrenaline (IV) and DOMA (V; 3,4-dihydroxymandelic acid). The amount of tritium found in each fraction has been expressed as a percentage of the total tritium overflowing from the tissue during the collection periods. The height of the columns represent the mean values from 4 experiments and the bars the standard errors. The asterisks indicate statistically significant differences ($P < 0.05$; paired t test) from corresponding values obtained in the absence of cocaine.

tritium in the resting and the evoked overflow from vas deferens and from brain slice in the absence or the presence of cocaine which was found in the various fractions is shown in Figure 8. None of the data has been corrected for the above recoveries and all values refer only to the tritiated compounds. In general the pattern in the two tissues is very similar. The resting overflow from both brain slice and vas deferens comprised mainly DOPEG (III; $\approx$45%) together with a smaller amount of noradrenaline (IV; $\approx$30%). Cocaine (10 μM) increased the proportion of noradrenaline and decreased the proportion of DOPEG overflowing from both tissues in a statistically significant manner ($P < 0.05$; paired t test). Evoked overflow consisted mainly of unchanged noradrenaline (IV; $\approx$60%) with a smaller proportion of DOPEG (III; $\approx$20%). Cocaine decreased the proportion of DOPEG (III; $\approx$10%) in the evoked overflow from both tissues and increased the proportion of noradrenaline to about 70–80%. As can be seen in Figure 8 small changes, some of which achieved statistical significance, were observed in other fractions, but the proportion of these materials present are so small as to make the changes of doubtful relevance.

In control tissues the amount of [^{3}H]-noradrenaline overflow (in d.p.m.) evoked by the initial period of stimulation was greater than that evoked by the succeeding period ($P < 0.05$; paired t test) as reflected by the ratios shown in Figure 9 which are less than unity for both vas deferens and brain slice. When cocaine (10 μM) was present during the later period the amount of [^{3}H]-noradrenaline overflowing in response to stimulation nearly doubled in brain slice and the S2/S1 ratio showed a statistically significant difference in comparison to that found in control tissues ($P < 0.05$; Mann-Whitney rank test). No such difference was observed in vas deferens ($P > 0.05$; Mann-Whitney rank test) (Figure 9).

Effect of cocaine on the overflow of unlabelled noradrenaline

Noradrenaline was not consistently measurable in the pooled samples containing the resting overflow from either brain slices or from vas deferens. In the first set of pooled samples containing the evoked overflow, noradrenaline was easily quantitated and averaged 0.34 $\pm$ 0.03 ng and 0.20 $\pm$ 0.03 ng in vas deferens ($n = 4$) and brain slice ($n = 6$) respectively. For

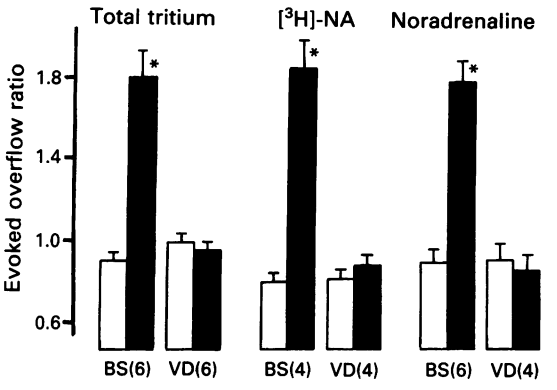

Figure 9 Ratio of the overflow evoked in a first (set of) stimulation period(s) when cocaine was absent, to that in a second (set) performed in the absence (open columns) or presence (filled columns) of cocaine (10 μM). In both vas deferens (VD) and in brain slice (BS) evoked overflow was measured by h.p.l.c. in terms of unlabelled noradrenaline or (in tissues previously incubated with [^{3}H]-noradrenaline) in terms of total tritium overflowing or of [^{3}H]-noradrenaline overflowing ([^{3}H]-NA). The columns represent the mean values and the bars show the standard errors. The number of experiments contributing to each mean value is shown below each pair of columns (n). * Statistically significant difference ($P < 0.05$; Mann-Whitney rank test) between control and cocaine-treated tissues.

each pair of pooled samples a ratio was calculated between the evoked overflow in the first set and that in the second set of pooled samples. In vas deferens the ratio obtained when cocaine was present during the second set of stimulations was not statistically significantly different from that obtained when cocaine was absent ($P > 0.05$; Mann-Whitney rank test). In brain slice the evoked overflow of noradrenaline from the tissues was nearly doubled in the presence of cocaine yielding a ratio which was statistically significantly different from that obtained when cocaine was absent during the second set of stimulations ($P < 0.05$; Mann-Whitney rank test) (Figure 9).

