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Arachidonic acid-induced bronchomotor responses are partially mediated by release of sensory neuropeptides from capsaicin-sensitive structures

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Administration of arachidonic acid (AA) both *in vitro* and *in vivo* elicited prominent contractile responses in guinea-pig airways, which were markedly reduced after capsaicin desensitization. Furthermore, AA superfusion elicited a significant calcitonin gene-related peptide-like immunoreactivity release from isolated bronchi. It is suggested that at least part of the bronchomotor actions of AA rely upon stimulation of capsaicin-sensitive primary afferents.

Introduction Recently, evidence has been presented suggesting that sensory neuropeptides released from capsaicin-sensitive nerves might play a crucial role in the pathophysiology of asthma (Barnes, 1986). In some tissues prostanoids have been shown to stimulate capsaicin-sensitive-structures (Yanagisawa *et al.*, 1986; Longhurst & Dittman, 1987; Manzini *et al.*, 1989) and Stewart *et al.* (1984) showed that leukotriene D_4 enhances histamine-induced bronchoconstriction through a mechanism involving capsaicin-sensitive afferent fibres. Eicosanoid (mainly leukotrienes)-mediated bronchomotor responses can be elicited by exogenous administration of arachidonic acid (AA) (Burka, 1985). In view of the above, we decided to investigate the effect of capsaicin desensitization on AA-induced bronchomotor responses produced both *in vivo* and *in vitro*. In addition, the ability of AA to evoke a release of sensory neuropeptides from guinea-pig isolated bronchi was also studied.

Methods Male albino guinea-pigs weighing 300–350 g were used throughout the study.

In vivo *experiments:* animals were anaesthetized with urethane (1.3 g kg^{-1}, i.p.) treated with (+)-tubocurarine (3 mg kg^{-1}, i.v.) and mechanically ventilated (60 strokes min^{-1}) through a tracheal cannula. Insufflation pressure was measured by means of a pressure transducer (Bentley Trantec) and monitored on a Basile Unirecord 7050. A polyethylene catheter was inserted in the left jugular vein for drug injection. Drugs were administered in a volume less than 0.5 ml kg^{-1}. Capsaicin pretreatment was carried out by injecting animals with a total dose of 55 mg kg^{-1} s.c. over two days, five days before the experiments.

In vitro *experiments:* main bronchi were dissected free from adhering tissues, and rings (2 mm wide) were mounted on two 'L'-shaped stainless steel holders and placed in a 5 ml organ bath containing physiological salt solution (at 37°C and oxygenated with 95% O_2 and 5% CO_2) of the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2.2H_2O$ 2.5, $MgCl_2.6H_2O$ 0.5, $NaH_2PO_4H_2O$ 1.0, $NaHCO_3$ 25, glucose 10. Contractile responses were recorded by means of an isometric force transducer and displayed on a Basile Unirecord 7050 polygraph. An optimal resting tension of 750 mg was applied to the preparations. Each preparation was allowed to equilibrate for at least 45 min before starting experiments. Indomethacin (10 μM) was added to the organ bath at least 45 min before the administration of AA.

Release experiments: epithelium-denuded main bronchi taken from 3–4 animals were pooled in a 2 ml organ bath (37°C) and superfused at a flow rate of 0.7 ml min^{-1} with physiological salt solution containing bovine serum albumin (1 g litre^{-1}). Epithelium was removed by means of a cotton-tipped applicator and effectiveness of this procedure was checked with histological methods and Evans blue staining. After a 30 min equilibration period, preparations were exposed to AA (0.1 mM) for 15 min. Superfusates were collected every 3 min in tubes containing enough acetic acid to give a final concentration of 2N. Calcitonin gene-related peptide-like immunoreactivity (CGRP-LI) was assayed as previously described (Manzini *et al.*, 1989). Experimental samples or samples containing rat-CGRP standard were incubated for 48 h at 4°C with an

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anti-human-CGRP rabbit antiserum (Peninsula, CA, U.S.A.) that demonstrates 100% cross-reactivity towards both α and β rat-CGRP. Then [^{125}I]-human-CGRP (Amersham, U.K.), was added and incubated for a further 48 h at 4°C. Separation of bound from free antigen was by double antibody precipitation. The lower detection limit was 2.5 fmol per tube.

All data are mean ± s.e. Statistical analyses were performed with Student's t test for paired or unpaired data as appropriate. The drugs used (with their sources) were as follows: arachidonic acid (Sigma), capsaicin (Serva), indomethacin (Serva), neurokinin A (Peninsula).

Results In anaesthetized guinea-pigs, intravenously administered AA (3 μmol kg^{-1}) produced increases in insufflation pressure, which rapidly (5–15 s) reached a maximum and then declined to basal value within 5–10 min. These increases in tracheal insufflation pressure induced by AA averaged 3.55 ± 0.30 kPa (n = 10) while administration of capsaicin (0.03 μmol kg^{-1}, i.v.) and neurokinin A (3 nmol kg^{-1}, i.v.) elicited similar bronchoconstrictor responses which averaged 2.69 ± 0.53 kPa (n=8) and 1.78 ± 0.30 kPa (n=8), respectively. In systemically (55 mg kg^{-1}, s.c.; five days before) capsaicin-pretreated guinea-pigs, bronchomotor responses to capsaicin were no longer observed (n=9), while responses to neurokinin A were unaffected (1.84 ± 0.25 kPa, n=9, NS). In such guinea-pigs, responses to AA were significantly reduced by 46.2% (being 1.91 ± 0.12 kPa, n=14, P < 0.0001 as compared to controls).

In isolated bronchi, AA (1 mM) produced at most, weak bronchomotor effects (n=8). However, in the presence of the cyclo-oxygenase inhibitor indomethacin (10 μM), AA consistently elicited a sustained contraction the amplitude of which, at steady state, was 316 ± 26 mg (n=18). Reproducible contractile responses to AA could be evoked at 90 min intervals (n=6). Capsaicin (10 μM) administration elicited a strong contraction which averaged 536 ± 46 mg (n=6) and could be evoked only once in each preparation, suggesting desensitization. After capsaicin exposure, AA-induced contractions were significantly (P < 0.001) reduced by 65.9% (108 ± 34 mg, n=6). In the same preparations, capsaicin exposure did not affect the amplitude of carbachol (3 μM)-induced contractions (n=6).

As shown in Figure 1, superfusion of isolated bronchi with AA (0.1 mM) produced an increase in the outflow of CGRP-LI. This effect was slowly-developing and became significant after 5–6 min from the beginning of AA administration (n=7). The total evoked release (TER) in 15 min was

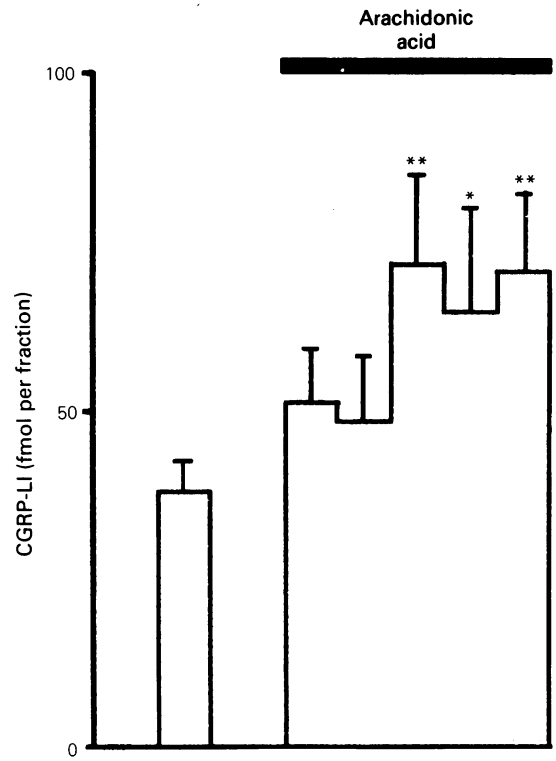

Figure 1 Calcitonin gene-related peptide-like immunoreactivity (CGRP-LI) outflow measured in the superfusates from guinea-pig isolated bronchi before and during (bar) exposure to arachidonic acid (0.1 mM). Each column represents a collecting period of 3 min and is the mean of 7 experiments.
*P < 0.05; **P < 0.01 as compared to basal values.

103.2 ± 36.7 fmol g^{-1} wet weight (n=7). When 30 min later exposure to AA was repeated, a similar CGRP-LI release could be detected, TER being 167 ± 78.4 fmol g^{-1} wet weight (n=7, NS). In other experiments, isolated bronchi were challenged with capsaicin 1 μM, which evoked a huge CGRP-LI release, the TER averaging 11.5 ± 3.2 pmol g^{-1}wet weight (n=6); a subsequent exposure to AA or capsaicin was no longer able to induce a detectable neuropeptide release.

Discussion Capsaicin induces bronchomotor responses through a local release of neuropeptides from primary afferent nerve endings (Lundberg & Saria, 1982). The stimulant action of capsaicin on these nerve endings is followed by a rapid desensitization, such that they become refractory in further activation by either capsaicin itself or other stimulatory agents (Maggi & Meli, 1988). Since, following capsaicin exposure, AA-induced contractions of iso-

lated bronchi were markedly reduced, while those to carbachol were unaffected, it is possible that at least part of the bronchomotor response to AA might be mediated indirectly via activation of capsaicin-sensitive structures. This proposal is supported by data obtained *in vivo* which show that systemic capsaicin pretreatment, with a procedure previously shown to deplete airway tissues of sensory neuropeptides (Manzini *et al.*, 1987), markedly reduced the increase in insufflation pressure elicited by i.v. administration of AA. This hypothesis is further confirmed by the release experiments which indicate that AA elicits a significant increase in the outflow of CGRP-LI, CGRP being a neuropeptide contained in capsaicin-sensitive sensory nerves (Martling, 1987) and which has been described as possessing potent bronchoconstrictor effects (Palmer *et al.*, 1987; Hamel & Ford-Hutchinson, 1988).

In the presence of AA, epithelial, smooth muscle and inflammatory cells (Hunter *et al.*, 1985) all generate substantial amounts of eicosanoids with bronchodilator (prostaglandins) and bronchoconstrictor (leukotrienes and thromboxanes) properties. In indomethacin-pretreated preparations, AA consistently elicits contractions, largely mediated by leukotriene C_4 and D_4 (Burka, 1985).

Although further studies are necessary to identify the eicosanoid(s) involved, our data suggest that products of the metabolic breakdown of AA through the lipoxygenase pathway might act on capsaicin-sensitive sensory nerves leading to a local release of neuropeptides. Interactions between sensory neuropeptides and leukotrienes might therefore create a vicious circle, of relevance in the genesis of airways hyperreactivity.

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The effects of L-arginine and N^G-monomethyl L-arginine on the response of the rat anococcygeus muscle to NANC nerve stimulation

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The effect of the competitive inhibitor of L-arginine, N^G-monomethyl L-arginine (L-NMMA) on the response of the rat anococcygeus muscle to non-adrenergic, non-cholinergic (NANC) inhibitory nerve stimulation has been examined. L-NMMA causes a rise in muscle tone and inhibition of the response to nerve stimulation. The stereoisomer D-NMMA is without effect. The rise in tone and inhibition of the nerve response is reversed by L-arginine. Another analogue, L-canavanine, which is effective against L-arginine utilization in the macrophage, was without effect on the rat anococcygeus. These results provide indirect evidence for nitric oxide (NO) or a substance releasing NO as the transmitter of the NANC nerves in this tissue.

Introduction The mode of action of endothelium-derived relaxing factor (EDRF) on vascular smooth muscle and the non-adrenergic, non-cholinergic (NANC) nerves in the bovine retractor penis (BRP) and rat anococcygeus muscles are similar in so far as both act by stimulating guanylate cyclase and both are blocked by haemoglobin (Bowman *et al.*, 1982; Rapoport & Murad, 1983; Bowman & Drummond, 1984; Martin *et al.*, 1985). There is good evidence that EDRF is NO and that the precursor from which it is synthesized is L-arginine (Palmer *et al.*, 1987; 1988). Part of the evidence for the involvement of L-arginine in the synthesis of EDRF is its ability to increase the release of NO from endothelial cells cultivated in its absence and the ability of the analogue, N^G-monomethyl L-arginine (L-NMMA), to inhibit release and also to inhibit the relaxant response to agents which release EDRF. We have recently reported that L-arginine failed to potentiate and L-NMMA failed to inhibit the response to NANC nerve stimulation in the BRP in concentrations up to 3×10^{-4} M (Gillespie & Xiaorong, 1989). In that abstract we drew attention to more recent experiments in the rat anoccygeus which gave quite different results. This paper describes these later results.

Methods Experiments were performed on isolated anococcygeus muscles (Gillespie, 1972) from male rats suspended in Ag/Ag Cl ring electrodes in 10 ml organ baths and bathed in Krebs saline solution containing (mM): Na^+ 145, K^+ 5.9, Ca^{2+} 2.5, Mg^{2+} 1.2, Cl^- 127, HCO_3^- 25, $H_2PO_4^-$ 1.2, dextrose 11, at 36°C and oxygenated with 95% O_2 plus 5% CO_2. Adrenergic motor nerves were blocked and tone raised by guanethidine sulphate (Ciba) 10^{-5} M added to the bath. Intramural nerves were stimulated by a fixed number of 20 pulses of supramaximal voltage and 0.5 ms duration at frequencies ranging from 0.1 to 10 Hz using a Grass S88 stimulator. The relaxant responses were recorded on a Grass (model 7) polygraph and expressed as a percentage reduction in the degree of tone existing at the moment of nerve stimulation. L- and D-NMMA were generously provided by Dr R.M.J. Palmer, Wellcome Research Laboratories. Solutions of NO (British Oxygen Corporation) were prepared as described by Palmer *et al.* (1987).

Results L-Arginine alone in concentrations up to 3×10^{-4} M had no effect on the response to NANC nerve stimulation. L-NMMA had two effects illustrated in Figure 1. First there was a further rise in tone almost immediately after adding the drug; secondly, there was a dose-related inhibition of the response to NANC nerve stimulation. Both effects occurred over the dose range 10^{-5} M to 3×10^{-4} M. Both effects were reversed by washing the drug out of the bath. The stereoisomer, D-NMMA, in concentrations up to 3×10^{-4} M was without effect on either tone or the response to nerve stimulation. L-Arginine, though itself unable to potentiate the response to nerve stimulation, was able to reverse the effects of L-NMMA, lowering tone and restoring the response to nerve stimulation (Figure 1). The simultaneous addition of L-arginine and L-NMMA prevented the effects of the latter on tone or the nerve response. These effects of L-NMMA are presumably prejunctional since, as Figure 1 illustrates, concentrations of the drug which inhibited the nerve response had no effect on the response to exogenously added NO. Another L-arginine analogue reported to inhibit nitrite formation by macrophages

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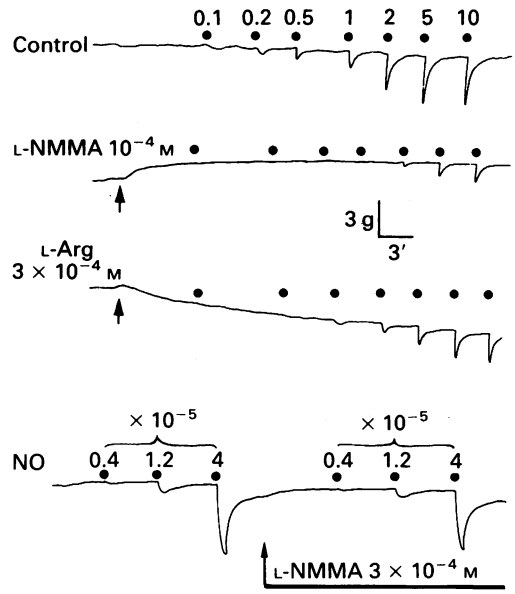

Figure 1 The effect of N^G-monomethyl L-arginine (L-NMMA) and L-arginine on the response of the rat isolated anococcygeus muscle to field stimulation of its NANC inhibitory nerves. The nerves were stimulated by 20 pulses of supramaximal voltage and 0.5 ms duration at the frequencies shown above each response. Guanethidine 10^{-5} M was present throughout to block the motor adrenergic nerves and raise tone. The top record is the control, the second, in the same tissue, shows the effect of adding L-NMMA 10^{-4} M; there was an additional rise in tone and inhibition of the response to nerve stimulation. The subsequent addition of L-arginine (L-Arg) 3×10^{-4} M with L-NMMA still present reversed both effects; tone was progressively reduced and the response to nerve stimulation partially restored. The bottom records show that L-NMMA had no inhibitory effect on the response to NO at the concentrations indicated.

(Iyengar *et al.*, 1987) is L-canavanine. This drug in concentrations up to 2×10^{-3} M had no effect on the response to NANC nerve stimulation in the rat anococcygeus muscle.

Discussion Garthwaite *et al.* (1988) were the first to describe the production of NO by neurones in the cerebellum. The present results, though indirect, suggest the NANC nerves in the rat anococcygeus may also liberate NO or a substance capable of releasing NO and that the substrate for its formation is the same as for EDRF, the guanidino-nitrogen(s) of L-arginine. The reversibility of the inhibition by L-arginine suggests that L-NMMA is acting competitively and the failure of the D-isomer indicates that the effect, as in the endothelial cell, is stereo-specific. The consistent rise in tone on adding L-NMMA suggests a constant background release of NO in concentrations capable of causing some relaxation of the tissue. Finally, the failure of L-canavanine to inhibit the nerve-evoked response suggests some difference; perhaps the enzymes for synthesis, in the endothelial cell and the NANC nerve terminal have different structural requirements from that in the macrophage.

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Electrical and mechanical responses of guinea-pig bladder muscle to nerve stimulation

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1 The electrical and mechanical responses to transmural stimulation of intrinsic nerves have been recorded from smooth muscle strips dissected from the dome of the guinea-pig bladder, by use of intracellular microelectrodes, and conventional tension recording techniques.

2 Stimulation of intrinsic nerves evoked action potentials in all cells studied. Hyperpolarization of the cells by extracellular current injection revealed subthreshold excitatory junction potentials (e.j.ps) in about a quarter of the cells studied.

3 Action potentials could still be evoked in the presence of atropine and neostigmine, but were abolished after desensitization of the cells to α,β-methylene ATP, a stable analogue of ATP.

4 In the presence of neostigmine, the evoked action potential was followed by a slow depolarization of the membrane. The mechanical response increased in amplitude and duration.

5 The contractile response to transmural nerve stimulation was reduced but not abolished in the presence of either atropine or desensitizing doses of α,β-methylene ATP. Atropine was more effective at high frequencies of stimulation ($\geqslant 30$ Hz), and α,β-methylene ATP at low frequencies ($\leqslant 15$ Hz). In combination the drugs abolished the response.

6 The results suggest that the mechanical response to excitatory nerve stimulation is biphasic. The early transient reponse is elicited by e.j.ps and evoked spikes, is resistant to atropine, but sensitive to desensitization of purinoceptors. The late response is mediated through muscarinic receptors, involves little membrane depolarization, and is unaffected by desensitization of purinoceptors. These responses are analogous to the responses seen in rabbit bladder, and in the sympathetically innervated rat tail artery and guinea-pig vas deferens.

Introduction

The atropine resistance of the contractile response to nerve stimulation of the mammalian urinary bladder was first recognized by Langley & Anderson (1885). The role of acetylcholine in excitatory transmission in the bladder was studied later in more detail by Henderson & Roepke (1934), who again noted that atropine does not completely abolish the response to nerve stimulation. Recent evidence has suggested that adenosine 5'-triphosphate (ATP) may be the neurotransmitter responsible for atropine resistant responses. ATP is released from guinea-pig bladder upon nerve stimulation, and mimics the non-cholinergic, non-adrenergic contractile response when applied locally to the muscle (Burnstock *et al.*, 1972; 1978). ATP has also been shown to be stored in guinea-pig bladder, although it is not known whether it is co-located with acetylcholine in cholinergic nerves, or is in separate purinergic nerves (Burnstock, 1983).

Creed *et al.* (1983) have recorded the electrical responses of rabbit bladder smooth muscle to single stimuli of the intrinsic nerves, and have observed an initial electrical response consisting of an excitatory junction potential (e.j.p.) giving rise to a spike, and a delayed more prolonged depolarization of 250–300 ms latency and 2 s duration. The initial response was unaffected by atropine or neostigmine, whilst the delayed response was abolished by atropine, and enhanced by neostigmine. Subsequently, Hoyle & Burnstock (1985) showed that the e.j.p. of rabbit bladder, recorded by use of sucrose-gap apparatus, was abolished after desensitization with α,β-methylene ATP. Also, Callahan & Creed (1986), using a similar technique, have recorded e.j.ps giving rise to

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action potentials in guinea-pig bladder that are insensitive to phentolamine and atropine.

These responses in mammalian bladder are reminiscent of the situation in several adrenergically innervated tissues, such as the guinea-pig vas deferens and rat tail artery. In these tissues similar biphasic responses to nerve stimulation have been recorded (Sneddon & Westfall, 1983; Sneddon & Burnstock, 1984) which can be differentially affected by α-adrenoceptor antagonists and α,β-methylene ATP, leading to the suggestion that ATP and noradrenaline acts as co-transmitters in these two systems (Sneddon & Burnstock, 1985).

The evidence suggests that in both the adrenergic and cholinergic systems discussed above, the biphasic response to nerve stimulation may be analogous. In each system there is an early tightly coupled electrical response in the form of an e.j.p. which may give rise to an action potential and can be abolished by purinoceptor desensitization, and a more latent response which is mediated by the classical neurotransmitter.

The experiments presented here were undertaken to characterize the electrical and mechanical responses of the urinary bladder of the guinea-pig to nerve stimulation and to investigate the possible contribution of a purinergic transmitter to the observed responses. Previous studies of the mechanical responses of guinea-pig bladder muscle have suggested that co-transmission in this structure may involve acetylcholine and a non-cholinergic non-adrenergic, possibly purinergic, transmitter (Mackenzie *et al.*, 1982; Theobald, 1982; Moss & Burnstock, 1985).

Methods

Male albino guinea-pigs weighing 300–600 g were stunned and bled. Strips of urinary bladder muscle were removed from the freely moveable intraperitoneal portion.

For mechanical recordings, strips measuring about $10 \times 1 \times 1$ mm were mounted in 0.2 ml organ baths containing platinum ring electrodes (Brading & Sibley, 1983). Tension was measured isometrically by use of Pioden transducers, with their output recorded on a six channel Watanabe pen recorder. Field stimulation of the intrinsic nerves was achieved with rectangular pulses from a Grass S48 stimulator (50 V, 50 μs, 1 s trains, variable frequency). After being mounted, tissues were allowed to equilibrate for one hour before exposure to a supramaximal concentration of carbachol (10 μM for 10 s). Subsequent responses were expressed as a percentage of this maximum response (mean $\pm$ s.e.mean).

Intracellular microelectrodes were pulled with resistances of 30–40 MΩ, filled with 3 M KCl. Tissue strips measuring $8 \times 5 \times 1$ mm were placed in a chamber equipped with an insulated partition for extracellular current application (Abe & Tomita, 1968), and an additional pair of silver ring electrodes round the tissue in the main bath for intrinsic nerve stimulation. The chamber was perfused with modified Krebs solution at 32–34°C, and responses were amplified and recorded on magnetic tape for subsequent reproduction on a digital oscilloscope and pen recorder.

A modified Krebs solution was used containing (mM): NaCl 120, KCl 5.9, $NaHCO_3$ 15.4, $MgCl_2$ 1.2, NaH_2PO_4 1.0, $CaCl_2$ 2.5, glucose 11, equilibrated with 97% O_2/3% CO_2, pH 7.4 at 36°C. Sucrose (15 g 100 ml^{-1}) was added to solutions used for all microelectrode experiments to dissociate action potentials from contraction and thus prevent electrode dislodgement. This treatment has little effect on the electrical properties of the membrane (Tomita, 1966; Creed, 1971).

All drugs were obtained from Sigma and dissolved in the perfusing solution. The concentrations stated are the final bath values. Student's t test was used to calculate the P values given in the paper; P values of less than 0.05 were considered to be significant.

Results

Membrane responses to electrical stimulation

The resting membrane potential of a sample of cells impaled in the course of these experiments was -38.5 ± 2.3 mV ($n = 25$). Most cells showed spontaneous spike activity. Stimulation of the intrinsic nerves was attempted with brief electrical pulses. With increasing duration (beyond 100 μs) an intensity of threshold could be found in each preparation at which a single pulse would evoke an action potential that could be blocked by tetrodotoxin (TTX) (0.2 μg ml^{-1}), and was presumably evoked by nerve stimulation. Brief trains of stimuli (2–60 Hz) were more effective at initiating action potentials, but with increasing duration and strength of stimulation TTX-resistant action potentials were evoked, presumably due to direct muscle activation.

Current pulses of 1000–1200 ms duration were applied by use of partition electrodes to hyperpolarize cells close to the insulated partition. Suprathreshold stimulation of the intrinsic nerves through the ring electrodes was applied during conditioning hyperpolarization. The evoked action potentials could be suppressed with increasing hyperpolarization. In about 1 in 4 or 5 of the cells penetrated, an excitatory junction potential (e.j.p.) could be

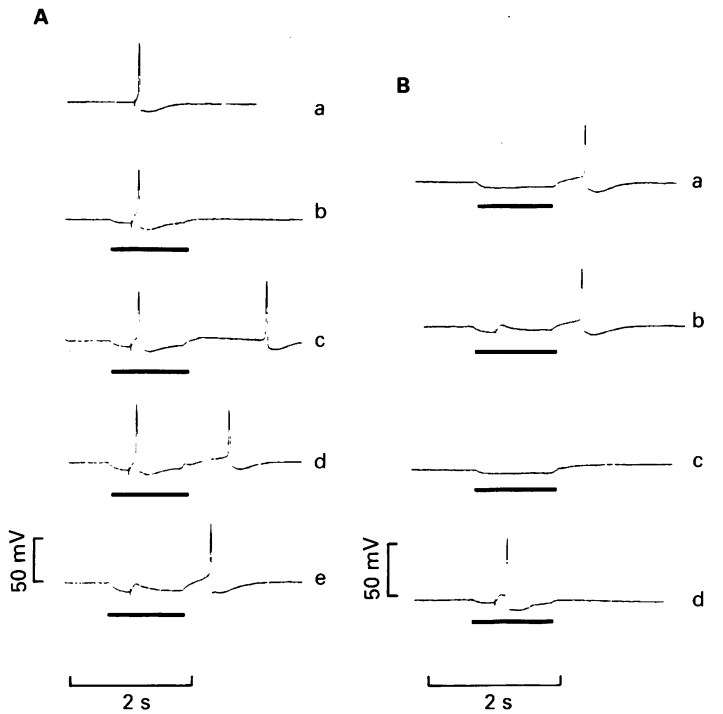

Figure 1 Microelectrode recordings of the effect of conditioning hyperpolarization on the membrane response to stimulation of intrinsic nerves with brief electrical stimuli. (A), (a) Control response with no conditioning hyperpolarization; (b–e) conditioning hyperpolarizing current of increasing strength from 0.2–0.8 mA was applied through the partition electrodes for 1.2 s beginning 300 ms before the nerves were stimulated (ring electrodes, 100 μs 100 V). In (e) the hyperpolarization is sufficient to prevent the e.j.p. reaching threshold for spike initiation. Note the appearance of anode break excitation with the larger hyperpolarizing currents. Recordings from the same cell. Resting membrane potential on initial impalement −38 mV. (B) In another cell; (a and c) conditioning hyperpolarization alone (1.2 s, 0.8 mA); (b) nerve stimulation (100 μs, 80 V) 300 ms after onset of the conditioning hyperpolarization results in an e.j.p.; (d) increasing the strength of the nerve stimulation to 100 V now results in an e.j.p. giving rise to an action potential. Resting membrane potential on impalement −38 mV.

recorded after the action potential had been suppressed. Figure 1A shows the effect of increasing the conditioning hyperpolarization on the evoked response of a single cell. In this cell a clear e.j.p. was visible after suppression of the action potential. In another cell (Figure 1B), after suppression of the action potential with conditioning hyperpolarization, increasing the strength of the nerve stimulation caused a return of the evoked action potential.

E.j.ps of a duration between 400–600 ms, and size from 8–12 mV in amplitude, could be recorded during conditioning hyperpolarization, but since there was considerable cell-to-cell variation in the absolute level of membrane potential at which the action potential was abolished, the driving force for the excitatory junctional current will be different in each case.

In a $0Ca^{2+}$ solution the e.j.ps and evoked action potentials were abolished before the spontaneous action potentials were affected (see Figure 2), consistent with the suggestion that the evoked responses are e.j.ps and not subthreshold direct membrane responses to the stimulus. Because of the relative infrequency and unpredictability of recording evoked e.j.ps, in the remainder of the experiments action potentials evoked by stimulus parameters chosen such that the responses were abolished by TTX (nerve-evoked action potentials), were recorded to assess the effects of drugs on nerve-mediated activity.

The effect of drugs on membrane activity

Atropine (1 μM), at a concentration sufficient to block the response to exogenous administered carbachol (10 μM), did not prevent nerve stimulation from evoking action potentials (see Figure 3). Application of neostigmine (1 μM) had little effect on the configu-

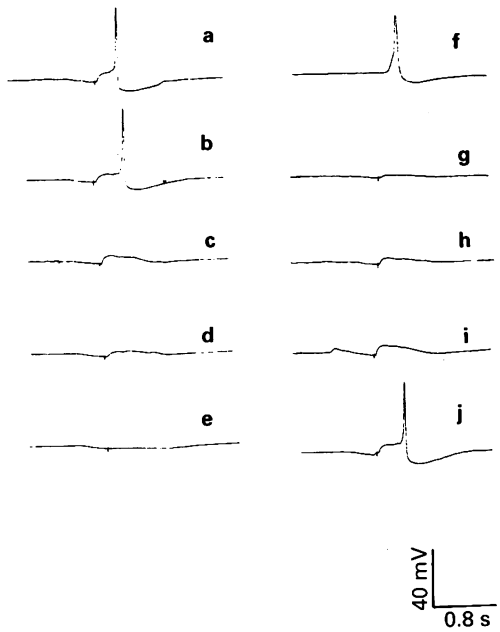

Figure 2 Effect of Ca^{2+} removal on the membrane responses to transmural nerve stimulation (100 μs, 100 V) applied 300 ms after the beginning of a conditioning hyperpolarizing current (1.2 s, 0.4 mA). Resting membrane potential on initial impalement −38 mV. (a) In normal Krebs solution an e.j.p. was elicited which gave rise to an action potential. (b) 90 s, (c) 2 min, (d) 2.5 min, (e) 3 min after changing to 0 Ca Krebs (6 mM Mg). Note progressive reduction in size of the e.j.p. and loss of the spike. (f) Spontaneous spike occurring as solution is changed to readmit normal Krebs solution. (g) 90 s, (h) 2 min, (i) 2.5 min, (j) 3 min after readmission of normal Krebs solution. Note the appearance of an e.j.p. and spike. Recordings from a single cell.

ration of the evoked spikes, although there was sometimes a small increase in spike amplitude (see Figure 3). Following exposure to 30 μM neostigmine, a slow depolarization of 5–10 mV and 15–20 s duration appeared after each action potential (see Figure 4). Superimposed on these were small spontaneous transient depolarizations.

To test the possibility that ATP might be responsible for the atropine-resistant response to nerve stimulation, tissues were desensitized to the non-hydrolyzable analogue of ATP, α,β-methylene ATP, by exposing them to progressively higher concentrations beginning with 1 μM (5 min), then 5 μM (5 min) and finally 30 μM (continuous). When the substance was first applied to the tissue, there was a transient membrane depolarization and an increased frequency of action potentials, which made it difficult to hold an impalement with microelectrode. Excitatory responses, as judged by the contractile behaviour, diminished with subsequent doses, and there was no effect on application of 30 μM. To study the effect of the drug on nerve-evoked action potentials, microelectrode recordings were made from cells before application of the drug, and further penetrations were then only attempted after desensitization was complete. Figure 5 illustrates the results of one such experiment in which tissues were stimulated by trains of very brief electrical stimuli (50 μs) at different frequencies before and after desensitization of the tissues to α,β-methylene ATP. Before treatment with the drug, action potentials were evoked with a latency that decreased with increasing stimuli to the intrinsic nerves. After treatment, actions potentials could not be evoked by any frequency, although spontaneous action potentials were still seen.

The effect of drugs on contractile activity

The effect of atropine (1 μM) on the contractile response to nerve stimulation was frequency-dependent (see Figure 6). There was little effect at low frequencies, but at higher frequencies atropine reduced the size of the contraction by a maximum of about 30%. Neostigmine enhanced the contractile responses at all frequencies of nerve stimulation as seen in Figure 7. Individual responses are illustrated in Figure 8, showing that not only is there an increase in the maximal size of the response, but it becomes greatly prolonged, and contains oscillatory components (also seen on application of acetylcholine, (Mostwin, 1985)). The fact that the effects of neostigmine are antagonized by atropine (see Figure 7 and 8), suggests that the effect is predominantly through muscarinic receptor activation, and thus postganglionic. After desensitization of the tissue to α,β-methylene ATP, the nerve-evoked contractile response was reduced at all frequencies, but the percentage reduction was greatest at low frequencies of stimulation (see Figure 6). A combination of desensitization to α,β-methylene ATP and atropine (1 μM) abolished the nerve-evoked response at all frequencies. The residual small contractile response evoked at high frequencies was also resistant to TTX (0.2 $\mu g\,ml^{-1}$), and thus presumably a response to direct muscle stimulation.

Discussion

These findings support and extend earlier observations that neuromuscular transmission in the smooth muscle of the guinea-pig bladder may involve both a muscarinic and a purinergic component.

The e.j.ps recorded are similar to e.j.ps recorded by microelectrode from other smooth muscles. In the guinea-pig vas deferens, e.j.ps of 8–10 mV amplitude

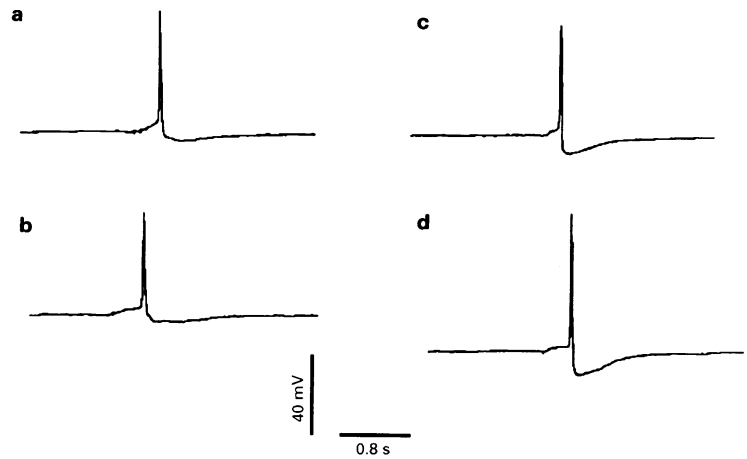

Figure 3 Effect of atropine (1 μM) and neostigmine (1 μM) on the electrical response to nerve stimulation. (a and c) Controls in normal Krebs solution; (b) 30 min after application of atropine; (d) 30 min after application of neostigmine. (a and b) From a single cell, (c and d) from another cell. There is little effect of atropine on the membrane response, but the action potential in the presence of neostigmine was increased in amplitude. Initial membrane potential of both cells −42 mV.

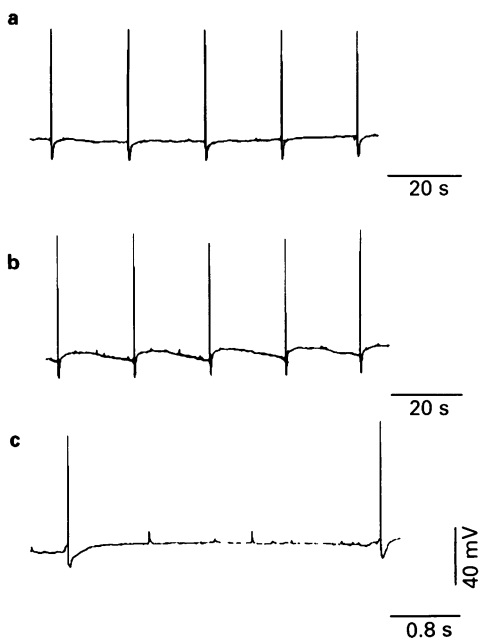

Figure 4 Effect of neostigmine on the membrane response to repetitive nerve stimulation (100 μs, 100 V every 20 s). Initial membrane potential −42 mV. The records are a continuation of Figure 3d. (a) After 40 min in neostigmine (1 μM), (b) 10 min after increasing the neostigmine concentration to 30 μM the action potential is followed by a slow depolarization of 5–10 mV, with brief spontaneous transient membrane depolarization superimposed. (c) Part of the recording in (b) with expanded time scale. All recordings from the same cell.

and 600 ms duration (Sneddon & Westfall, 1983) or 15 mV amplitude and 500–600 ms duration (Sneddon & Burnstock, 1984) have been recorded, and in the rat tail artery Sneddon & Burnstock (1985) have recorded e.j.ps of 8–10 mV amplitude. In the guinea-pig bladder, isolated e.j.ps were not found unless a positive conditioning current was used, which suggests that the e.j.ps normally cross the threshold for the initiation of action potentials. The proposal that the resting membrane potential of guinea-pig bladder is close to the threshold action potential excitation has been made previously (Brading *et al.*, 1986). The finding that discrete e.j.ps could not be recorded from every cell penetrated suggests that close connections with nerve varicosities may not occur for every smooth muscle cell. The density of innervation in bladder smooth muscle has been assessed by Gosling *et al.* (1982) who have reached a similar conclusion from electron microscopy.

The observations presented here are analogous to previous findings in rat tail artery and guinea-pig vas deferens, two tissues innervated by sympathetic adrenergic nerves, in which electrophysiological evidence for co-transmission of noradrenaline and ATP has been found. In each tissue nerve, stimulation results in an early rapid transient component which is abolished by arylazidoaminopropionyl ATP ($ANAPP_3$) or desensitization to α,β-methylene ATP, and a prolonged (30 s in rat tail artery) latent depolarization which is sensitive to phentolamine (Sneddon & Westfall, 1983; Sneddon & Burnstock, 1984; 1985). In the guinea-pig bladder, the co-transmission involves a cholinergic, parasympathetic innervation. There is no evidence for a direct sympa-

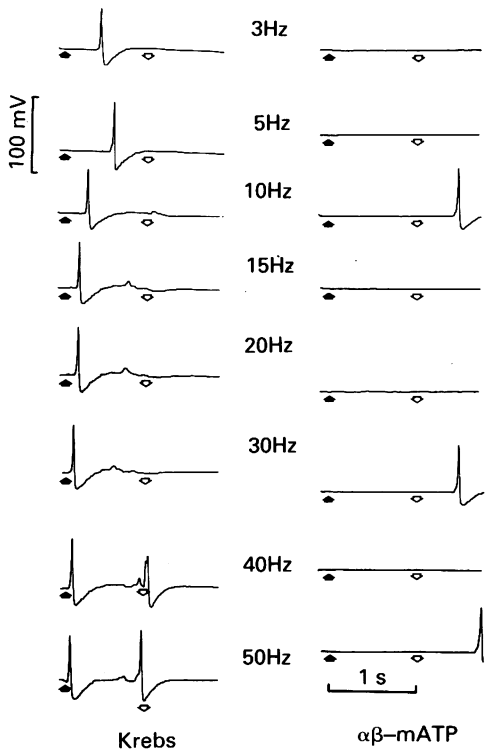

Figure 5 Effect of desensitization to α,β-methylene ATP (α,β-mATP) on the membrane response to nerve stimulation elicited by 1 s trains of increasing frequency (50 μs, 50 V, indicated by the arrows on the baseline of each tracing). Response in Krebs solution on the left, and after desensitization to α, β-methylene ATP on the right. The isolated action potentials among the tracings on the right are spontaneous. Records on the right from a single cell, and on the left from another cell in the same preparation. Initial resting membrane potential of both cells −38 mV.

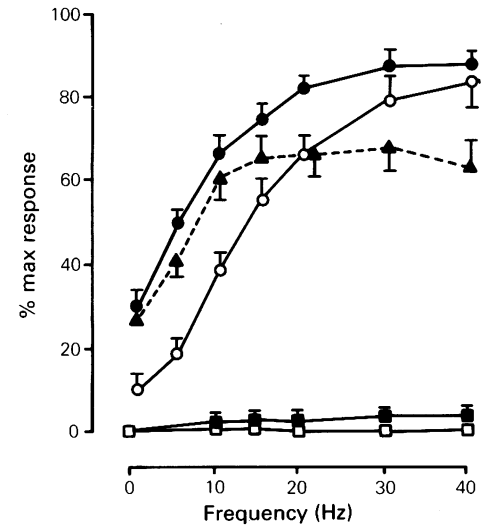

Figure 6 Effect of atropine and desensitization to α,β-methylene ATP on the contractile response of bladder strips to stimulation of the intrinsic nerves with trains of stimuli (50 μs, 50 V, 1 s train duration), stimulation frequency on abscissa scale. (●) Control responses in Krebs solution, (▲) responses in atropine (1 μM), (○) responses after desensitization of the preparation to α,β-methylene ATP (30 μM). The differences between the control responses and those in the presence of atropine are significant at 30 and 40 Hz, and between the control responses and after desensitization with α,β-methylene ATP and atropine (■). There is no significant difference between these responses and those in TTX (0.2 μg ms^{-1}) (□). Each point represents the mean (n = 5) and vertical lines show s.e.mean.

thetic innervation of the dome of the guinea-pig bladder. The smooth muscle is not responsive to α- or β-adrenoceptor agonists (Edvarsen & Setekleiv, 1968; Dumsday, 1971), and the e.j.p. and action potential recorded by the sucrose-gap method are unaffected by phentolamine (Callahan & Creed, 1986).