Discussion

Fractional resting and evoked tritium overflows from vas deferens and brain slice were comparable with published values (Marshall, 1983; Pelayo *et al.*, 1980). In brain slice a small fall in fractional evoked overflow was detectable in the third stimulation period and a marked fall in period 4; experiments were therefore limited to two periods of stimulation. In vas deferens the first period of stimulation evoked an overflow which was often unrepresentative of those occurring later but stimulation periods 2 to 7 evoked reproducible overflows giving Sx/S2 ratios of approximately unity.

In brain slice but not in vas deferens cocaine increased fractional evoked overflow probably by blocking uptake$_1$ since desmethylimipramine produced the same effect at about 10 times lower concentration. Desmethylimipramine has a very different spectrum of activity from cocaine but shares an ability to block uptake$_1$ and is 10 to 30 times more potent (Iversen, 1965; Ross & Renyi, 1967).

The differential effect of cocaine is unlikely to have been caused by the different experimental conditions used for the tissues since in vas deferens cocaine did not increase fractional evoked overflow when the experiments were conducted in the modified Krebs bicarbonate saline or the apparatus normally used for brain slices. Although the stimulation current was higher the differential effect of cocaine was not changed qualitatively when the current was varied over a wide range.

Increasing frequency of stimulation increased fractional evoked overflow since stimulation was for a fixed period and thus involved a greater number of pulses at higher frequencies. In vas deferens, at low frequencies, in agreement with others, fractional overflow per pulse was found to change little with increasing frequency (Henderson & Hughes, 1974; Marshall, 1983). In brain slice a decrease in fractional evoked overflow with increasing frequency was observed in agreement with Montel *et al.* (1974). The reason for this difference between the tissues is not clear but at each frequency tested cocaine was without effect in vas deferens and increased fractional evoked overflow from brain slice (although the 6 Hz point did not achieve statistical significance). The small difference in the frequency of the stimulation used in the two tissues cannot therefore account for the differential effect of cocaine; neither can it be due to different amounts of noradrenaline being released which will vary considerably with the different frequencies. The observation does suggest there is a qualitative difference between the tissues at the level of the nerve endings.

Total tritium overflow was usually measured which might not reflect changes in noradrenaline overflow (Marshall *et al.*, 1978). If tritiated noradrenaline forms only a small proportion of the total tritium released from one tissue then blockade of re-uptake may not give a detectable rise in total tritium overflow. Fractionation of the tritium overflow suggests this is not the case since similar proportions of noradrenaline and its major metabolite DOPEG were found in the effluent from both tissues. Cocaine decreased the proportion of the overflowing tritium present as DOPEG and that present as noradrenaline increased indicating that cocaine is blocking the re-uptake of released noradrenaline. The differential effect of cocaine was still apparent whether total tritium, [^{3}H]-noradrenaline or unlabelled noradrenaline was measured.

One explanation for the differential effect of cocaine is that in vas deferens α_2-adrenoceptors alter release to compensate for the change in synaptic noradrenaline concentration resulting from uptake blockade. However, functionally similar presynaptic α_2-adrenoceptors are present in both tissues since yohimbine and idazoxan each increased evoked overflow to a similar extent. When activation of presynaptic α_2-adrenoceptors is blocked, cocaine produced a rise in fractional evoked overflow of tritium in both tissues suggesting that the differential effect of cocaine is due to some difference in the functioning of the presynaptic α_2-adrenoceptors. If, in the two tissues, there was a difference in the number of presynaptic α_2-adrenoceptors and a difference in the receptor reserve then an increase in synaptic noradrenaline levels might produce a large alteration in feedback control in one tissue (well able to compensate for the increased synaptic concentration) but little compensatory change in the other. If this were so it would not be expected that presynaptic receptor blockade would produce similar effects over a similar concentration range in both tissues.

One possible difference could be in the physical arrangement of the re-uptake sites, the release sites and the presynaptic α_2-adrenoceptors. If the re-uptake sites were located between the release sites and the presynaptic α_2-adrenoceptors (i.e. within the negative feedback loop) then blockade of re-uptake would result in a higher concentration of noradrenaline reaching the presynaptic α_2-adrenoceptors which would reduce noradrenaline release. Overflow would therefore be little altered. Alternatively if the re-uptake sites are not between the release points and the presynaptic α_2-adrenoceptors (i.e. are outside the negative feedback loop) this would prevent compensation taking place when uptake was blocked and overflow would increase. This would also explain why uptake blockade increases overflow in both tissues when the presynaptic α_2-adrenoceptors are blocked. This is not an appealing explanation since it suggests a rigidity of organisation in the membrane of the varicosity which seems unlikely.