The effect of atropine on the contractile response seen in these experiments was greater at stimulation frequencies ⩾ 30 Hz. The effect of desensitization to α,β-methylene ATP was greater at stimulation frequencies < 15 Hz. This is in general agreement with previous studies of guinea-pig bladder (De Sy, 1971; Moss & Burnstock, 1985), rabbit and pig bladder (Sibley, 1984) in which atropine resistance has been observed at stimulation frequencies < 10 Hz. Creed et al. (1983) have also noted that the late atropine-sensitive component of the electrical response of rabbit bladder to nerve stimulation increased in amplitude and duration with higher frequencies of stimulation. Kasakov & Burnstock (1983) showed that α,β-methylene ATP abolished the contractile response at stimulation frequencies > 2–4 Hz. Moss & Burnstock (1985) demonstrated the effects of atropine and α,β-methylene ATP on the contractile responses of guinea-pig, marmoset and ferret urinary bladder: desensitization to α,β-methylene ATP produced the greatest inhibition at frequencies of < 10 Hz, and with the simultaneous addition of atropine the response to field stimulation was abolished at all frequencies between 2–40 Hz. All these results, and the present data are consistent with a model in which single nerve stimuli evoke e.j.ps through release of ATP, which initiate action potentials and contraction of the detrusor. At higher frequencies, acetylcholine release triggers contraction through a mechanism which may be less dependent on depolarization of the cell membrane. The latent muscarinic depolarization seen clearly in the rabbit

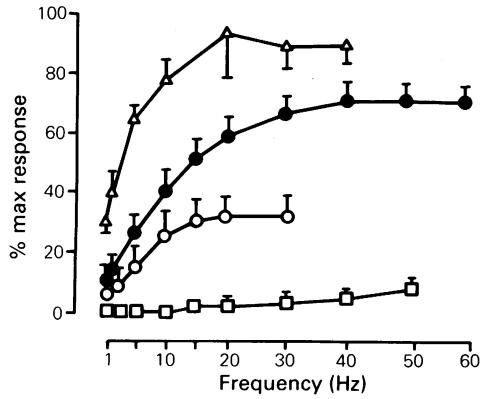

Figure 7 Effect of neostigmine in the presence and absence of atropine and α,β-methylene ATP on the contractile responses of strips of bladder to intrinsic nerve stimulation by trains of stimuli (50 μs, 50 V, 1 s train duration, stimulation frequency on abscissa scale). (●) Control responses in Krebs solution. (△) Responses in neostigmine (1 μM). (○) Responses in the presence of neostigmine and atropine. (□) Responses in the presence of neostigmine and atropine, after desensitization of the tissue to α,β-methylene ATP (30 μM).

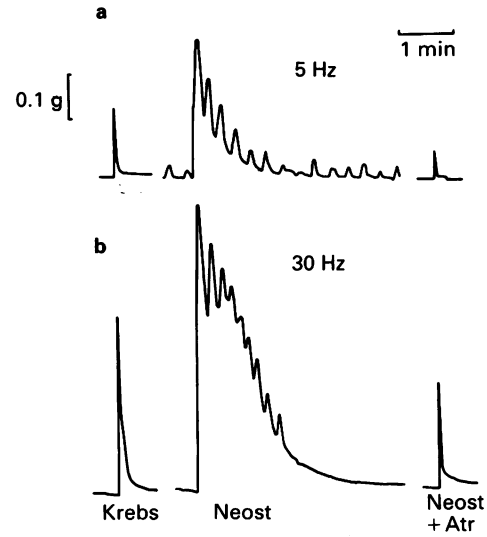

Figure 8 Effect of neostigmine on the mechanical responses of two strips of bladder to stimulation of the intrinsic nerves with 1 s trains (50 μs, 50 V) at 5 Hz (a) and 30 Hz (b). Left column shows responses after 20 min in neostigmine (Neost, 1 μM). Right column shows responses 20 min after exposure to atropine (Atr, 1 μM) in the continued presence of neostigmine.

is only seen in the presence of cholinesterase inhibitors in the guinea-pig, although there is a significant muscarinic component to contraction in their absence.

During preparation of this manuscript, further work carried out on this problem and extending the above results has been published (Fujii, 1988).

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Vasodilator actions of HA1077 *in vitro* and *in vivo* putatively mediated by the inhibition of protein kinase

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1 The *in vitro* and *in vivo* vasorelaxant effects of HA1077, 1-(5-isoquinolinesulphonyl)-homopiperazine HCl, a novel vasodilator were examined.

2 The inhibitory effects of HA1077 on contractile responses to various agonists were examined on strips of rabbit aorta. The concentration-response curves to 5-hydroxytryptamine, prostaglandin $F_{2\alpha}$, histamine, angiotensin II, noradrenaline and dopamine were concentration-dependently shifted to the right in the presence of HA1077 (0.3–3.0 μM).

3 The *in vivo* vasodilator effects of HA1077 were examined in the constant-pressure autoperfused coronary vascular bed of dogs. Intra-coronary administration of HA1077 (3–30 μg per dog) dose-dependently increased coronary blood flow (CBF), with no effect on mean blood pressure (MBP) or heart rate (HR). Intra-coronary infusion of atropine, propranolol or diphenhydramine did not modify the *in vivo* coronary vasodilator response to HA1077.

4 To determine the flow profile for HA1077 in dogs, blood flow in four vascular beds was measured, by use of noncannulating electromagnetic flow probes. HA1077 (0.01–0.3 mg kg^{-1}, i.v.) dose-dependently decreased MBP and increased vertebral blood flow (VBF), CBF, renal blood flow (RBF) and femoral blood flow (FBF).

5 A haemodynamic analysis showed that continuous i.v. infusion of HA1077 (0.01 and 0.033 mg kg^{-1} min^{-1}) dose-dependently decreased peripheral vascular resistance and increased cardiac output. There were no significant changes in right atrial pressure, dP/dt or ventricular minute work.

6 The effects of HA1077 on various enzymes considered to be related to the regulation of smooth muscle contraction were examined. HA1077 had little effect on cyclic nucleotide phosphodiesterases, yet it potently inhibited protein kinases such as cyclic nucleotide dependent protein kinases and Ca^{2+}/calmodulin dependent myosin light chain kinase.

7 The present study demonstrates that HA1077 is a novel type of arterial vasodilator.

Introduction

There is no definitive treatment for delayed cerebral vasospasm (Allen & Bahr, 1979; Gioia *et al.*, 1985) which often occurs following subarachnoid haemorrhage secondary to rupture of an intracranial aneurysm. We found that HA1077, 1-(5-isoquinolinesulphonyl)-homopiperazine HCl, and HA1004, N-(2-guanidinoethyl)-5-isoquinolinesulphonamide HCl, given intravenously, led to cerebral vasodilatation in a canine subarachnoid two-haemorrhage model (Takayasu *et al.*, 1986). Although their potential usefulness for the treatment of cerebral or coronary vasospasm in man has not been shown, the canine two-haemorrhage model used in our study mimics the vasospasm seen in patients with subarachnoid haemorrhage (Varsos *et al.*, 1983). Varsos *et al.* (1983) found no relief of cerebral vasospasm with comparable i.v. doses of nifedipine in the two-haemorrhage canine model.

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Nimodipine given intravenously produced a persistent hypotensive effect without affecting vasospasm in the two-haemorrhage dog model (Gioia *et al.*, 1985).

Because systemic i.v. application of HA1077 was more effective than HA1004 in a two-haemorrhage model (Takayasu *et al.*, 1986), the possible mechanism of action of HA1077 in the vascular-relaxation process was examined in our previous study (Asano *et al.*, 1987). HA1077 produced a competitive inhibition of Ca^{2+}-induced contractions of strips of depolarized aorta of the rabbit and significantly inhibited phenylephrine-induced contraction in Ca^{2+}-free solution. The contraction in response to adrenoceptor stimulation in Ca^{2+}-free solution is attributed almost entirely to release of intracellular Ca^{2+} (Fleckenstein, 1977; Weiss, 1981). Cultured vascular smooth muscle cells treated with HA1077 showed a dose-dependent loss or a decrease in size and length of the actin-containing microfilament structure (Sasaki *et al.*, 1987). Neither verapamil nor diltiazem in doses up to 100 μM induced reorganization of the actin microfilament structure. These results suggested that the mechanism of the vasodilator action of HA1077 differed from that of conventional calcium entry blockers and that its effect may be dependent upon an action on internal sequestering sites.

We describe here the pharmacology of HA1077 *in vitro* and its haemodynamic action, *in vivo*. Biochemical studies were also done to examine possible mechanisms of its vasodilator action.

Methods

Recording of mechanical responses

The aorta was excised from male rabbits (2–3 kg) and segments of the vessel were then trimmed of fat and connective tissue. The intimal surface of the preparation was rubbed gently to remove the endothelium and spirally cut strips 3 mm × 25 mm were prepared. The inhibitory effect of HA1077 on agonist-induced contractile response was studied in these preparations.

Each strip was mounted isometrically under 1.5 g of tension in a 20 ml tissue bath (37°C) containing normal physiological salt solution (pH 7.4) and aerated with 5% CO_2 in O_2. Force generation was monitored with an isometric transducer (Nihon Kohden, TB-611T) coupled to a polygraph recorder (Nihon Kohden, RM-6000). The normal physiological salt solution was composed of (mM): NaCl 115, KCl 4.7, $CaCl_2$ 2.5, $MgCl_2$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2 and glucose 10.0. A 1.5 h equilibration period was allowed before starting the experiments. During this period, the solutions were replaced every 30 min.

Cumulative concentration-response curves were constructed for 5-hydroxytryptamine, prostaglandin $F_{2\alpha}$, histamine, angiotensin II, noradrenaline and dopamine, as described by van Rossum (1963). Concentration-response curves for each agonist were constructed in the absence and in the presence of HA1077 (0.1–3 μM). Antagonist (HA1077) contact time was 5 min. Although the contact time was short, equilibrium was reached. The pD_2 values (i.e., the concentration of each agonist required to produce 50% of the maximal response) were calculated for each individual concentration-response curve to agonists. Details of the experimental procedures were as described by Hidaka *et al.* (1978).

In vivo *studies*

Mongrel dogs of either sex (10–15 kg) were anaesthetized with sodium pentobarbitone (35 mg kg^{-1}, i.v.). The trachea was intubated and ventilation was carried out with room air delivered from a respirator (Harvard, 607A). The ventilation rates (12–16 cycles min^{-1}) and tidal volumes (22–27 ml kg^{-1}) were adjusted to maintain the arterial blood pH, P_{CO_2} and P_{O_2}, within physiological limits. Body temperature was maintained at 37–38°C with a heating pad. Catheters were placed in the right femoral artery and vein to measure the pulsatile arterial blood pressure and i.v. injection of drug solution, respectively. Blood pressure (mmHg) was recorded from the femoral artery with a pressure transducer (Sanei, 45266). Mean blood pressure (MBP; mmHg) was calculated as diastolic blood pressure plus one third pulse pressure. Heart rate (HR; beats min^{-1}) was monitored with a cardiotachometer (Sanei, 2336A) triggered by a lead II electrocardiogram.

Arterial blood taken from the left femoral artery was led to the circumflex branch of the left coronary artery by a short extracorporeal loop. An electromagnetic flow probe of the extracorporeal type (Nihon Kohden, MFV-1200) was inserted into this circuit. Perfusion pressure was regulated by the use of pneumatic resistance. The coronary vascular bed was perfused extracorporeally under a controlled mean pressure at 100 mmHg. The drugs, in a volume of 0.03 ml, were given by a microinjector into a rubber tube connected close to the arterial cannula. Even though the drugs were given into the coronary artery and at low doses there were no alterations in systemic MBP or in HR. Heparin sodium (400 u kg^{-1}, i.v.) was administered to prevent clotting.

Mean blood flow in four vascular beds was measured with a noncannulating electromagnetic flow probe (Nihon Kohden, MFV-1100): left vertebral artery, circumflex branch of the left coronary artery, left renal artery and left femoral artery. A flow probe of adequate size was placed around each artery.

A Miller-microtransducer was introduced into the left ventricle via the left femoral artery to measure left ventricular systolic pressure, LVP (mmHg). Maximum left ventricular *dP/dt*, *dP/dt* (mmHg ms^{-1}) was determined by electronic differentiation of the pressure signal. Cardiac output, CO ($l\,min^{-1}$) was determined by the thermodilution technique using a Swan-Ganz catheter, a 5 ml iced injectate of 5% dextrose in H_2O, and a cardiac output computer (Edwards, 9520A). The Swan-Ganz catheter was introduced into a femoral vein and advanced to the pulmonary artery via the right ventricle and the right atrium. Right atrial pressure, RAP (mmHg) was measured with a pressure transducer (Sanei, 45266) via a catheter, which was introduced into a femoral vein and advanced to the right atrium. Additional values were obtained in the following manner: total peripheral resistance, TPR (mmHg min/l) = (MBP − RAP)/CO; left ventricular minute work, LVMW (Kp × m/min) = MBP × CO. TPR, CO and LVMW were expressed as 'index' (calculated for 10 kg body weight).

The vehicle for each drug was purified water or saline. The vehicle was administered to the controls and given in a volume equivalent to that used for the administered drugs. Significant changes in the cardiovascular parameters never occurred.

Enzyme assay and determinations

Myosin light chain, prepared by the method of Perrie & Perry (1970), was purified from chicken gizzard by the method of Adelstein & Klee (1981). The partially purified holoenzyme of adenosine 3′:5′-cyclic monophosphate (cyclic AMP)-dependent protein kinase I (second DE-52 step) and its purified catalytic subunits were prepared from rabbit skeletal muscle, by the method of Beavo *et al.* (1974). Cyclic GMP-dependent protein kinase from pig lung was partially purified by the method of Kuo & Greengard (1974). Actomyosin from rabbit skeletal muscle was prepared according to Ebashi & Ebashi (1964).

Cyclic AMP-dependent protein kinase activity was assayed in a reaction mixture containing, in a final volume of 0.2 ml, 50 mM Tris-HCl (pH 7.0), 10 mM magnesium acetate, 2 mM EGTA, 1 μM cyclic AMP or absence of cyclic AMP, 3.3 to 20 μM [r-^{32}P]ATP (4×10^5 c.p.m.), 0.5 μg of the enzyme, 100 μg of histone H2B and each compound.

Cyclic GMP-dependent protein kinase activity was assayed in a reaction mixture containing, in a final volume of 0.2 ml, 50 mM Tris-HCl (pH 7.0), 50 mM magnesium acetate, 2 mM EGTA, 1 μM cyclic GMP or absence of cyclic GMP, 3.3 to 20 μM [r-^{32}P]ATP (4×10^5 c.p.m.), 2.4 μg of the enzyme, 100 μg of histone H2B and each compound.

Myosin light chain kinase activity was assayed, under the conditions described (Tanaka *et al.*, 1980), in a reaction mixture containing, in a final volume of 0.2 ml, 50 mM Tris-HCl (pH 7.0), 10 mM magnesium acetate, 0.1 mM calcium chloride or 1 mM EGTA, 100 ng of calmodulin, 5 to 100 μM [r-^{32}P]ATP (4×10^5 c.p.m.), 20 μM smooth muscle 20,000-dalton myosin light chain and 0.6 μg of myosin light chain kinase.

The mixture was incubated at 30°C for 5 min. The reaction was terminated by adding 1 ml of ice-cold 20% trichloroacetic acid after adding 500 μg of bovine serum albumin as a carrier protein. The sample was centrifuged at 3000 r.p.m. for 15 min, the pellet was resuspended in ice-cold 10% trichloroacetic acid solution and the centrifugation-resuspension cycle was repeated three times. The final pellet was dissolved in 1 ml of 1 N NaOH and radioactivity was measured with a liquid scintillation counter.

Actomyosin adenosine triphosphatase (ATPase) was assayed at 25°C in a volume of 0.2 ml containing 40 mM Tris-maleate buffer (pH 7.0), 60 mM KCl, 1 mM $MgCl_2$ 100 μM $CaCl_2$ and 0.2 to 1.0 mM ATP. The reaction was initiated by adding ATP and was terminated by adding 1 ml of 20% trichloroacetic acid. Inorganic phosphate was measured according to Martin & Dotty (1949).

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

Phosphodiesterase activity was measured as described (Hidaka & Shibuya, 1974). The reaction mixture (0.5 ml) contained 50 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, [^{3}H]-cyclic AMP or [^{3}H]-cyclic GMP, various concentrations of inhibitors and an appropriate amount of enzyme. After 15 min incubation at 30°C, the reaction was terminated by boiling for 5 min; 50 μg of snake venom was added and the mixture was incubated for another 10 min. Then 3 ml of water was added and the preparation applied to a small cation-exchange resin column (AG50-x 4, 200–400 mesh, 0.7 × 1.5 cm). The product, [^{3}H]-adenosine or [^{3}H]-guanosine, was eluted with 1.5 ml of 3 N ammonium hydroxide after the column had been washed with 15 ml of deionized water. The amount of product was determined in a liquid scintillation counter (Beckman, LS-7500).

Statistics

The values are given as mean ± s.e.mean. The significance of the difference was calculated by Student's t test or paired t test. P values of 0.05 or less were considered to represent significant differences.

Drugs and chemicals

HA1077 1-(5-isoquinolinesulphonyl)-homopiperazine HCl, HA1004, N-(2-guanidinoethyl)-5-isoquinolinesulphonamide HCl and trapidil HCl were synthesized and provided by Drs T. Sone and A. Morikawa in the Life Science Laboratories, Asahi Chemical Industry, Tokyo, Japan. The drugs used were nicardipine HCl (Yamanouchi, Tokyo, Japan), 5-hydroxytryptamine creatinine sulphate, prostaglandin $F_{2\alpha}$ tris salt, histamine diphosphate, angiotensin II acetate salt, noradrenaline bitartrate salt, dopamine HCl, acetylcholine chloride, diphenhydramine HCl, propranolol HCl, atropine sulphate salt, isoprenaline HCl (Sigma, St. Louis, MO, U.S.A.) and sodium pentobarbitone (Pittman-Moore, Indianapolis, IN, U.S.A.).

The doses of the drugs used for the *in vivo* experiments refer to the weights of the salts.

Results

Inhibition of agonist-induced contraction

5-Hydroxytryptamine, prostaglandin $F_{2\alpha}$, histamine, angiotensin II, noradrenaline and dopamine caused a concentration-dependent contraction in rabbit aortic strips. The contractile responses of the aorta to all agonists tested were significantly altered, concentration-dependently, in the presence of HA1077 (Figure 1 and Table 1). HA1077 in a concentration of 0.1 μM did not shift to the right the concentration-response curve of any agonist tested. With an increase in the concentration of HA1077 (0.3–3.0 μM), the concentration-response curves for agonists tested were shifted significantly, with a concomitant increase in pD_2 values or a decrease in the maximally developed tension.

HA1077 (1 μM) depressed significantly the maxima of concentration-response curves to 5-hydroxytryptamine (76.3 ± 6.6% vs. control, $P < 0.05$), with no effect on the pD_2 values (Table 1). HA1077 (1 μM) displaced to the right the concentration-response curves for all agonists tested, except for 5-hydroxytryptamine.

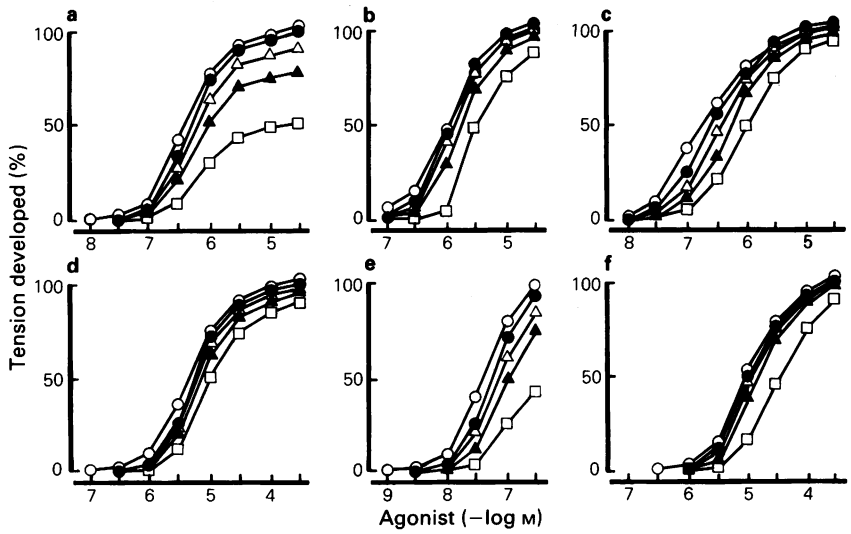

Figure 1 Effects of HA1077 on cumulative dose-response curves to 5-hydroxytryptamine (a, 5-HT), prostaglandin $F_{2\alpha}$ (b, $PGF_{2\alpha}$), noradrenaline (c, NA), histamine (d), angiotensin II (e, Ang II), and dopamine (f, DA) in rabbit aortic strips. Mean maximum responses were 2.99 ± 0.21 g ($n = 24$, 3×10^{-5} M 5-HT), 3.48 ± 0.13 g ($n = 24$, 3×10^{-5} M $PGF_{2\alpha}$), 3.70 ± 0.16 g ($n = 24$, 3×10^{-5} M NA), 5.25 ± 0.19 g ($n = 24$, 3×10^{-4} M histamine), 3.23 ± 0.20 g ($n = 24$, 3×10^{-7} M Ang II) and 4.37 ± 0.21 g ($n = 24$, 3×10^{-4} M DA), respectively. Each graph shows concentration-response curves obtained in the absence (○, $n = 24$) and in the presence of HA1077 [10^{-7} M (●, $n = 6$), 3×10^{-7} M (△, $n = 6$), 10^{-6} M (▲, $n = 6$) and 3×10^{-6} M (□, $n = 6$)]. For clarity only the mean values are given; s.e.mean ranges between 1 and 5%.

Table 1 Effects of HA1077 on the concentration-response curves of rabbit aortic strips for various agonists

Agonist	pD_2 *of HA1077* 0 μM	0.1 μM	1.0 μM	3.0 μM
			× 0.1 μM	
5-HT	4.40 ± 0.39	5.28 ± 0.89	17.32 ± 7.01	34.17 ± 7.55*
			× 1 μM	
$PGF_{2\alpha}$	1.13 ± 0.10	1.13 ± 0.16	1.85 ± 0.26*	3.70 ± 0.81*
			× 1 μM	
Histamine	4.77 ± 0.45	6.13 ± 1.15	7.37 ± 1.14*	11.85 ± 2.46*
			× 0.01 μM	
Ang II	4.13 ± 0.31	6.00 ± 0.86	13.52 ± 3.02*	24.07 ± 3.88*
			× 0.1 μM	
NA	1.97 ± 0.12	2.60 ± 0.29	5.72 ± 0.45*	11.53 ± 1.26*
			× 10 μM	
DA	0.91 ± 0.03 (n = 24)	1.08 ± 0.09 (n = 6)	1.49 ± 0.15* (n = 6)	3.58 ± 0.48* (n = 6)

Data are expressed as mean ± s.e.mean. ANOVA revealed significant differences between the effects of HA1077 on the concentration-response curves for each agonist ($P < 0.05$). The statistical significances between control and HA1077-treated groups are calculated by Student's t test (* $P < 0.05$). Abbreviations; 5-hydroxytryptamine, 5-HT; prostaglandin $F_{2\alpha}$, $PGF_{2\alpha}$; noradrenaline, NA; angiotensin II, Ang II; dopamine, DA.