In rabbit ear artery cocaine increased overflow evoked by propagated nerve impulses but not that evoked by field stimulation (Rand *et al.*, 1988). Transmural stimulation was used throughout the work described here but differences in tissue geometry may result in invasion of more varicosities by conducted impulses in brain slice than in vas deferens. Alterna-

tively, the order in which the varicosities are activated along the nerve may be important.

Schomig *et al.* (1989) have shown that the outward transport of noradrenaline was three times greater in rat atria than in vas deferens. We have observed previously that atria, like brain slice, give increased evoked overflow when exposed to cocaine (El-Mas & Hughes, 1990). It is not clear however if these two sets of observations have a common cause or what that cause might be.

We would like to express our thanks to the Egyptian Government for financial support to M. El-M.

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(Received May 4, 1990
Accepted June 27, 1990)

British Journal of Pharmacology

VOLUME 101 (3) NOVEMBER 1990

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Papers will be considered for publication on all aspects of pharmacology, including chemotherapy.

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The *British Journal of Pharmacology* is published monthly by the Scientific & Medical Division, Macmillan Press Ltd.

The journal is covered by *Current Contents*, *Excerpta Medica* and *Index Medicus*.

All business correspondence and reprint requests should be addressed to the Scientific & Medical Division, Macmillan Press Ltd., Houndmills, Basingstoke, Hampshire RG21 2XS, UK. Telephone: (0256) 29242; Fax: (0256) 842754.

Annual subscription prices for 1991 EEC £375, elsewhere £415/US$750 (sterling rate is definitive). Orders must be accompanied by remittance. Cheques should be made payable to Macmillan Press, and sent to: Macmillan Press Ltd., Subscription Department, Brunel Road, Houndmills, Basingstoke, Hampshire RG21 2XS, UK.

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ISSN 0007–1188

0007–1188/90 $3.50 + $0.00

IMPORTANT ANNOUNCEMENT

EDITORIAL OFFICE
BRITISH JOURNAL OF PHARMACOLOGY

CHANGE OF ADDRESS

Dr G. M. Lees' term of office as Secretary to the Editorial Board of the *British Journal of Pharmacology* will end later this year. Dr Lees is to be succeeded by Dr R. W. Horton and Dr W. A. Large, as Joint Secretaries to the Editorial Board. Consequently, the Editorial Office is to be moved from Aberdeen to London. The transfer will occur in stages.

From October 15, 1990 ***only new manuscripts*** should be sent to:

The Editorial Office
British Journal of Pharmacology
St. George's Hospital Medical School
Cranmer Terrace
London SW17 0RE
Tel: 081 767 6765; Fax: 081 767 5645

All other manuscripts, correspondence and enquiries will be dealt with by the Editorial Office in Aberdeen until October 26, when the Office will close and the records then be transferred to London.

From November 1, 1990 *all manuscripts, correspondence and enquiries* should be directed to Dr Horton or Dr Large at the new Office.

A. T. Birmingham
Chairman, Editorial Board

SPECIAL REPORTS

The purpose of *Special Reports*, which are superseding 'Short Communications', is to provide rapid publication for **new** and **important** results which the Editorial Board considers are likely to be of special pharmacological significance. (Please note that Short Communications are no longer acceptable for publication.) *Special Reports* will have publication priority over all other material and so authors are asked to consider carefully the status of their work before submission.

In order to speed publication there is normally no revision allowed beyond very minor typographical or grammatical corrections. If significant revision is required, the Board may either invite rapid resubmission or, more probably, propose that it be re-written as a Full Paper and be re-submitted for consideration. In order to reduce delays, proofs of *Special Reports* will be sent to authors but **essential corrections must reach the Press Editor within 48 hours of receipt.** Authors should ensure that their submitted material conforms exactly to the following requirements.

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BRITISH JOURNAL OF PHARMACOLOGY VOLUME 101 (3) 1990

Filmsetting by Santype International Limited, Salisbury, Wiltshire
and printed in Great Britain by Unwin Brothers Ltd., The Gresham Press, Old Woking, Surrey