Coronary circulation

Intra-coronary injection of HA1077, like other vasodilators, produced a dose-dependent increase in CBF, with no effect on MBP or HR (Figure 2). HA1077 (30 μg i.a.) produced an approximate 50% increase in CBF (predose CBF, 18.2 ± 2.8 ml min^{-1}; postdose CBF, 26.6 ± 3.3 ml min^{-1}). HA1077 was about 5 times more potent in increasing CBF than was HA1004. The rank order of potency for vasodilators tested was: nicardipine > HA1077 > trapidil > HA1004.

Continuous intra-coronary infusion of atropine (2 μg min^{-1}), propranolol (1 μg min^{-1}) and diphenhydramine (20 μg min^{-1}) led to no significant effect on the HA1077-induced vasodilatation. In contrast, infusion of either blocker completely or partially blocked the arterial response to acetylcholine (0.1 μg), isoprenaline (0.1 μg) and histamine (0.1 μg), respectively (Figure 3).

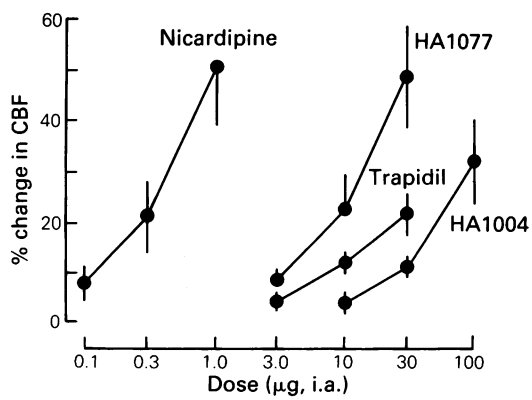

Figure 2 Effect of intra-coronary injection of HA1077 and various vasodilators on coronary blood flow (CBF) in pentobarbitone-anaesthetized dogs. The data used to calculate the percentage of changes were the maximum observed changes elicited after drug injection. Values represent mean of five experiments; vertical lines show s.e.mean.

Blood flow profile

HA1077 (0.01, 0.03, 0.1 and 0.3 mg kg^{-1}, bolus, i.v.) dose-dependently decreased MBP and increased HR, VBF, CBF, RBF and FBF (Table 2). In saline-treated animals, none of the parameters varied during the experiment.

Maximum changes in haemodynamic parameters occurred from 1 to 5 min after a bolus injection of HA1077 (Figure 4). The lowest dose of HA1077 (0.01 mg kg^{-1}, i.v.) produced significant increases in both VBF and CBF, with no significant effect on MBP, HR, RBF or FBF, although the increase in blood flow in the coronary and vertebral artery beds was minimal (<10%). There was a dose-related increase in both VBF and CBF, despite a significant decrease in MBP.

Nicardipine (3.0 μg kg^{-1}, i.v.) also produced a decrease in MBP and an increase in VBF, CBF and FBF. However, nicardipine produced no significant increases in RBF. In some dogs, there was a reduction in RBF after the i.v. administration of nicardipine (Table 2).

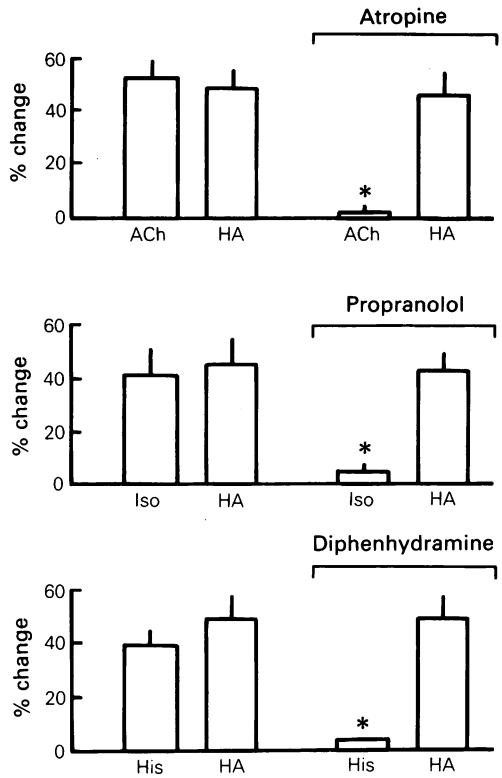

Figure 3 Maximal changes in coronary blood flow from control produced by relatively low intra-coronary injections of HA1077 (HA, 30 μg), acetylcholine (ACh, 0.1 μg), isoprenaline (Iso, 0.1 μg) and histamine (His, 0.1 μg) in pentobarbitone-anaesthetized dogs, in which specific blockers were infused. The continuous infusion of atropine (2 μg min^{-1}), propranolol (1 μg min^{-1}) and diphenhydramine (20 μg min^{-1}) failed to inhibit the HA1077-induced vasodilatation. Values represent mean ± s.e.mean of five experiments. The asterisks indicate significant differences from control (* $P < 0.05$; Student's t test).

Haemodynamics

HA1077 was administered by continuous i.v. infusion (0.01 and 0.033 mg kg^{-1} min^{-1} for 30 min; total dose = 0.3 and 1.0 mg kg^{-1}). Figure 4 shows the time courses of the changes in MBP, cardiac output, total peripheral resistance and HR in the saline infusion and the low (0.01 mg kg^{-1} min^{-1}), and high (0.033 mg kg^{-1} min^{-1}) dose groups. In the saline-treated animals (0.1 ml kg^{-1} min^{-1} for 30 min; total dose = 3 ml kg^{-1}), the parameters did not vary during the experiment. In dogs given a low dose of HA1077 (total dose = 0.3 mg kg^{-1}), there was no immediate hypotensive episode because HA1077 was

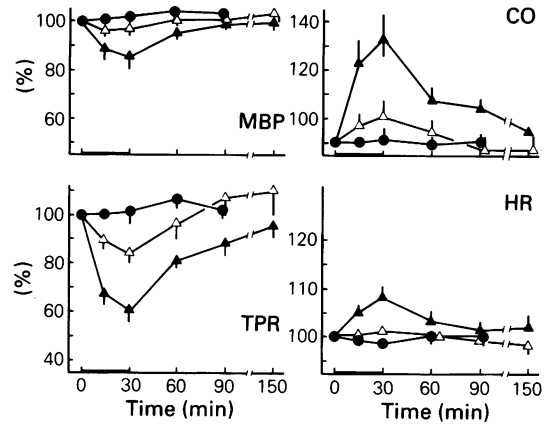

Figure 4 Time course of the effect of HA1077 infusion (0.01–0.033 mg kg^{-1} min^{-1} for 30 min; total dose = 0.3–1.0 mg kg^{-1}) on mean blood pressure (MBP), cardiac output (CO), total peripheral resistance (TPR), and heart rate (HR) in pentobarbitone-anaesthetized dogs. HA1077 (●, saline; △, 0.3 mg kg^{-1}; ▲, 1.0 mg kg^{-1}) was infused intravenously over 30 min (indicated by bar), and each animal was observed for 150 min. Values represent mean of four to six experiments; s.e.mean shown by vertical lines.

infused over a 30 min period rather than as a bolus. The bolus administration of HA1077 (0.3 mg kg^{-1}) caused a severe hypotension (Figure 5).

The maximal increase in cardiac output occurred at 30 min after start of the i.v. infusion and it increased from 1.74 l min^{-1} at basal to 2.38 l min^{-1} within 30 min of the start of the high-dose HA1077 infusion. The calculated total peripheral resistance reflects these changes; it fell significantly in a dose-dependent manner following the infusion of HA1077.

The haemodynamic response to i.v. infusion of HA1077 in dogs is summarized in Table 3. HA1077 produced a significant fall in MBP, left ventricular systolic pressure and total peripheral resistance with an increase in HR and cardiac output. There were no significant changes in right atrial pressure, dP/dt or left ventricular minute work.

Protein kinase inhibition

The effect of HA1077 on various enzymes considered to be related to the regulation of smooth muscle contraction were examined (Table 4). HA1077 had little effect on cyclic nucleotide phosphodiesterases, but the protein kinases were potently inhibited. The K_i values of HA1077 for cyclic nucleotide-dependent protein kinases and Ca^{2+}/calmodulin-dependent myosin light chain kinase were 1.6 and 36 μM, respectively. Actomyosin ATPase activity was not inhibited in concentrations up to 100 μM.

Table 2 Change in blood pressure, heart rate and blood flow after bolus i.v. administration of HA1077 to pentobarbitone-anaesthetized dogs

Parameter		*HA1077* 0.01 mg kg^{-1} (n = 7)	0.03 mg kg^{-1} (n = 7)	0.1 mg kg^{-1} (n = 12)	0.3 mg kg^{-1} (n = 12)	*Nicardipine* 3.0 μg kg^{-1} (n = 12)
MBP (mmHg)	Base	127.9 ± 6.9	127.3 ± 7.7	121.0 ± 5.6	13.4 ± 5.1	105.9 ± 4.0
	Δmax	0.0 ± 0.0	−4.0 ± 0.6*	−14.1 ± 1.5*	−31.5 ± 3.0*	−22.3 ± 2.8*
HR (beats min^{-1})	Base	124.0 ± 4.0	122.0 ± 4.0	122.6 ± 3.8	114.8 ± 3.7	112.5 ± 3.1
	Δmax	0.1 ± 0.1	1.3 ± 0.5*	5.9 ± 1.0*	9.3 ± 1.2*	4.5 ± 1.7*
VBF (ml min^{-1})	Base	18.2 ± 2.8	18.2 ± 3.2	23.1 ± 5.1	21.7 ± 6.0	18.1 ± 4.3
	Δmax	1.7 ± 0.6*	4.0 ± 1.1*	17.6 ± 4.3*	22.2 ± 5.4*	20.3 ± 3.0*
CBF (ml min^{-1})	Base	32.2 ± 9.6	27.8 ± 8.4	38.7 ± 8.6	35.1 ± 7.8	23.9 ± 4.5
	Δmax	0.8 ± 0.2*	4.5 ± 1.7*	11.8 ± 2.2*	22.6 ± 3.1*	23.4 ± 4.7*
RBF (ml min^{-1})	Base	78.9 ± 12.9	97.0 ± 8.3	104.1 ± 9.7	104.1 ± 7.1	109.3 ± 12.6
	Δmax	1.1 ± 0.6	10.0 ± 1.4*	19.8 ± 3.0*	23.8 ± 4.2*	−2.0 ± 8.9
FBF (ml min^{-1})	Base	53.5 ± 14.5	51.0 ± 22.0	62.3 ± 10.7	47.3 ± 9.7	31.0 ± 7.0
	Δmax	1.0 ± 1.0	4.5 ± 0.5*	11.3 ± 2.1*	14.7 ± 6.2*	15.5 ± 3.1*

Data are expressed as mean ± s.e.mean. Number in parentheses represents the number of dogs. The asterisks indicate significant differences from representative control before HA1077 administration (* $P < 0.05$; paired t test). Abbreviations: MBP, mean blood pressure; HR, heat rate; VBF, vertebral blood flow; CBF, coronary blood flow; RBF, renal blood flow; FBF, femoral blood flow.

Discussion

The present *in vitro* study revealed that HA1077 is a vasodilator with a potentially novel mechanism of action. The concentrations of HA1077 inhibiting agonist-induced contractions of rabbit aorta were similar to those inhibiting protein kinase.

HA1077 inhibited various agonist-induced vascular contractions, at concentrations of 0.3–3.0 μM (Figure 1). These results are consistent with a foregoing assumption that the mechanism of the vasodilator action of HA1077 differed from that of conventional calcium entry blockers. These blockers selectively shifted concentration-response curves to KCl, as opposed to concentration-response curves to receptor agonists such as noradrenaline and prostaglandin $F_{2\alpha}$ (Asano & Hidaka, 1984).

Inhibition of the 5-hydroxytryptamine-induced

Table 3 Summary of effects of i.v. infusion of HA1077 on haemodynamic parameters in pentobarbitone-anaesthetized dogs

Parameter		*HA1077* (mg kg^{-1} min^{-1}) 0	0.01	0.033
MBP (mmHg)	Base	131.7 ± 6.0	116.8 ± 8.5	122.2 ± 9.2
	Δmax	1.3 ± 1.2	−8.3 ± 1.5*	−15.8 ± 4.8*
HR (beats min^{-1})	Base	142.2 ± 4.8	126.8 ± 8.7	126.4 ± 11.0
	Δmax	−3.0 ± 0.6	1.4 ± 1.8	11.2 ± 3.4*
RAP (mmHg)	Base	5.4 ± 0.5	10.6 ± 4.9	5.4 ± 0.5
	Δmax	0.0 ± 0.0	0.4 ± 0.4	0.2 ± 0.2
LVP (mmHg)	Base	138.4 ± 6.5	127.8 ± 10.3	131.8 ± 11.2
	Δmax	2.4 ± 1.4	−9.8 ± 1.8*	−16.0 ± 3.8*
dP/dt (mmHg ms^{-1})	Base	2.4 ± 0.2	2.4 ± 0.3	2.5 ± 0.2
	Δmax	0.0 ± 0.0	0.0 ± 0.1	0.4 ± 0.3
CO (l min^{-1})	Base	1.18 ± 0.12	1.55 ± 0.40	1.74 ± 0.47
	Δmax	0.00 ± 0.04	0.22 ± 0.10	0.64 ± 0.09*
TPR (kdyn s cm^{-1})	Base	9.1 ± 1.3	6.8 ± 1.3	6.7 ± 1.2
	Δmax	0.1 ± 0.4	−1.1 ± 0.3*	−2.8 ± 0.7*
LVMW (kp m min^{-1})	Base	2.08 ± 0.14	2.32 ± 0.40	2.71 ± 0.50
	Δmax	0.03 ± 0.08	0.16 ± 0.12	0.49 ± 0.40

Data are expressed as mean ± s.e.mean of five experiments. CO, TPR and LVMW are expressed as 'index' (10 kg^{-1}). The asterisks indicate significant differences from representative control before saline or HA1077 administration (* $P < 0.05$; paired t test). For further abbreviations see 'Methods'.

Table 4 Effect of HA1077 on various enzymes related to regulation of smooth muscle contraction

Regulators of intracellular Ca^{2+}	K_i μM
Cyclic AMP-dependent protein kinase	1.6
Cyclic GMP-dependent protein kinase	1.6
Ca^{2+}/calmodulin-dependent myosin light chain kinase	36
Actomyosin ATPase	>100
Cyclic AMP phosphodiesterase	>100
Cyclic GMP phosphodiesterase	>100

Values of K_i were obtained from Dixon plots using three concentrations of substrate and four to five concentrations of HA1077.

contraction differs, from findings with other agonists. We have no obvious explanation for the different profile against 5-hydroxytryptamine.

The present *in vivo* study confirms that HA1077 has a potent dilator effect on both cerebral and coronary vascular beds in dogs. Intra-coronary administration of HA1077 (3–30 μg) dose-dependently increased CBF, with no effect on MBP or HR. We have already reported that the intra-vertebral administration of HA1077, at the same doses, increased VBF (Asano *et al.*, 1987).

The vasodilator effects of the intra-coronary injections of HA1077 to anaesthetized dogs were not altered by atropine, propranolol, or diphenhydramine treatment. The *in vivo* action of HA1077 is clearly different from other agents such as acetylcholine, isoprenaline, or histamine. Similarly, the intra-vertebral injection of HA1077 dose-dependently increased VBF, which was not prevented by these blockers (data not shown).

Intra-coronary administration of HA1077 was significantly more potent in increasing CBF than was HA1004 (Figure 2). This is consistent with previous observations that systemic i.v. application of HA1077 was more effective than HA1004 in cases of canine chronic vasospasm (Takayasu *et al.*, 1986).

One notable difference between the blood flow profile for HA1077 and nicardipine concerned renal haemodynamic alterations. In our previous study, nicardipine (0.01–10 μg kg^{-1}, i.v.) caused a dose-dependent decrease in RBF, with a significant reduction in MBP (Asano & Hidaka, 1985). Hof (1983) also found that the increase in RBF produced by nicardipine was not significant. In addition, there was a reduction in RBF after i.v. injections of nicardipine (3 μg kg^{-1}), in some dogs in the present study. On the other hand, i.v. administration of HA1077 caused a significant and dose-dependent increase in RBF, despite a reduction in MBP (Table 2).

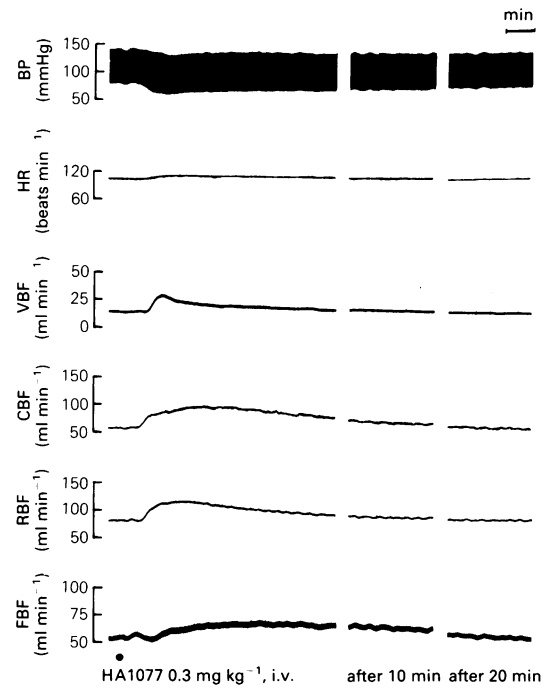

Figure 5 Effects of bolus i.v. administration of HA1077 on blood pressure (BP), heart rate (HR), vertebral blood flow (VBF), coronary blood flow (CBF), renal blood flow (RBF) and femoral blood flow (FBF) as illustrated by polygraph tracings obtained from a typical experiment. HA1077 (0.3 mg kg^{-1}) was given i.v. as bolus administration.

Infusion of HA1077 i.v. decreased total peripheral resistance and increased cardiac output, despite significant decreases in MBP. HA1077 given as an i.v. bolus had qualitatively the same effects on these cardiovascular haemodynamic parameters as did the i.v. infusion (data not shown). Haemodynamic analysis indicates a direct relaxant effect of HA1077 on arterial vascular smooth muscle.

Basal values for right atrial pressure in Table 3 seem rather high. One possible explanation is that cardiac output was determined simultaneously by the thermodilution method. Here, a Swan-Ganz catheter was introduced into the pulmonary artery via the right ventricle and the right atrium as described in Methods.

Analysis of the present biochemical effects of HA1077 revealed that this drug inhibits cyclic nucleotide-dependent protein kinases at concentrations similar to those causing inhibition of contractile response to vasoconstrictor agonists. These results suggest that the vasodilator effect of HA1077 may be related to its ability to inhibit specifically

cyclic nucleotide-dependent protein kinases. This would appear to be one of the major findings of the present study. However, there are data suggesting that activation of these kinases is related to vascular smooth muscle relaxation (Kuo & Greengard, 1969; Adelstein & Eisenberg, 1980). The relaxation of vascular smooth muscle induced by many vasodilators is thought to be mediated by cyclic AMP and/or cyclic GMP accumulation, and subsequent activation of cyclic nucleotide-dependent protein kinases (Adelstein & Eisenberg, 1980). Thus, although the present data suggest a putative involvement of cyclic nucleotide-dependent protein kinases in the HA1077-induced vasodilatation in vascular smooth muscle, more evidence is required to establish clearly any functional relationship.

The concentrations of HA1077 inhibiting cyclic AMP- and cyclic GMP-dependent protein kinases are similar to those inhibiting the agonist-induced contraction of vascular smooth muscle. Protein phosphorylation plays a significant role in modulating various cellular processes. The importance of protein kinase activity in the vascular contraction-relaxation cycle is now being investigated with HA1077.

We thank Drs T. Sone and A. Morikawa of Life Science Laboratories (Asahi Chemical Industry) for the synthesis of HA1077, M. Ohara for comments and K. Nishimura and M. Yanagita for expert technical assistance.

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The effects of verapamil upon noradrenaline-induced contraction of the rat isolated aorta following acute and prolonged alterations in PO_2

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1 Noradrenaline (NA; ED_{90}) caused a contraction of the rat aorta which could be separated into two components, a rapid response mediated by release of intracellular Ca^{2+} and a more slowly developing contraction which relied principally upon Ca^{2+} influx.

2 Exposure to acute (30 min) hypoxia has been previously shown to reduce the NA-induced contraction (by 28.0 ± 2.7%, $n = 168$) which recovered completely upon re-oxygenation (recovery response). In the present study, prolonged exposure to hypoxia (70 h) caused a more pronounced reduction (39.7 ± 3.0%, $n = 90$) of the NA-induced contraction, but, re-oxygenation then produced incomplete recovery to 77.9 ± 3.9% ($n = 90$) of the control response.

3 Prolonged exposure to 95% O_2 caused a 36.5 ± 3.1% ($n = 42$) reduction of NA-induced contractions, whereas prolonged exposure to 21% O_2 only caused a small (12.6 ± 3.4%, $n = 6$) depression of these responses.

4 The component of the NA-induced contraction mediated by release of intracellular Ca^{2+} is 39.8 ± 1.3% ($n = 83$) of the NA contraction in Ca-containing Krebs solution and was previously found to be unaffected by acute hypoxia. However, following prolonged exposure to either hypoxia or 21% O_2, this component only reached 30.7 ± 2.2% ($n = 32$) or 28.3 ± 0.9% ($n = 6$) of the control response, respectively. Prolonged exposure to 95% O_2 caused a more pronounced reduction of this component of contraction which then reached 19.1 ± 2.1% ($n = 12$) of the control response.

5 Verapamil (10 nM–10 μM) produced similar concentration-dependent reductions of NA-induced contractions elicited during control conditions or acute hypoxia; under these conditions, 1 μM verapamil caused a 34.1 ± 6.9% ($n = 6$) and a 41.8 ± 2.9% ($n = 18$) reduction of these responses respectively. However, recovery responses caused by re-oxygenation of tissues exposed to acute hypoxia were more sensitive to verapamil which, at a concentration of 1 μM, caused a 59.2 ± 2.7% ($n = 18$) reduction of these responses. Verapamil (10 nM–10 μM) also caused similar pronounced concentration-dependent reductions of contractions elicited during prolonged exposure to normoxia or hyperoxia and of recovery responses obtained following re-oxygenation of tissues exposed to prolonged hypoxia; 1 μM verapamil caused a 62.5 ± 1.1% ($n = 6$), 77.2 ± 3.8% ($n = 12$) and a 68.0 ± 4.3% ($n = 12$) reduction of these responses respectively. In contrast, contractions elicited during prolonged hypoxia were less sensitive to verapamil which at a concentration of 1 μM only caused a 16.2 ± 2.2% ($n = 12$) reduction of these responses.

6 The present study indicates that prolonged exposure of the rat aorta to either hypoxic or oxygenated conditions causes attenuation of NA-induced contraction. However, these effects are also accompanied by changes in tissue Ca^{2+} handling which differ under each condition and might account for the observed modifications in tissue sensitivity to the calcium-entry blocker verapamil.

Introduction

Contraction of vascular muscle *in vitro* has been shown to be modulated by changes in the Po_2 of the environment surrounding the vessel. A majority of studies have investigated major systemic arteries, chiefly the aorta, where acute exposure (< 120 min) of vascular muscle to hypoxia caused a reduction of contractions to both adrenoceptor agonists and KCl, while a return to control Po_2 restored contractile

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responses (Griesmer & Coret, 1960; Coret & Hughes, 1964; Detar & Bohr, 1968; Ebeigbe *et al.*, 1980a; Marriott, 1987). In contrast, Detar & Bohr (1972) found that when rabbit aorta had been exposed to hypoxia for 80 h, the magnitude of contraction produced by adrenaline was then as great as that produced under the original control conditions and that re-oxygenation produced relaxation rather than further contraction; this change in behaviour during prolonged exposure to hypoxia was termed 'adaptation'.

When rabbit aorta was exposed to acute episodes of hypoxia followed by re-oxygenation, Ebeigbe and co-workers (1980b) demonstrated a correlation between hypoxic depression of contractions to noradrenaline or KCl and a reduction in ^{45}Ca uptake. Moreover, these Po_2-dependent modifications in contractility of the rabbit aorta could also be mimicked by a reduction in the concentration of extracellular Ca^{2+} (Ebeigbe *et al.*, 1980a). In accord with these results, a previous study (Marriott, 1987), indicated that an acute reduction in Po_2 preferentially reduced the component of the contraction of the rat aorta which was mediated by influx of extracellular Ca^{2+} and that the vasodilator actions of the Ca^{2+} entry blocker, diltiazem were modified under these conditions.

In view of the findings described above, the present study was designed to investigate whether there are changes in the utilization of activator Ca^{2+} by vascular muscle during prolonged exposure to hypoxia and whether modifications in Ca^{2+}-entry blocker activity occur under such conditions.

Methods

Cylindrical segments of thoracic aortae (8 mm in length) were obtained from male Wistar rats (200–280 g) killed by cervical dislocation. In all experiments the preparations were initially mounted on parallel stainless steel wire holders and equilibrated at 37°C for 60 min under a resting tension of 3 g in Krebs solution of the following composition (mM): NaCl 118.40, KCl 4.75, $CaCl_2$ 2.50, $MgSO_4$ 1.18, KH_2PO_4 1.19, $NaHCO_3$ 25.00 and glucose 11.66, gassed with 95% O_2 plus 5% CO_2 (control conditions). EDTA (10 μM) and ascorbic acid (50 μM) were included in the Krebs solution in order to reduce the degradation of NA (Maxwell *et al.*, 1983).

In all cases, contractions to 1 μM NA were obtained at the beginning of the experiment under control conditions, repeated responses were obtained until successive peak contractions varied by less than 10% (control response). Pilot concentration-response experiments showed that 1 μM NA produced approximately 90% of the maximal response.

Experimental protocols

Acute exposure to hypoxia After equilibration, tissues were exposed to hypoxic conditions for 30 min (acute hypoxia) by gassing vigorously with 5% CO_2 in N_2 before stimulation with NA. Maintained responses were obtained and then tissues were rapidly re-oxygenated (by use of 95% O_2/5% CO_2) and further tension changes recorded. The reproducibility of contractions under such conditions has been described previously (Marriott, 1987).

Prolonged exposure to different levels of Po_2 Following equilibration, preparations were carefully removed from their holders and placed in 10 ml containers (surface area 3.14 cm^2) and stored at 10°C for 70 h either in an atmosphere of 5% CO_2 in N_2 (prolonged hypoxia), 5% CO_2 in O_2 or 21% O_2/5% CO_2 in N_2.

After storage, vessels were replaced upon their tissue holders and re-equilibrated under tension at 37°C for 60 min in Krebs solution, gassed with either 5% CO_2 in N_2 (hypoxic protocol) or 5% CO_2 in O_2 (oxygenated protocols). Responses were then elicited to NA and repeated until successive peak contractions varied by less than 10%.

In the experiments conducted after prolonged hypoxia, responses to NA reached a stable peak tension after approximately 20 min (hypoxic response, see Figure 1) at which point the gassing mixture was rapidly switched to 5% CO_2 in O_2 and the tension changes were recorded for a further 30 min (recovery response, see Figure 1). The gas mixture was then changed back to 5% CO_2 in N_2 and NA was washed away from the tissues. Before further responses were elicited, tissues were equilibrated for 30 min under hypoxic conditions. Following prolonged exposure to oxygenated conditions,

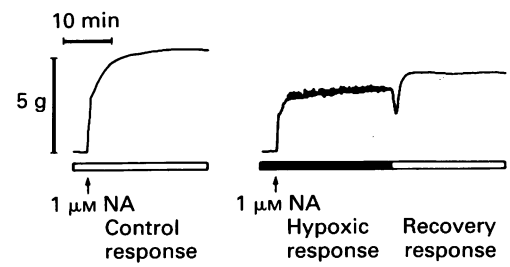

Figure 1 Representative traces of contractions of the rat aorta induced by noradrenaline (NA, 1 μM) during control oxygenated conditions and following exposure to prolonged hypoxia (hypoxic response) followed by re-oxygenation (recovery response). Hypoxic preparations were exposed to hypoxia for 70 h before addition of NA.

responses to NA were obtained which also reached a stable peak tension after approximately 20 min. With the protocols described, contractions to NA following prolonged exposure to either oxygenated conditions or hypoxia with an associated recovery response upon re-oxygenation could be reproduced over a period of 8 h with less than 10% variation in size.

Investigation of the effects of Ca^{2+}-free conditions Preparations were exposed to Ca^{2+}-free conditions under control conditions by pre-incubating for 4 min with a modified Krebs solution from which Ca^{2+} had been omitted and 0.5 mM EGTA added. Contractions were then elicited to 1 μM NA (EGTA-resistant response) and Ca^{2+}-free conditions maintained during the contraction. EGTA-resistant responses could be elicited over an 8 h period with less than 10% variation in size providing tissues were equilibrated for 30 min between successive responses in Krebs containing Ca^{2+}. EGTA-resistant responses were also elicited after incubation for 4 min in Ca^{2+}-free conditions following prolonged exposure of tissues to 95% N_2/5% CO_2, 95% O_2/5% CO_2 or 21% O_2/5% CO_2 in N_2 in the presence of Ca^{2+}. In these cases, all solutions were equilibrated under conditions appropriate to the relevant protocol.

Investigation of the effects of verapamil Verapamil was included in the bathing solution of both oxygenated and hypoxic tissues. Each concentration of verapamil was incubated for 30 min before a contraction to NA was elicited.

Measurement of oxygen tensions

Partial pressures of oxygen were continuously measured from one of the organ baths by either a Clarke-type catheter electrode (diameter 1.35 mm) positioned within 1 mm of the tissue or Clarke-type cell (Rank Bros., UK). Vigorous gassing of bathing solutions with 5% CO_2 in O_2 or 5% CO_2 in N_2 for 30 min produced oxygen tensions of 394 $\pm$ 6 (n = 36) and 76 $\pm$ 5 (n = 24) mmHg respectively. These Po_2 values represent an equilibrium, reached in approximately 5 min, between the composition of the gas mixture used to bubble the bathing medium and atmospheric air. Other studies frequently described higher and lower Po_2 values for solutions bubbled with 5% CO_2 in O_2 and N_2 respectively than those obtained in the present study. These differences probably reflect variations in gassing rate (see Ebeigbe *et al.*, 1980a). Prolonged exposure of the tissue bathing medium to atmospheres of 5% CO_2 in N_2, 5% CO_2 in O_2 or 21% O_2/5% CO_2 in N_2 for 70 h produced oxygen tensions measured at the end of the storage period of 40 $\pm$ 5 (n = 27), 387 $\pm$ 6 (n = 32) and 109 $\pm$ 4 mmHg (n = 12) respectively.

Statistical analyses

Values are presented as arithmetic means with the associated s.e.mean for the number (n) of animals examined. Statistical evaluation of results was carried out by Student's paired or unpaired t test where appropriate. Values of $P < 0.05$ were considered significant.

Drugs and chemicals

Noradrenaline bitartrate and EGTA-sodium salt were obtained from Sigma. Verapamil hydrochloride was a gift from Abbott. All other agents were obtained from BDH.

Results

The effects of acute exposure to hypoxia upon contractile responses and their sensitivity to verapamil

Under control conditions, contractions to NA of the rat aorta were found to consist of a rapid contraction followed by a slower increase in tension resulting in a maintained peak response (see Figure 1, control response). NA-induced contractions have been previously shown to be reduced during acute hypoxia (28.0 $\pm$ 2.7%, n = 168 reduction): in these tissues re-oxygenation caused a recovery of tone (recovery response) which reached 102.7 $\pm$ 3.6% (n = 168) of the control response to NA (see Marriott, 1987). In a Ca^{2+}-free Krebs solution containing 0.5 mM EGTA, NA caused a transient response (EGTA-resistant response) reaching 39.8 $\pm$ 1.3% (n = 83) of the control contraction to NA. However, the EGTA-resistant response has been previously shown to be unaffected by acute exposure to hypoxia (0.9 $\pm$ 3.2% reduction (n = 12), Marriott, 1987).

Verapamil (10 nM–10 μM) produced concentration-dependent reductions of NA-induced contractions elicited under control conditions, during acute hypoxia and of recovery responses obtained after re-oxygenation of tissues exposed to acute hypoxia (see Figure 2a). However, there was no significant difference between the effects of verapamil upon NA-induced responses elicited during hypoxia or control conditions, but verapamil (> 10 nM) produced significantly ($P < 0.001$) greater reductions of recovery responses (Figure 2a). For example, 1 μM verapamil reduced the NA-induced contraction by 34.1 $\pm$ 6.9% (n = 6) and 41.8 $\pm$ 2.9% (n = 18) during control and

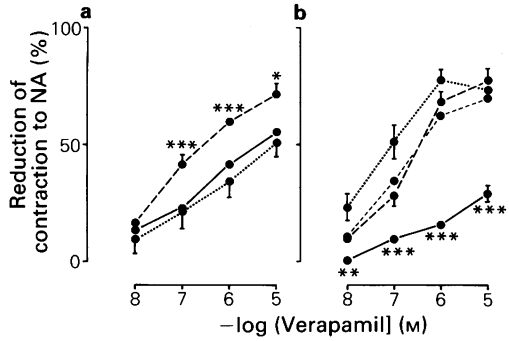

Figure 2 The effects of verapamil (10 nM–10 μM) on contractions of the rat aorta induced by 1 μM noradrenaline (NA) under conditions of (a) acute hypoxia (●———●) ($n = 18$), subsequent recovery upon re-oxygenation (●- - - -●) ($n = 18$) and control conditions (●- - - - -●) ($n = 6$). (b) Under conditions of prolonged hypoxia (●———●) ($n = 12$), subsequent recovery upon re-oxygenation (●— —●) ($n = 12$) and prolonged exposure to 95% O_2 (●- - - - -●) ($n = 12$), or 21% O_2 (●- - - -●) ($n = 6$). Asterisks indicate significant differences, * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ from, in (a), the results obtained in hypoxic tissues and, in (b), the effects upon recovery responses. Vertical bars represent the s.e.mean.

hypoxic conditions respectively but caused a 59.2 ± 2.7% ($n = 18$) reduction of the recovery response (Figure 2a).

The effects of prolonged exposure to hypoxic, hyperoxic or normoxic conditions upon contractile responses

Preliminary experiments ($n = 6$) demonstrated that removal and replacement of tissues upon their holders caused less than 10% variation in the magnitude of NA-induced contractions or in their sensitivity to verapamil when compared to controls which were undisturbed.

In the presence of Ca^{2+} Contractions to NA following prolonged exposure to hypoxia retained a biphasic appearance similar to control responses, but were significantly reduced ($P < 0.001$), reaching at peak only 60.3 ± 3.0% ($n = 90$) of control responses obtained at the beginning of the experiment (see Figure 1, hypoxic response). This reduction was significantly ($P < 0.01$) greater than that seen after exposure to acute hypoxia. In approximately 50% of tissues, small spontaneous contractions were superimposed upon the maintained tension induced by NA during hypoxia (Figure 1a shows an example). In such cases, the magnitude of the hypoxic response was estimated as the mid-point between the maximum and minimum tensions. Re-oxygenation of tissues stimulated with NA during prolonged hypoxia caused further contractions (recovery responses) though to tensions significantly ($P < 0.01$) lower than the control responses, reaching at peak 77.9 ± 3.9% ($n = 90$) of the control response to NA (see Figure 1, recovery response).

Following prolonged exposure to 95% O_2/5% CO_2, the shape of the responses to NA remained unchanged, but peak tensions were significantly ($P < 0.001$) smaller than control responses (63.5 ± 3.1% ($n = 42$) of the control response). This reduction in the NA-induced contraction was not significantly different from that seen following exposure of tissues to prolonged hypoxia.

Contractions to NA following prolonged exposure to 21% O_2/5% CO_2 in N_2 reached 87.4 ± 3.4% ($n = 6$) of the control response: the reduction incurred was significantly ($P < 0.05$ and 0.01 respectively) smaller than those observed following prolonged exposure to hypoxia or 95% O_2/5% CO_2 (see above).

Under Ca^{2+}-free conditions Exposure to prolonged hypoxia caused a reduction of the EGTA-resistant response, to 30.7 ± 2.2% ($n = 32$) of the control response to NA obtained in the presence of Ca^{2+}. However, re-oxygenation did not produce any change in the EGTA-resistant response elicited during hypoxia. The reduction of the EGTA-resistant response following prolonged exposure to 21% O_2/5% CO_2 in N_2 to 28.3 ± 0.9% ($n = 6$) of the control response to NA was not significantly different from that seen following prolonged hypoxia. However, EGTA-resistant responses elicited in tissues exposed to either prolonged hypoxia or 21% O_2/5% CO_2 were significantly ($P < 0.001$ and $P < 0.05$ respectively) smaller than those elicited under control conditions. Prolonged exposure to 95% O_2/5% CO_2 caused a significantly greater depression of the EGTA-resistant response ($P < 0.01$ from hypoxic and 21% O_2 results) to 19.1 ± 2.1% ($n = 12$) of the control response to NA obtained in the presence of Ca^{2+}.

In the presence of verapamil Verapamil (10 nM–10 μM) also caused a concentration-dependent reduction of contractions to NA in tissues exposed to either prolonged hypoxia or prolonged oxygenated conditions (see Figure 2b). However, there was no consistent significant difference between the effects of verapamil upon NA-induced contractions elicited following prolonged exposure to 95% O_2/5% CO_2 or 21% O_2/5% CO_2 in N_2 or recovery responses obtained following re-oxygenation of tissues previously exposed to prolonged hypoxia (see Figure 2b); 1 μM verapamil caused a 77.2 ± 3.8% ($n = 12$), 62.5 ± 1.1% ($n = 6$) and a 68.0 ± 4.3%

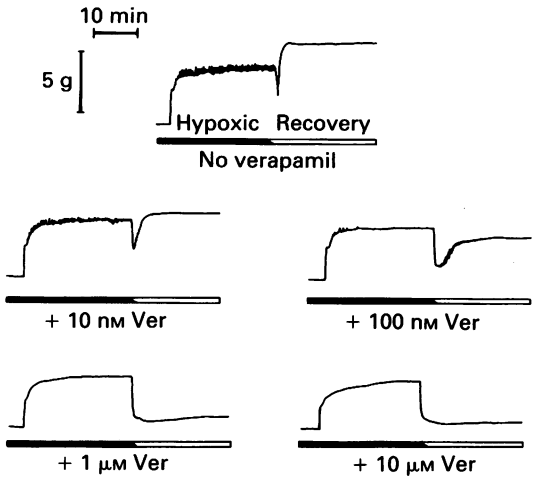

Figure 3 Representative traces of contractions of the rat aorta to noradrenaline (NA, 1 μM) during hypoxia (70 h) and following re-oxygenation (recovery). Contractions elicited in the presence of verapamil (Ver, 10 nM–10 μM) are also shown. Time and tension scales apply to all contractions.

($n = 12$) reduction of these responses respectively. In contrast, NA-induced contractions elicited during prolonged hypoxia were significantly ($P < 0.001$) less sensitive to verapamil than those evoked under the oxygenated conditions described above (see Figure 2b). For example, 1 μM verapamil only reduced the NA-induced contraction during prolonged hypoxia by 16.2 $\pm$ 2.2% ($n = 12$). Examples of the differences between the effects of verapamil (10 nM–10 μM) upon contractions induced by NA during prolonged hypoxia with subsequent re-oxygenation are shown in Figure 3.

Comparisons between the effects of verapamil in tissues exposed to acute and prolonged changes in Po_2

NA-induced contractions elicited in tissues subjected to prolonged 21% O_2/5% CO_2 in N_2 (prolonged normoxia) were significantly ($P < 0.01$) more sensitive to verapamil (>100 nM) than those elicited under control conditions (compare b and a in Figure 2). For example, 1 μM verapamil caused a 62.5 $\pm$ 1.1% ($n = 6$) reduction of NA-induced contractions following prolonged normoxia (see Figure 2b) yet only caused a 34.1 $\pm$ 6.9% ($n = 6$) reduction of these contractions under control conditions (Figure 2a). In contrast, during prolonged hypoxia NA-induced contractions were significantly less sensitive to verapamil ($P < 0.01$, <100 nM; $P < 0.001$, >100 nM) than under corresponding conditions of acute hypoxia (compare b and a in Figure 2); 1 μM verapamil reduced these responses by 41.8 $\pm$ 2.9% ($n = 18$, Figure 2a) and 16.2 $\pm$ 2.2% ($n = 12$, Figure 2b) respectively. However, recovery responses obtained following re-oxygenation of tissues exposed to either acute or prolonged hypoxia showed similar sensitivities to verapamil (compare a and b in Figure 2); under these conditions 1 μM verapamil caused a 59.2 $\pm$ 2.7% ($n = 18$, Figure 2a) and a 68.0 $\pm$ 4.3% ($n = 12$, Figure 2b) reduction respectively.

Qualitatively similar results to those described in the present study have also been obtained using the Ca^{2+}-entry blockers diltiazem, nifedipine and flunarizine which are from chemical sub-groups distinct from verapamil.

Discussion

The present study has demonstrated that prolonged alterations in oxygen tension can modify the magnitude of NA-induced contractions in the rat isolated aorta. These findings extend a previous study in which acute hypoxia was found to attenuate contractility in this vessel (Marriott, 1987). Since NA-induced contractions of the rat aorta can be resolved into components which are mediated by either release of intracellular Ca^{2+} or influx of Ca^{2+} from the extracellular environment (Yamashita *et al.*, 1977), the Po_2-dependency of these processes has also been examined in the present study by use of verapamil or Ca^{2+}-deprivation, procedures which reduce the availability of extracellular Ca^{2+} for contraction.

Mechanisms responsible for Po_2*-dependent changes in contractility*

Several mechanisms have been proposed in order to account for hypoxic depression of vascular contractility and these include release of endogenous vasodilators, activation of oxygen receptors, a reduction in available energy, and a reduction in membrane permeability to ions important for contraction (Pittman, 1981).

In the present investigation and a previous study (Marriott, 1987), the reduction in NA-induced contractions of the rat aorta observed during acute hypoxia appeared to be consistent with a preferential attenuation of contractile processes mediated by influx of extracellular Ca^{2+}. Contraction thought to rely exclusively upon release of intracellular Ca^{2+} being unaffected by these hypoxic conditions (see Marriott, 1987). In addition, the complete recovery of NA-induced contractions resulting from re-oxygenation of tissues exposed to acute hypoxia, seen in this and a previous study (Marriott, 1987), appear to result from activation of processes which depend upon Ca^{2+} influx, since recovery responses were abolished in the absence of extracellular Ca^{2+}.

These observations support the findings of Ebeigbe (1982) who described a reduction and an increase in ^{45}Ca uptake of NA-stimulated rabbit aorta during acute hypoxia and following re-oxygenation respectively.

The possibility that changes in Ca^{2+} availability might mediate modifications in vascular contractility following prolonged alterations in oxygen tension has also been examined in the present study. However, in order to estimate the degenerative effects of tissue storage upon contractility, preparations were also maintained for a prolonged period at a Po_2 normally encountered in arterial blood (normoxia produced by storage in an atmosphere of 21% O_2/5% CO_2 in N_2). Such normoxic conditions were found to reduce NA-induced contractions mediated by both intra- and extracellular Ca^{2+} and these reductions are taken to reflect the effects of storage procedures upon tissue viability.

Prolonged exposure of tissues to hypoxic or normoxic conditions produced similar reductions in contractions mediated by intracellular Ca^{2+}, indicating that such effects result from degenerative processes during storage.

However, in the presence of extracellular Ca^{2+}, prolonged exposure to hypoxia caused a reduction in contractility, in excess of that expected to result from storage procedures. Consequently, it is possible that exposure of rat aorta to prolonged periods of hypoxia produces a preferential interference with contractile processes which depend upon Ca^{2+} influx. It is not clear if these processes are similar to those responsible for the hypoxia-induced reduction in ^{45}Ca uptake of NA-stimulated rabbit aorta found by Ebeigbe and co-workers (1980b).

The effects of prolonged exposure to hypoxia upon contractility also appeared to be readily reversible in the present study, since re-oxygenation of tissues previously exposed to prolonged hypoxia caused a recovery of NA-induced contraction. As these recovery contractions were not apparent in a Ca^{2+}-free medium, such responses were also likely to depend upon influx of extracellular Ca^{2+}. In addition, these recovery contractions were of a similar size to responses elicited in tissues exposed to prolonged normoxia. This indicates that degenerative processes operating during storage prevented complete recovery of responses to control values as observed in corresponding tissues exposed to acute hypoxia (see Marriott, 1987 and above).

It is interesting that the findings from the present study contrast markedly with those of Detar & Bohr (1968, 1972), who obtained a paradoxical relaxation to oxygen in adrenaline-stimulated rabbit aorta exposed to proionged hypoxia. The reasons for these discrepancies remain obscure. However, differences in study design, namely species, form of preparation and agonist employed might be critical in producing the hypoxic 'adaptation' in vascular contractility described by Detar & Bohr (1972).

The present study also examined the effects of prolonged exposure to a high oxygen tension (hyperoxygenation) upon NA-induced contraction of the rat aorta. In contrast to the preferential depression of contractions mediated by extracellular Ca^{2+} observed during prolonged hypoxia, prolonged hyperoxygenation caused similar reductions of contractions mediated by either intra- or extracellular Ca^{2+}; these reductions were greater than the expected loss of tissue viability due to storage alone. It is therefore possible that prolonged exposure of rat aorta to a high oxygen tension produces a general reduction of contractile processes, by a mechanism differing from that operating during prolonged hypoxia. It is, however, impossible to speculate upon the nature of such processes at the present time.

Mechanisms of Po_2-dependent modifications in verapamil sensitivity

Verapamil is known to block stimulated entry of Ca^{2+} into vascular muscle, whereas, passive leaks of Ca^{2+} into these cells appear to be insensitive to this agent (Flaim, 1982). Vascular contractility has also been shown to be reduced by verapamil through mechanisms independent of calcium entry blockade (Raddino *et al.*, 1987), but these actions are associated with the use of higher concentrations ($>10\,\mu M$) than those utilised in the present study (Triggle, 1982). However, in the rat aorta the effects of selective blockade of Ca^{2+} entry processes by verapamil need not be identical to those of extracellular Ca^{2+} withdrawal, since different pathways for activated Ca^{2+} entry do not appear to be equally sensitive to verapamil (Marriott, 1988).

The present study has demonstrated that the ability of verapamil to block NA-induced contractions of the rat aorta is modified by both acute and prolonged changes in Po_2. A change in vascular sensitivity to calcium entry blockade might result from (a) differences in the relative contribution of Ca^{2+} from an intracellular source to contraction, and/or (b) changes in the populations of channels gating Ca^{2+} entry (Van Breemen *et al.*, 1982), or (c) through modification in the binding of the calcium entry blocker to its active site. The latter mechanism would appear unlikely in the present study, since calcium entry blockers from different chemical subgroups appear to bind to unique sites (Spedding, 1985), yet all appear to undergo similar changes in activity following alterations in Po_2 (Marriott, 1987).

Clearly, those vascular contractions which are mediated by release of intracellular Ca^{2+} should be

insensitive to calcium entry blockade, unless release processes are triggered by influx of extracellular Ca^{2+}. Previous studies have demonstrated that EGTA-resistant responses of the rat aorta are indeed resistant to verapamil, supporting the suggestion that these responses are mediated by intracellular Ca^{2+} (Heaslip & Rahwan, 1983; Marriott, 1988). Nevertheless, it is not certain that the relative contribution of Ca^{2+} from an intracellular source ultimately determines the verapamil sensitivity of sustained NA-induced contractions elicited in the presence of Ca^{2+}, since in the rat aorta, intracellular Ca^{2+} is only transiently involved during the early part of NA-induced contractions (Yamashita *et al.*, 1977).

Although in the present study verapamil generally produced larger reductions of contractions, in which the contribution of intracellular Ca^{2+} was depressed (i.e. following storage under oxygenated conditions), no graded relationship between the sensitivity of contractions to verapamil and their dependency upon intracellular Ca^{2+} could be found. For example, the relative contribution of intracellular Ca^{2+} to NA-induced contraction appeared similar during both acute and prolonged hypoxia; EGTA-resistant responses amounted to approximately 50% of the size of corresponding contractions in the presence of Ca^{2+} in each case. However, tissues were significantly more sensitive to verapamil under the former conditions. Consequently, such findings indicate that in the rat aorta the relative contribution of intracellular Ca^{2+} to contraction is not a major determinant of verapamil sensitivity following Po_2 changes.

It is therefore possible that a modification in systems gating Ca^{2+} entry are responsible for the Po_2-dependent changes in verapamil sensitivity observed in the present study. However, the nature of such changes remain obscure. It is interesting that McGrath & Ugwu (1986) have previously indicated that oxygen tension might control the populations of channels gating Ca^{2+} entry, since they found that nifedipine-sensitive channels were more important during NA-induced contraction of the rat tail artery following an increase in O_2 content.

The present study has shown that NA-induced contractions of the rat aorta are reduced following prolonged exposure to hypoxia and oxygen. However, it is possible that different mechanisms are responsible in each case. In addition, these modifications in contractility are accompanied by changes in tissue sensitivity to verapamil, probably due to Po_2-dependent effects upon Ca^{2+} influx pathways. Such findings may be relevant in predicting the therapeutic effects of calcium entry blocking agents in conditions involving chronic ischaemia and reoxygenation-induced vasospasm. In addition, these findings might be of value in attempts to preserve tissue viability during transplant or grafting procedures.

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An *in vitro* study of the relationship between GABA receptor function and propulsive motility in the distal colon of the rabbit

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1 The effects of γ-aminobutyric acid (GABA), 3-aminopropane sulphonic acid (3-APS) and baclofen on spontaneous, electrically-induced and propulsive motility were investigated in rabbit distal colon.

2 In unstimulated longitudinal (LMPs) and circular muscle strip preparations (CMPs) 3-APS (10–200 μM) and GABA caused a clear-cut relaxation susceptible to desensitization. Baclofen (10–200 μM) caused relaxation in a minority (30%) of preparations. The 3-APS response was sensitive to tetrodotoxin (TTX; 1 μM), SR 95531 (a novel competitive $GABA_A$-receptor antagonist) (10 μM), picrotoxinin (30 μM), and insensitive to hyoscine (1 μM) and to a combination of prazosin (1 μM) and propranolol (1 μM). The baclofen response was antagonized by 5-aminovaleric acid (DAVA, 500 μM), TTX and hyoscine and resistant to $GABA_A$-receptor and adrenoceptor blockade. $GABA_A$-receptors were therefore associated with non-adrenergic non-cholinergic (NANC) inhibitory nerve activation while $GABA_B$-receptors were involved in depression of cholinergic tone of smooth muscle. GABA (10–200 μM) elicited both above mentioned effects.

3 In LMPs, baclofen (10–200 μM) dose-dependently inhibited submaximal responses to both cholinergic and NANC inhibitory nerve stimulation. This effect was resistant to SR 95531 and picrotoxinin and prevented by DAVA and baclofen desensitization. GABA (10–200 μM) mimicked the action of baclofen. GABA inhibitory effects persisted in the presence of $GABA_A$-receptor blockade.

4 In segments of distal colon, GABA and baclofen (1–200 μM), but not 3-APS (1–200 μM), dose-dependently decreased the velocity of propulsion of an intraluminally-distended balloon. This effect was antagonized by DAVA and GABA or baclofen desensitization and resistant to SR 95531 and picrotoxinin. These antagonists *per se* had no effect on propulsion. In preparations in which propulsion was slowed by hyoscine (1 μM), baclofen caused no consistent further depression of propulsive activity.

5 Our results show that $GABA_A$- and $GABA_B$-receptors are present in rabbit colon. $GABA_A$-receptor stimulation activates NANC inhibitory nerves without apparently affecting propulsion. $GABA_B$-receptors are associated with a reduction of neural (mainly cholinergic) activity subserving muscular tone and peristalsis and appear to be located on both cholinergic and NANC inhibitory nerves. However, the persisting propulsive activity during suppression of $GABA_A$- and $GABA_B$-receptor function suggests that GABA in enteric neurones is not crucial for the neural circuitry subserving colonic peristalsis in this species.

Introduction

Autoradiographic (Jessen *et al.*, 1979), immunohistochemical (Krantis & Harding, 1987), biochemical (Tanaka, 1985) and transmitter release studies (Taniyama *et al.*, 1983) indicate that γ-aminobutyric acid (GABA)-containing neurones are not restricted to the CNS, but are also distributed in the periphery including the enteric plexuses of mammals. As far as the large intestine is concerned, a number of studies

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have documented the presence of GABA in the colon of rat (Hills *et al.*, 1987), guinea-pig (Kerr & Krantis, 1983; Miki *et al.*, 1983; Krantis *et al.*, 1986; Hills *et al.*, 1987), cat (Taniyama *et al.*, 1982; Miki *et al.*, 1983; Davanger *et al.*, 1987; Taniyama *et al.*, 1987) and man (Miki *et al.*, 1983). However, the effects of GABA have been investigated in some detail only in the cat and guinea-pig colon. In the circular muscle of cat colon $GABA_A$-receptor stimulation causes excitatory cholinergic responses (Taniyama *et al.*, 1987). In the guinea-pig, activation of colonic $GABA_A$-receptors induces non-adrenergic non-cholinergic (NANC) inhibitory responses (Krantis *et al.*, 1980; Onori *et al.*, 1984), while $GABA_B$-receptors are associated with a consistent decrease of the basal cholinergic tone supplying the longitudinal muscle layer (Giotti *et al.*, 1985) and the cholinergic rhythmic contractions of the circular coat (Ong & Kerr, 1983). Studies designed to evaluate the possible functional role of GABA-containing neurones on the propulsive activity of guinea-pig colon by using $GABA_A$-receptor antagonists and desensitization procedures have given conflicting results. In fact, $GABA_A$-receptors were considered not to be involved in the modulation of distension-initiated enteric reflexes (Krantis *et al.*, 1980), to reduce (Frigo *et al.*, 1987), or, together with $GABA_B$-receptors (Ong & Kerr, 1983) to enhance the efficiency of peristalsis (Krantis & Kerr, 1981a).

In this study, we investigated the effects of GABA and the $GABA_A$- and $GABA_B$-receptor agonists 3-aminopropane sulphonic acid (3-APS) and baclofen on the spontaneous, electrically-induced and propulsive motility of rabbit distal colon. The possible involvement of endogenous GABA in peristalsis was evaluated after suppression of GABA function by SR 95531, a novel competitive $GABA_A$-receptor antagonist (Wermuth & Bizière, 1986; Tonini *et al.*, 1989), or picrotoxinin (a blocker of the chloride channel associated with $GABA_A$-receptors; Krantis & Kerr, 1981b), and in the presence of the non-specific $GABA_B$-receptor antagonist D-aminovaleric acid (DAVA) (Muhyaddin *et al.*, 1982; Ong & Kerr, 1987; Dickenson *et al.*, 1988) as well as $GABA_A$- and $GABA_B$-receptor desensitization.

Methods

Male albino rabbits, weighing 1800–2200 g were killed by stunning and bleeding. A 12 cm segment of distal colon was excised with the oral end cut 1 cm above the pubis symphysis and transferred to a Petri dish containing prewarmed Tyrode solution to remove the intraluminal content. From this specimen three kinds of preparation were obtained.

Longitudinal muscle strip preparations (LMPs)

A 3 cm segment was slit open at the mesenteric border and pinned out with the mucosa uppermost. After removal of the mucosa, 2 cm wide segments were cut with the long axis directed towards longitudinal muscle fibres. LMPs were mounted in an organ bath containing 10–20 ml Tyrode solution maintained at 37°C and continuously bubbled with 95% O_2 + 5% CO_2. One end of the preparation was attached to a glass hook at the base of the bath and the free end was connected by a thread to an isotonic transducer (load 0.5 g). The movements of the longitudinal muscle were recorded 60–90 min later by a U. Basile 7070 microdynamometer or by a KO 380 Battaglia-Rangoni polygraph. During the equilibration period, the solution was changed at 15 min intervals.

LMPs were used to detect the effects of drugs on spontaneous motility and tone, and on electrically-induced excitatory and inhibitory responses. In unstimulated LMPs, GABA, baclofen, and 3-APS (each at 10–200 μM) were administered at 20 min intervals during which the preparations were carefully washed to avoid GABA-receptor desensitization. In each preparation, the relaxing effect of only one agonist concentration was tested and expressed as percentage of the response to 10 μM isoprenaline. Separate experiments with 3-APS (100 μM) were also performed in the absence and in the presence of 10 min exposure to the antagonists hyoscine, TTX, picrotoxinin and SR 95531. Any drug-induced change in 3-APS response was expressed as a % change with respect to the agonist basal response.

Submaximal cholinergic nerve-mediated contractions were elicited every 10 min by stimulating low tone preparations with 10 pulses at 0.5 Hz (pulse duration: 1 ms, at 60 V) via two parallel silver wire electrodes (1 cm apart). These contractions were 55.9 ± 4.5% (n = 16) of those evoked by 50 pulses at 5 Hz. Submaximal relaxations to NANC nerve stimulation were elicited at 3 min intervals by delivering 5 pulses at 1.5 Hz (pulse duration: 0.5 ms, at 15–25 V) in the presence of hyoscine (1 μM) and guanethidine (5 μM). These responses were 77.2 ± 2.4% (n = 16) of those evoked at 60 V. The actions of GABA (1–200 μM) and baclofen (1–200 μM) on each type of electrically-induced response were evaluated by exposing the tissue for 30 s to a single concentration of the drug. The amplitude of responses in treated preparations was expressed as a percentage of the control response. Antagonists were added to the bathing solution 10–20 min before the agonist. In auto- or cross-desensitization experiments, each agonist was first administered at a high concentration (100, 200 μM). Subsequently (6–10 min later), a second dose of agonist (100, 200 μM) was administered in the presence of the priming dose.

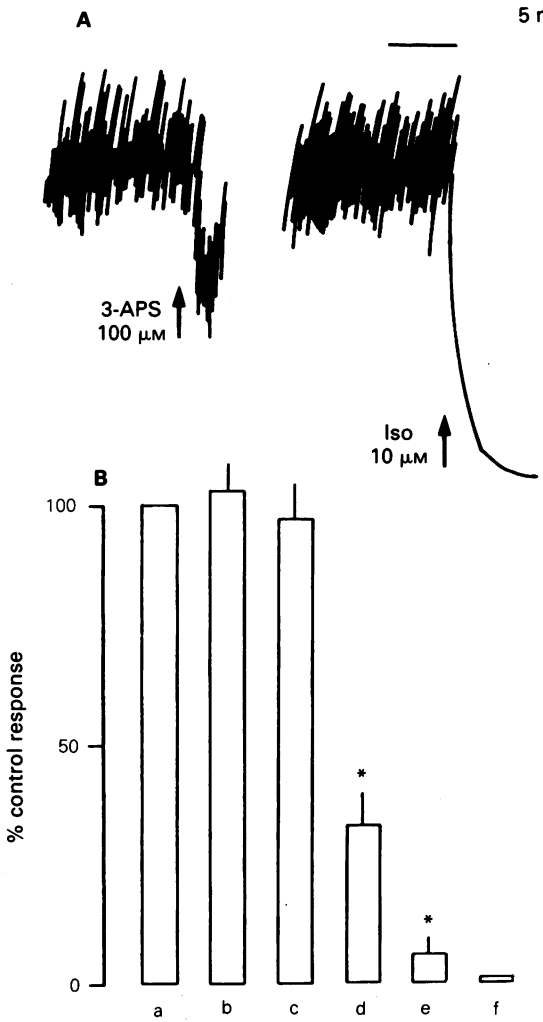

Figure 1 Characterization of the relaxation induced by 3-aminopropane sulphonic acid (3-APS) in longitudinal muscle strip preparations of rabbit distal colon. In (A) relaxation caused by 3-APS (100 μM) compared to that caused by 10 μM isoprenaline (Iso). W, indicates washing. In (B), (a) control response to 100 μM 3-APS; (b) with hyoscine (1 μM, $n = 8$); (c) in the presence of α- and β-adrenoceptor blockade (1 μM prazosin + 1 μM propranolol, $n = 7$); (d) antagonism by picrotoxinin (10 μM, $n = 5$); (e) 3-APS response in auto-desensitized preparations after 6 min exposure to an equimolar priming concentration of 3-APS ($n = 6$); (f) antagonism caused by tetrodotoxin (1 μM, $n = 6$). *$P < 0.005$ (vs control).

Circular muscle strip preparations (CMPs)

Two mm wide segments (rings) were dissected from a 10 mm long piece of intestine and cut along the mesenteric border to obtain circular muscle strip preparations (CMPs). After removal of the mucosa, CMPs were mounted in the same way as described for LMPs. Compared to LMPs, CMPs usually had a relatively low tone and their use in this study was limited because the main effect of GABA and GABA agonists was inhibition of both muscular tone and motility. For this reason, these preparations were used mainly for qualitative purposes.

Intact segments of distal colon: induction of propulsive activity

Segments of distal colon, 8 cm long, were transferred to an organ bath containing 100 ml Tyrode solution prewarmed and gassed as described above. Each segment was set up horizontally with the oral end tied to a fixed perspex holder and the free aboral end connected to an isotonic transducer loaded at 2 g. Propulsion was elicited as previously described in this species (Tonini *et al.*, 1982), by using a thin rubber balloon which was inserted intraluminally 1 cm below the oral side and distended by filling with 0.6 ml water. The velocity of propulsion was evaluated by recording the time (s) required by the balloon to travel the entire distance (mm) from the oral to the aboral end of the preparation. The mean velocity calculated from two consecutive propulsions was taken as the control value.

GABA and GABA mimetic compounds were administered 30 s before the induction of peristalsis. Any drug-induced change in the velocity of bolus transport was expressed as a percentage of the control value. In any preparation, only one agonist concentration was used if this exceeded 50 μM, otherwise two randomly selected concentrations (range 1–50 μM) were used, the first administration being followed by a thorough washing. Antagonists preceded agonist administration by 10–20 min. For desensitization experiments, a procedure similar to that described for LMPs was used. This consisted of the administration of a high priming agonist concentration (100, 200 μM). A second dose of the agonist was tested in the presence of the priming dose.

Statistical analysis

Differences between mean values were analysed by Student's t test for paired and unpaired data; $P < 0.05$ was taken as statistically significant.

Solutions and drugs

The composition of Tyrode solution was (mM): NaCl 136.9, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1.04, NaH_2PO_4 0.4, $NaHCO_3$ 11.9, glucose 5.5. The drugs used were: γ-aminobutyric acid (GABA), 3-amino-1-propane

sulphonic acid (3-APS), picrotoxinin, (−)-scopolamine hydrochloride (hyoscine), tetrodotoxin (TTX), (−)-isoprenaline (all from Sigma); 2-(3-carboxypropyl)-3-amino-6-(4-methoxyphenyl)-piridazynium bromide (SR 95531) (RBI), (±)-baclofen and guanethidine sulphate (Ciba), 5-amino-n-valeric acid (DAVA) (Fluka), prazosin hydrochloride (Pfizer), (±)-propranolol hydrochloride (ICI). All drugs were administered as aqueous solutions except picrotoxinin which was dissolved in ethanol and baclofen which was dissolved in 0.1 M HCl to obtain stock solutions. These were further diluted with distilled water. Drugs were administered in volumes not exceeding 1% of the bath volume.

Results

Interaction experiments in longitudinal muscle strip preparations

LMPs showed various patterns of mechanical activity. Few preparations were completely inactive or had periods of spontaneous contractions interposed with periods of inactivity. A greater proportion of preparations showed phasic contractions and had a relatively high tone.

In the LMPs, the $GABA_A$-receptor agonist 3-APS (10–200 μM) caused a dose-dependent transient inhibition of muscular tone, ranging from 5 to 35% of that induced by 10 μM isoprenaline, associated with some reduction of the amplitude of phasic contractions. As shown in Figure 1, the relaxant response caused by 100 μM 3-APS (29.5 ± 4.0% of that induced by 10 μM isoprenaline, $n = 12$) persisted unchanged in the presence of muscarinic receptor (1 μM hyoscine: $n = 8$) or α- and β-adrenoceptor blockade (1 μM prazosin + 1 μM propranolol, $n = 7$), underwent desensitization ($n = 5$), was virtually abolished by 1 μM TTX ($n = 5$), antagonized by 10 μM picrotoxinin ($n = 5$) and (Figure 2) by the novel competitive $GABA_A$-receptor antagonist SR 95531 (0.1–10 μM).

Baclofen (10–200 μM), a $GABA_B$-receptor agonist, had little effect on the resting mechanical activities of the LMPs. A slight inhibitory action on the tone, 19.4 ± 1.9% ($n = 6$) of that caused by 10 μM isoprenaline, was observed in 30% of the preparations tested with baclofen (200 μM). This effect was resistant to 10 μM SR 95531 ($n = 3$) and to α- and β-adrenoceptor blockade (1 μM prazosin + 1 μM propranolol, $n = 4$) but was antagonized by 500 μM DAVA ($n = 3$), TTX ($n = 4$) and hyoscine ($n = 3$).

In the LMPs, GABA (10–200 μM) caused a transient inhibitory response which involved both phasic contractions and muscular tone (Figure 3). The relaxant effect induced by 100 μM GABA was 40.9 ± 3.5% ($n = 11$) of that caused by 10 μM isoprenaline. SR 95531, at a concentration (10 μM) which invariably abolished the relaxant effect to 100 μM 3-APS, suppressed the GABA (100 μM) response in 12 out of 20 preparations. In the 8

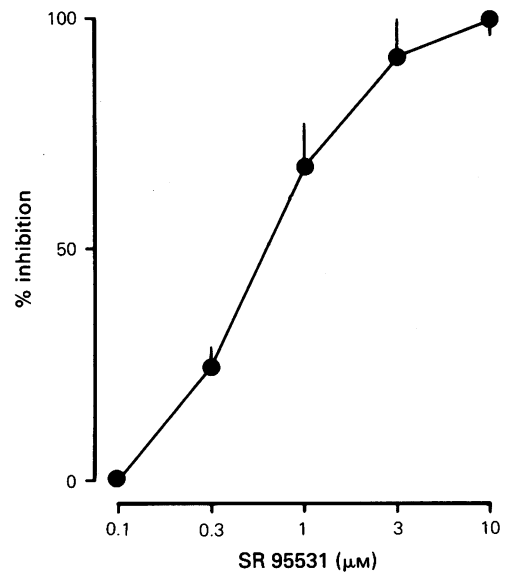

Figure 2 Concentration-response curve for SR 95531-induced antagonism of the relaxant response to 100 μM 3-aminopropane sulphonic acid (3-APS) in longitudinal muscle strip preparations of rabbit distal colon. Values are means with vertical lines indicating s.e. mean of 5–7 experiments.

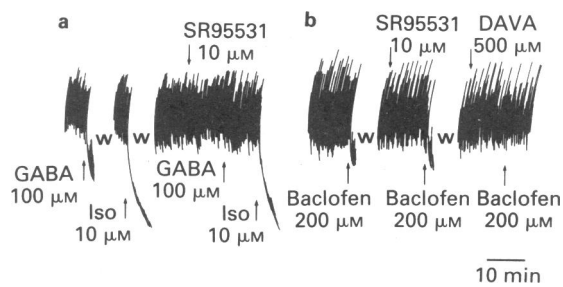

Figure 3 Relaxant effect of GABA and baclofen in longitudinal muscle strip preparations (a) and circular muscle strip preparations (b). In (a), GABA-induced relaxation (100 μM) is compared with the relaxation caused by isoprenaline (Iso, 10 μM). Ten min exposure to SR 95531 (10 μM) abolished the GABA response (in this example GABA did not show any appreciable $GABA_B$-receptor-mediated effect), while the relaxation caused by Iso was unaffected. In (b), relaxation to baclofen (200 μM) was unaffected by SR 95531 (10 μM) and abolished by 10 min exposure to 500 μM 5-aminovaleric acid (DAVA). W, indicates washing.

remaining preparations, the residual response to GABA (37.6 ± 8.0% of the control value) was abolished by DAVA (500 μM) pretreatment.

Interaction experiments in circular muscle strip preparations

CMPs had a motility pattern resembling that of LMPs but their muscular tone was usually much lower. In these preparations, the inhibitory effects of 3-APS, baclofen and GABA were qualitatively similar to those observed in LMPs (Figure 3).

Electrically-induced excitatory and inhibitory responses

Submaximal contractions to electrical stimulation of LMPs were abolished by 1 μM TTX ($n = 4$) or 1 μM hyoscine ($n = 4$). These nerve-mediated cholinergic responses were invariably inhibited (>90% of preparations affected) by both GABA (1–200 μM) or baclofen (1–200 μM), added to the bath 30 s before stimulation. The inhibitory effects caused by both agonists were similar in magnitude and were resistant to picrotoxinin (10 μM) or SR 95531 (10 μM) pretreatment (Figure 4). These antagonists did not affect electrically-induced contractions.

Both auto- and cross-desensitization to the effect of GABA and baclofen were observed. In fact, a second addition of GABA or baclofen in the presence of the priming agonist concentration did not induce any effect, irrespective of the agonist used for the priming. Desensitization was readily reversed upon removal of the agonist.

In hyoscine (1 μM) and guanethidine (5 μM) pretreated LMPs, submaximal electrical stimulation elicited TTX (1 μM)-sensitive relaxations, due to non-adrenergic non-cholinergic (NANC) inhibitory nerve activation. Submaximal NANC relaxations were depressed by baclofen (exposure time 30 s) in only 60% of preparations, irrespective of the baclofen concentration used (Figure 5). Maximal inhibition was obtained with 200 μM baclofen and was 37.8 ± 8.1% ($n = 6$). Baclofen-induced inhibition underwent desensitization. In the continuing presence of baclofen (100 μM), a second response to electrical stimulation (3 min later) showed only a partial recovery, while a ⩾90% recovery was observed 6 min later at the third stimulation. If a second dose of baclofen (100 μM) was applied after this period, it did not have any effect.

GABA was not routinely used in these experiments because GABA caused relaxant responses by activating *per se* on NANC inhibitory nerves. However, after suppression of this effect by SR 95531, 100 μM GABA reduced the electrical response by approximately 26.5 ± 5.7% ($n = 7$) through a mechanism which underwent auto- (100 μM GABA, $n = 3$) and cross-desensitization with baclofen (100 μM, $n = 4$).

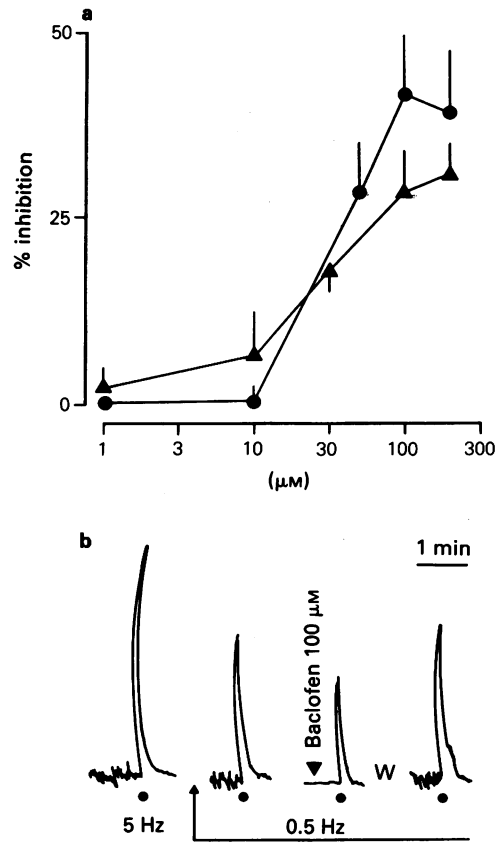

Figure 4 Concentration-response curve for GABA (●) and baclofen (▲) in inhibiting submaximal cholinergic contractions to electrical stimulation of longitudinal muscle strip preparations of rabbit distal colon (a). Values represent means and vertical lines show s.e.mean of 5–7 experiments. Inhibition was significant at concentrations ⩾30 μM for baclofen ($P < 0.005$) and ⩾50 μM for GABA ($P < 0.05$). (b) A representative tracing illustrating the inhibitory effect of 100 μM baclofen on submaximal cholinergic contractions (evoked at 0.5 Hz every 10 min). W, indicates washing.

Cholinergic and NANC inhibitory nerve-mediated responses induced by electrical stimulation of CMPs (same parameters used for LMPs) were also sensitive to the action of baclofen. Baclofen (100 μM) reduced both these responses by 30.1 ± 5.1% ($n = 4$) and by 28.5 ± 6.4% ($n = 3$) respectively, thus confirming the moderate modulation exerted by $GABA_B$-receptors on these pathways.

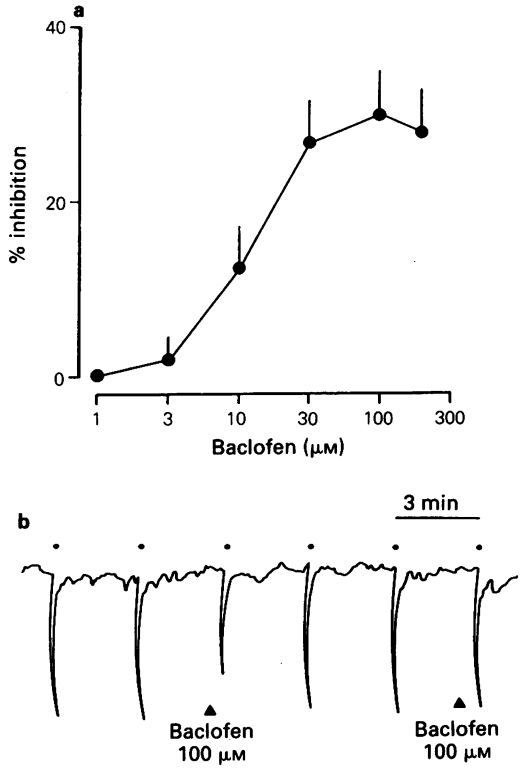

Figure 5 Concentration-response curve for baclofen-induced inhibition of nerve-mediated NANC relaxations after electrical stimulation of longitudinal muscle strip preparations of rabbit distal colon (a). Values are means and vertical lines s.e.mean of 5–7 experiments. Significant inhibition ($P < 0.05$) was obtained at concentrations $\geqslant 30\,\mu$M baclofen. A representative tracing illustrating the inhibitory effect of $100\,\mu$M baclofen on NANC responses is shown in (b). Note that a second exposure to baclofen was ineffective. Submaximal stimulation (dots) of NANC inhibitory nerves was performed every 3 min.

Induction of propulsive activity

In preparations set up to study peristalsis, inflation of the balloon caused, after a latency of 3–5 s, the appearance of a caudally moving wave of contraction which propelled the balloon aborally. The control velocity of propulsion was 1.89 ± 0.13 mm s^{-1} ($n = 36$). A 30 s exposure to GABA and baclofen (both at 1–200 μM) inhibited dose-dependently the velocity of propulsion. A subsequent inflation of the balloon after GABA and baclofen had been left in the bath for 10 min elicited normal propulsion. The minimal effective concentration for both agonists was 10 μM. The maximum inhibition was obtained in the presence of 200 μM of each com-

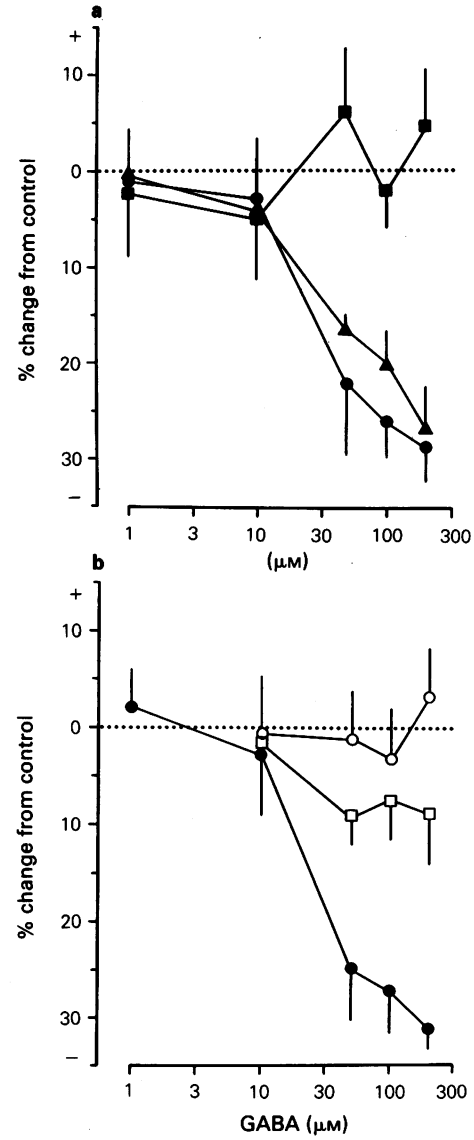

Figure 6 Effect of GABA (●), baclofen (▲) and 3-aminopropane sulphonic acid (3-APS) (■) on the velocity of propulsion of an intraluminally distended balloon in segments of rabbit distal colon (a). Note the inhibitory effect of GABA and baclofen ($P < 0.05$ for each agonist at concentrations $\geqslant 50\,\mu$M) and the ineffectiveness of 3-APS. In (b), influence of GABA desensitization (○) and 5-aminovaleric acid (DAVA) (□) on the inhibitory effect of GABA (●) on the velocity of propulsion. The difference between GABA-induced inhibition in the absence and in the presence of DAVA is significant ($P < 0.05$) in the range 50–200 μM. Values are means and vertical lines s.e.mean of 4–9 experiments.

pound. By contrast, 3-APS (1–200 μM) had no consistent effect (<10% change) on the velocity of propulsion after either 15 or 30 s of exposure to the agonist (Figure 6a). GABA-induced inhibition was counteracted by GABA or baclofen desensitization (200 μM of each agonist left in contact with the tissue for 10 min) and by 500 μM DAVA, which *per se* did not affect basal propulsion (Figure 6b). Picrotoxinin and SR 95531 had no effect on basal propulsive activity (Figure 7a). The inhibitory effect of GABA on propulsion was not modified by pretreatment with either of these antagonists at a concentration of 10 μM (Figure 7b).

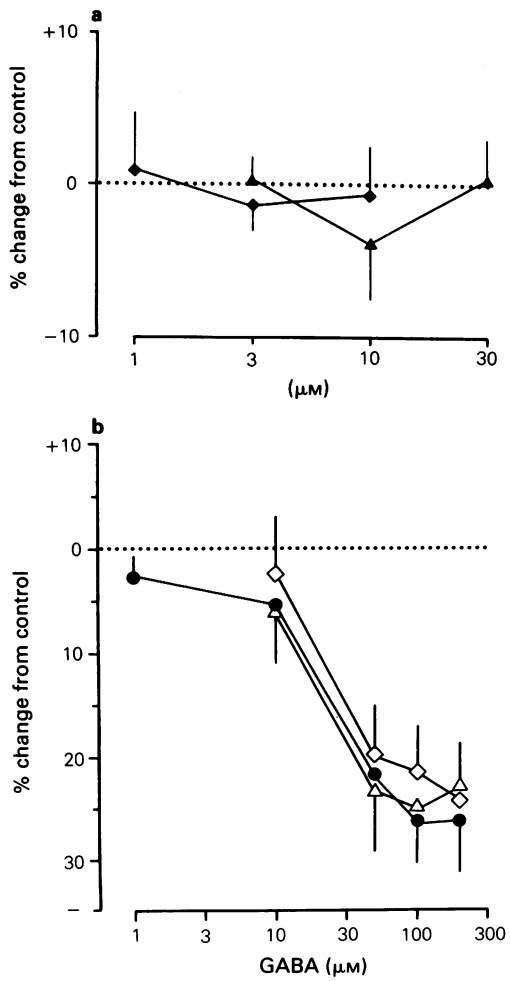

Figure 7 Failure of SR 95531 (◆) and picrotoxinin (▲) to affect the basal velocity of propulsion (a). In (b) failure of 10 μM SR 95531 (◇) and 10 μM picrotoxinin (△) to antagonize the inhibitory effect of GABA (●) on the velocity of propulsion. Values are means and vertical lines s.e.mean of 4–10 experiments.

In separate experiments ($n = 9$), 1 μM hyoscine blocked propulsive activity in 4 preparations and caused a decrease in the velocity of propulsion by 43.3 ± 8.3% in the remaining 5 preparations. In the latter preparations, GABA (100 μM, $n = 5$) did not further modify the velocity of propulsion.

Discussion

In order to ascertain the effect of $GABA_A$- and $GABA_B$-receptor stimulation on the motility of the distal colon of rabbit, the effects of GABA, 3-APS and baclofen were investigated on spontaneous, electrically-induced and propulsive activity. The possible physiological role of endogenous GABA on peristalsis was studied by means of GABA-receptor function blockade or desensitization.

In unstimulated LMPs, GABA and 3-APS caused a transient, desensitizing relaxation sensitive to TTX, picrotoxinin and SR 95531, a novel competitive $GABA_A$-receptor antagonist (Wermuth & Bizière, 1986) also effective at the intestinal level (Tonini *et al.*, 1989). Relaxation was presumably due to stimulation of $GABA_A$-receptors located on NANC inhibitory nerves. This suggests that the effect of $GABA_A$-receptor activation in the rabbit colon is similar to that observed in the colon of guinea-pig (Krantis *et al.*, 1980; Onori *et al.*, 1984) or in rat duodenum (Maggi *et al.*, 1984; Krantis & Harding, 1987) but differs from that in the guinea-pig ileum (Krantis *et al.*, 1980; Kaplita *et al.*, 1982; Krantis & Kerr, 1981b; Giotti *et al.*, 1983; Ong & Kerr, 1987; Tonini *et al.*, 1987) and the cat colon (Taniyama *et al.*, 1987), in which stimulation of this type of receptor induces a cholinergically-mediated contractile response. However, while in the presence of $GABA_A$-receptor blockade the 3-APS response was always abolished, a residual GABA inhibitory response sensitive to DAVA, a $GABA_B$-receptor antagonist, and hyoscine was observed in 40% of preparations. As expected, baclofen had little or no effect on resting LMPs activity. A slight reduction in muscular tone sensitive to desensitization, hyoscine and DAVA was observed in 30% of preparations. A similar but more consistent $GABA_B$-receptor-mediated inhibition of spontaneous cholinergic tone has been previously described in the guinea-pig resting intestine (Giotti *et al.*, 1983; 1985; Ong & Kerr, 1983; 1987).

Activation of $GABA_B$-receptors by GABA or baclofen was invariably associated with a depression of submaximal cholinergic contractions to transmural stimulation. On a molar basis, these agonists were approximately equipotent and their inhibitory effect was susceptible to auto- and cross-desensitization. These data are qualitatively and

quantitatively in agreement with those found for the guinea-pig intestine, in which electrically-stimulated cholinergic nerves are moderately sensitive to GABA action through $GABA_B$-receptors (Kaplita *et al.*, 1982; Kleinrok & Kilbinger, 1983; Ong & Kerr, 1983).

In our experiments, an effect mediated by $GABA_B$-receptors was also associated with depression of the response to electrical stimulation of NANC inhibitory nerves in 60% of preparations. This finding is difficult to explain. It is possible that a variable proportion of NANC inhibitory neurones represents a population of cells with low $GABA_B$-receptor density and is therefore resistant to the effect of GABA.

Our findings extend to the rabbit colon the observation that $GABA_B$-receptors are associated with inhibition of resting and, to a greater extent, electrically-stimulated autonomic nerve activity both at the intestinal and extra-intestinal level (Bowery *et al.*, 1981; Kaplita *et al.*, 1982; Kleinrok & Kilbinger, 1983; Giotti *et al.*, 1983; Ong & Kerr, 1983; 1987; Ronai *et al.*, 1987).

As far as peristalsis is concerned, GABA and baclofen inhibited the velocity of propulsion, while 3-APS was ineffective. The effect of GABA was antagonized by DAVA (which *per se* was ineffective on peristalsis) and by GABA or baclofen desensitization, but was insensitive to picrotoxinin or SR 95531. The latter antagonists also had no effect on the basal velocity of propulsion. These results indicate that $GABA_B$-receptors are mainly involved in the inhibitory effect of GABA on propulsive activity. This effect could involve an action on both excitatory cholinergic and inhibitory nerves subserving peristalsis.

Based on previous findings indicating that in the rabbit colon the excitatory component of the peristaltic reflex is not entirely dependent on the activation of cholinergic nerves (MacKenna & McKirdy, 1972; Tonini *et al.*, 1982), the effect of GABA on propulsion was tested in the presence of hyoscine. In a preparation in which hyoscine had been shown to reduce the velocity of propulsion, GABA did not induce a further significant inhibition. Therefore, the inhibitory action of GABA on peristalsis appears to involve mainly cholinergic nerves without any apparent effect on non-cholinergic excitatory and NANC inhibitory pathways. We have no satisfactory explanation for the apparent lack of effect of GABA on the non-cholinergic excitatory component (probably substance P-containing neurones: Furness & Costa 1987) which contribute to propagated contraction behind the bolus, in the presence of muscarinic receptor blockade. The lack of effect of GABA on NANC inhibitory nerves during propulsion may be explained by the experimental procedure used (e.g. the presence of hysoscine). It is known that in the intestine, including the rabbit colon, radial distension of the gut wall activates, besides NANC inhibitory nerves, an additional descending pathway which inhibits cholinergic motor neurones presynaptically (Hirst *et al.*, 1975; Julé, 1980). Hyoscine-induced relaxation in front of the bolus, mediated by blockade of muscarinic receptors in the muscle, could balance the possible decrease of reflex relaxation caused by GABA acting on NANC inhibitory nerves.

The functional role of $GABA_A$-receptors on colonic peristalsis of other animal species (e.g. guinea-pig) was previously investigated in the presence of GABA desensitization or $GABA_A$-receptor antagonists. The results, however, were conflicting since endogenous GABA was found not to be involved in peristaltic reflexes (Krantis *et al.*, 1980) or to induce both excitatory (Krantis & Kerr, 1981a; Ong & Kerr, 1983) or inhibitory effects (Frigo *et al.*, 1987) on propulsion, via an effect on $GABA_A$-receptors. In our experiments, suppression of $GABA_A$-receptor function by SR 95531, picrotoxinin or GABA desensitization was without effect on the velocity of propulsion, suggesting that $GABA_A$-receptors are unlikely to have an important physiological role in rabbit colon peristalsis. These findings are in agreement with those recently obtained by our group in the guinea-pig ileum (Tonini *et al.*, 1989). Direct stimulation of $GABA_A$-receptors by 3-APS also failed to produce a consistent effect on peristaltic activity. This may be due to the rapidly occurring desensitization to the $GABA_A$-receptor-mediated effect. Alternatively, the activation of NANC inhibitory nerves by 3-APS may not be important for the mechanism leading to propulsive activity, even though these nerves, when adequately stimulated, were found to prevail on intestinal excitatory reflexes which occur orally of distension of the circular coat (Costa & Furness, 1976).

As mentioned above, activation of $GABA_B$- receptors resulted in slowing of propulsive activity. $GABA_B$-receptors have been described on nerve endings of myenteric neurones close to the site where endogenous GABA should be released. In fact, in the intestine GABA-containing fibres are present predominantly in the intramural nerve bundle innervating the circular muscle rather than in the ganglia of the myenteric plexus (Hills *et al.*, 1987; Davanger *et al.*, 1987). Since released GABA does not affect smooth muscle directly (Krantis *et al.*, 1980), its only consistent effect should be interference with the neurotransmitter releasing properties of motor neurones. Therefore, based on our results, if GABA has a physiological role in rabbit colon propulsion, this should be of moderate degree, mediated by

$GABA_B$-receptors and inhibitory in nature. However, our results with $GABA_B$-receptor desensitization and DAVA experiments, failed to show a $GABA_B$-receptor-mediated modulator effect of endogenous GABA on propulsive activity. Since desensitization procedures may not be adequate for the assessment of the functional involvement of a released neurotransmitter, selective $GABA_B$-receptor antagonists are needed to clarify this point.

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The mode of inhibition of the Na^+-K^+ pump activity in mast cells by calcium

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1 The inhibition by calcium of the Na^+-K^+ pump in the plasma membrane of rat peritoneal mast cells was studied in pure populations of the cells by measuring the ouabain-sensitive uptake of the radioactive potassium analogue, ^{86}rubidium ($^{86}Rb^+$).

2 Exposure of the cells to calcium induced a time- and concentration-dependent decrease in the ouabain-sensitive K^+($^{86}Rb^+$)-uptake of the cells without influencing the ouabain-resistant uptake. The development of the inhibition required the presence of potassium in the medium in the millimolar range (1.5–8.0 mM), and it did not occur at a concentration of potassium (0.24 mM) that is probably rate limiting for the pump activity. In the presence of 1 mM calcium full inhibition developed almost immediately and was not readily reversed. The inhibition was not significantly reduced by 15 min incubation with 1.2 mM EGTA.

3 The inhibitory action of calcium did not develop when the mast cells were incubated in a potassium-free medium, which is known to block Na^+-K^+ pump activity and allow accumulation of sodium inside the cells. Likewise, increasing the sodium permeability of the plasma membrane by monensin abolished the inhibition of the pump activity. In both cases, incubation of the cells with 4.7 mM potassium and tracer amounts of $^{86}Rb^+$ resulted in a very large uptake of K^+($^{86}Rb^+$) into the cells (up to 2 nmol per 10^6 cells min^{-1}), indicating a high activity of the Na^+-K^+ pump.

4 These observations support the view that long-term incubation of rat peritoneal mast cells in a calcium-free medium increases the permeability of the plasma membrane to sodium, and the consequent increase in the intracellular concentration of sodium causes an increase in the activity of the pump. Addition of calcium to the cell suspension decreases the sodium permeability, and hence the pump activity. This hypothesis is supported by the stimulation of pump activity produced by monensin, which is not inhibited by calcium. The enhancement of pump activity after exposure of calcium-deprived cells to EGTA might be the result of a further increase in the sodium permeability of the plasma membrane.

Introduction

The enhancement by digitalis glycosides of secretory responses of various tissues, including rat mast cells, is well documented (Frossard *et al.*, 1983; Amellal *et al.*, 1984; 1985 and references therein; Binck *et al.*, 1985). However, the effect of ouabain on histamine secretion is evident in the absence, but not in the presence, of extracellular calcium. Recently, the Na^+-K^+ pump mediating ouabain-sensitive K^+($^{86}Rb^+$)-uptake into intact rat peritoneal mast cells has been characterized (Knudsen & Johansen, 1989). In addition, a high sensitivity of pump activity to extracellular calcium was demonstrated, in that there was a marked decrease after exposure of the mast cells to micromolar concentrations of calcium.

The present investigation was performed in order to study the mechanism of inhibition of the Na^+-K^+

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pump by calcium. The dependence of the inhibition on the presence of extracellular potassium, the interaction between calcium and EGTA and the effect on the inhibition of increasing the plasma membrane permeability for sodium by monensin were examined. It is suggested that modulation by calcium of the permeability of the plasma membrane to sodium leads to a variation in the stimulation of the pump from inside the cell.

Methods

Isolation of mast cells

Male Sprague-Dawley rats, 215–405 g, were used for the experiments. Peritoneal mast cells were isolated from mixed peritoneal cells by differential centrifugation on a self-generated gradient of Percoll, essentially as described previously (Kundsen & Johansen, 1989). Briefly, the rats were killed by decapitation under light ether anaesthesia. Ten ml of Krebs-Ringer solution prewarmed to 37°C was injected into the abdominal cavity through a small incision in the abdominal wall. After 1 min of gentle massage of the abdominal wall, the mixed peritoneal cells were removed and cooled in an ice-chilled waterbath. The remaining part of the cell isolation was carried out at 4°C. After the differential centrifugation in Percoll the mast cells were concentrated in the lower third of the gradient, whereas the peritoneal leucocytes and macrophages were located in the upper third of the gradient. The latter was removed and the mast cells were washed three times with Krebs-Ringer solution by centrifugation at 220 *g* for 10 min and resuspension in order to remove the remaining Percoll. Then for the experiments the cells were suspended in a Krebs-Ringer solution containing bovine serum albumin, 1 mg ml^{-1}, and glucose, 1 mg ml^{-1}, pH 7.4.

A coulter counter (Model 134, Analys Instrument AB, Sweden) was used to count the number of cells, and inspection of a stained smear (Toluidine Blue) was used to determine the fraction of mast cells: 97.6 ± 2.7% (mean ± s.d., $n = 35$) of the cell suspensions.

Incubation procedure

Mast cell suspensions pooled from 5 to 6 rats were divided into samples with the same cell density in a volume of 0.4 ml. Each sample contained between 6.7×10^4 and 3.05×10^5 cells. After a preincubation period at 37°C the samples, in duplicate, were incubated with potassium and ^{86}rubidium ($^{86}Rb^+$) for determination of the cellular $K^+(^{86}Rb^+)$-uptake as described below. The final incubation volume was 0.5 ml. In order to measure the ouabain-resistant $K^+(^{86}Rb^+)$-uptake, samples containing 1 mM ouabain were run in parallel. For further details of the preincubation periods and drug additions, see legends to figures.

Measurements of cellular $K^+(^{86}Rb^+)$-uptake and Na^+-K^+ pump activity

The cells were incubated for 5 min in a medium containing potassium, 4.7 mM, or 0.24 to 8.0 mM (Figure 2), and with tracer amounts of the radioactive potassium analogue ^{86}rubidium ($^{86}Rb^+$) present (the concentration of ^{86}Rb was less than 5 μM). Incubations were terminated by addition of 9.5 ml of ice-cold Krebs-Ringer solution. Then the cells were washed twice by centrifugation at 600 *g* for 15 min at 4°C and resuspension in order to remove the extracellular ^{86}Rb. Following the last centrifugation cell lysis was performed by adding 0.5 ml of an aqueous solution of NaOH (47.6 mM) to the samples. The whole of each sample was then transferred to a scintillation vial (5 ml) and mixed with Ecoscint. Cellular uptake of $^{86}Rb^+$ was measured in a Mark III Liquid Scintillation Spectrometer (Nuclear Chicago) using the preset window for ^{32}P. Counting efficiency was 90%. The specific activity of potassium in the extracellular medium was used to calculate the cellular uptake of $K^+(^{86}Rb^+)$. The activity of the Na^+-K^+ pump is equivalent to the fraction of the $K^+(^{86}Rb^+)$ that is blocked by 1 mM ouabain.

Statistics

Statistical analysis was performed by use of Student's two-tailed *t* test for two groups of data. For comparison of the effect of potassium (Figure 2) and EGTA (Figure 4) on the $K^+(^{86}Rb^+)$-uptake Duncan's multiple comparison test was used. $P < 0.05$ was considered statistically significant.

Solutions

The calcium-free Krebs-Ringer solution, pH 7.4, had the following composition (mM): NaCl 133.8, KCl 4.7, $MgSO_4$ 1.2, Tris-HCl 12.5, and contained bovine serum albumin, 1 mg ml^{-1}, and glucose 1 mg ml^{-1}. Changes in the concentrations of calcium and potassium in the Krebs-Ringer solution were balanced by changes in the concentration of NaCl in order to maintain isotonicity.

Materials

Bovine serum albumin and monensin were supplied by Sigma Chemical Company (St. Louis, U.S.A.),

Percoll by Pharmacia Fine Chemicals (Sweden), Ecoscint by BN plastic (Helsinge, Denmark), ouabain from MecoBenzon (Copenhagen, Denmark) and ^{86}Rb by Amersham (Buckinghamshire, England). The radioactive rubidium was used within 3 months of manufacture in order to minimize complications resulting from the progressive increase in ^{134}caesium relative to ^{86}Rb. EGTA (ethyleneglycol-bis(beta-amino-ethylether)-N,N'-tetraacetic acid) and all other chemicals were of analytical grade.

Monensin was dissolved in ethanol (stock solution 1 mM) and diluted in Krebs-Ringer solution for the experiments. The final concentration of ethanol was 1% (v/v). Control experiments were performed as described in the legend to Figure 6 for the experiments with monensin without calcium. The ouabain-sensitive $K^+(^{86}Rb^+)$-uptake of the cells was 175 and 308 pmol per 10^6 cells min^{-1} in the absence and presence of ethanol, 1% (v/v), respectively (mean values from two experiments). The ouabain-resistant uptake was not changed by ethanol (data not shown). Monensin, 10^{-5} mol l^{-1}, was not toxic to the cells, since more than 96% of the cells excluded Trypan Blue. EGTA was dissolved in Krebs-Ringer solution, and the pH adjusted to 7.4 with NaOH. After exposure of the cells to 3 mM EGTA for 15 min 97% of the cells excluded the dye. These cells were preincubated for 30 min at 37°C in the presence of potassium, but in the absence of calcium.

Results

Calcium and potassium

Whereas the addition of calcium (0.1–1 mM) had no effect on the ouabain-resistant $K^+(^{86}Rb^+)$-uptake of the mast cells, the Na^+-K^+ pump activity was almost completely blocked after preincubation of the cells for 1 min with 1 mM calcium (Figure 1). A ten times lower concentration of calcium (0.1 mM) seemed to cause a slow reduction of pump activity, but the modest decreases observed after preincubation for 15 to 60 min (the longest period of observation) were not significantly different from control values from cells preincubated in a calcium-free medium.

In the presence of 1 mM calcium the $K^+(^{86}Rb^+)$-uptake by ouabain-treated cells tended to be enhanced at 30 and 60 min compared to the values obtained from cells incubated with calcium alone (Figure 1). However, neither of these differences was statistically significant nor were the small differences between $K^+(^{86}Rb^+)$-uptake into ouabain-treated cells in the presence and absence of calcium at 30 and 60 min. In view of the lack of effect of calcium on ouabain-resistant $K^+(^{86}Rb^+)$-uptake, in some of the subsequent experiments only the total uptake of $K^+(^{86}Rb^+)$ into the mast cells was measured.

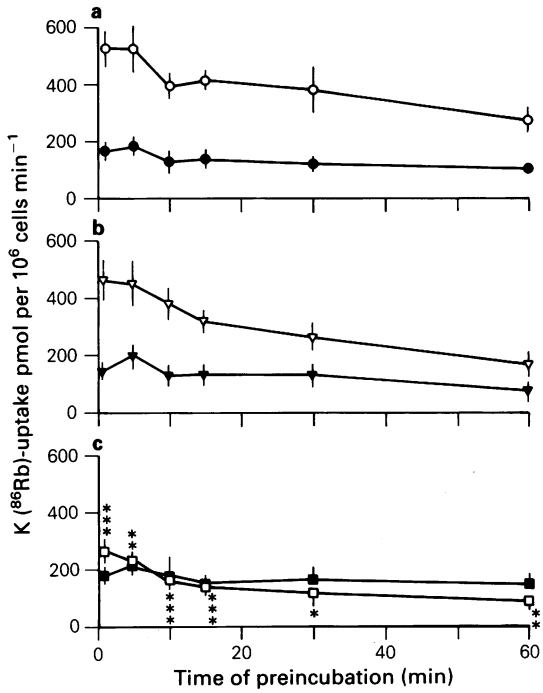

Figure 1 Time course of the decrease in the $K^+(^{86}Rb^+)$-uptake due to exposure of the cells to calcium, 0.1 mM (b) and 1.0 mM (c). Control samples were run in parallel in a calcium-free medium (a). Before the addition of calcium, the cells were preincubated for 30 min in a calcium-free medium. Abscissa scales: time of exposure of the cells to calcium (min) before the incubation with $K^+(^{86}Rb^+)$. Ordinate scales: cellular $K^+(^{86}Rb^+)$-uptake in the absence (open symbols) and presence (closed symbols) of 1 mM ouabain. Ouabain and potassium, 4.7 mM, were present throughout. The Na^+-K^+ pump activity was obtained by subtracting the ouabain-resistant uptake from the total cellular uptake of $K^+(^{86}Rb^+)$. Mean values from 5–10 experiments are shown; vertical lines show s.e.mean. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ indicate the levels of significance for the comparison between the total $K^+(^{86}Rb^+)$-uptake in samples and controls.

Inhibition of pump activity by 0.5 mM calcium was not counteracted by varying the extracellular concentration of potassium from 1.5 to 8.0 mM, i.e. above and below the physiological level of extracellular potassium (Figure 2). However, calcium (0.5 mM) was without effect in the presence of 0.24 mM potassium. The pump activity was significantly increased in the presence of 1.5 mM potassium, both in the presence ($P < 0.01$) and absence ($P < 0.05$) of calcium, and the activity in the presence

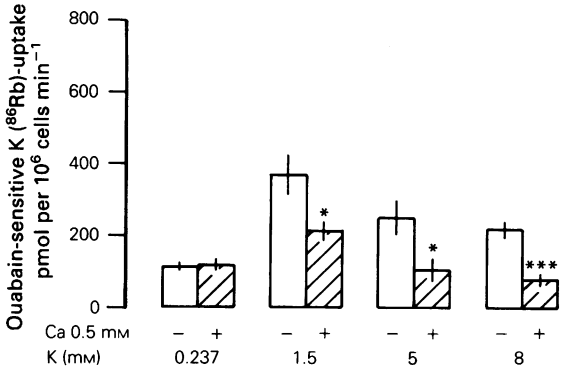

Figure 2 Effect of extracellular potassium on the inhibition by calcium (0.5 mM) of Na^+-K^+ pump activity. The cells were temperature equilibrated for 10 min at 37°C (no calcium, no potassium), and before the incubation with $K^+(^{86}Rb^+)$ the cells were preincubated for 30 min with calcium and potassium as indicated on the abscissa scale. Samples with and without 1 mM ouabain were run in parallel in order to calculate the ouabain-sensitive $K^+(^{86}Rb^+)$-uptake, i.e. the pump activity. Open columns show the pump activity in the absence of calcium and hatched columns show the pump activity in the presence of calcium. Mean values and s.e.mean (vertical lines) from 5 experiments. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ indicate the levels of significance for the comparison between the pump activity in the presence and absence of calcium

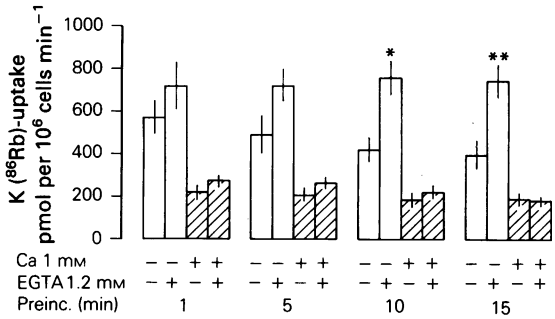

Figure 3 Time-dependence of the effect of EGTA on the cellular uptake of $K^+(^{86}Rb^+)$ in the presence and absence of calcium. The cells were preincubated for 30 min at 37°C with 4.7 mM potassium with or without the addition of 1 mM calcium. After exposure to 1.2 mM EGTA for 1 to 15 min the cells were incubated with $K^+(^{86}Rb^+)$ for 5 min. Control samples were treated identically but without the addition of EGTA to the medium. Abscissa scale: time of exposure of the cells to EGTA (min). Open columns show the uptake in the absence of calcium and hatched columns show the uptake in the presence of calcium. Mean values and s.e.mean (vertical lines) from 5 experiments. * $P < 0.05$; ** $P < 0.01$ indicate the level of significance for the effect of EGTA on the $K^+(^{86}Rb^+)$-uptake into calcium-deprived cells.

of 0.24 mM potassium was lower than the values obtained with 1.5 mM and 5.0 mM potassium ($P < 0.05$) in a calcium-free medium. The ouabain-resistant $K^+(^{86}Rb^+)$-uptake was proportional to the extracellular concentration of $K^+(^{86}Rb^+)$ from 6 to 312 pmol per 10^6 cells min^{-1}, and there was no significant difference between the values determined in the presence and absence of calcium ($P < 0.5$).

Calcium and EGTA

The subsequent addition of EGTA had no effect on the inhibition by calcium of the cellular uptake of $K^+(^{86}Rb^+)$ (Figure 3). The final concentration of EGTA was only 0.2 mM in excess of the concentration of calcium. In contrast, 1.2 mM EGTA enhanced the uptake into mast cells incubated in a calcium-free medium after exposure of the cells to EGTA for 10–15 min.

The low value of cellular $K^+(^{86}Rb^+)$-uptake into calcium-treated cells was increased modestly, but significantly, after exposure of the cells to 3 mM EGTA for 1 ($P < 0.05$) and 15 min ($P < 0.01$) (Figure 4). In a calcium-free medium EGTA, 0.1–3.0 mM caused a large increase of the $K^+(^{86}Rb^+)$-uptake at 1 ($P < 0.05$) and 15 min ($P < 0.01$), but the effect of 0.1 mM EGTA did not differ significantly from that of 3.0 mM.

Potassium-free medium

Incubation of mast cells in a calcium-containing medium (0.5 mM) for up to 90 min had no inhibitory effect on the cellular $K^+(^{86}Rb^+)$-uptake when potassium was omitted from the medium. This is demonstrated in Figure 5 by the curves obtained in the presence and absence of calcium. The small differences between the two curves were not statistically significant, except at 15 min.

Monensin

Monensin is an anionic carrier substance that forms electroneutral complexes with Na^+ in a 10 fold preference over K^+ and transports Na^+ down the chemical gradient across the plasma membrane into the cell (Pressman, 1968). In the presence of 10 μM monensin calcium no longer inhibited Na^+-K^+ pump activity (Figure 6). Moreover, monensin alone increased the pump activity by a factor of 3.5 (which includes an effect of ethanol, the vehicle used to dissolve monensin, see data in the Materials section) compared to the value observed in the absence of calcium. Monensin had no effect on the ouabain-resistant $K^+(^{86}Rb^+)$-uptake. In the calcium-free medium the values were 84 ± 15 and 71 ± 15 pmol per 10^6 cells min^{-1} (mean ± s.e.mean, 5 experiments) in the absence and presence of monensin, respec-

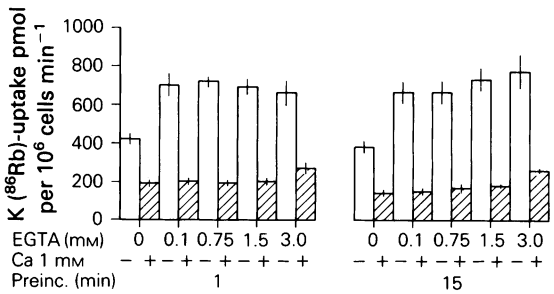

Figure 4 Dose-dependence of the effect of EGTA on the cellular uptake of K^+($^{86}Rb^+$) in the presence and absence of calcium. The cells were preincubated with potassium and with or without calcium (as indicated in legend to Figure 3). Then the cells went through a second preincubation period of 1 or 15 min with increasing concentrations of EGTA (from 0.1 to 3.0 mM as indicated). In the calcium-containing medium the concentration of EGTA was adjusted to the values indicated, but in excess of the concentration of calcium, i.e. 1 mM larger than the values indicated on the abscissa scale. Open columns show uptake in the absence of calcium and hatched columns show the uptake in the presence of calcium. Mean values and s.e.mean (vertical lines) from 5 experiments.

tively, and in the calcium-containing medium the uptake values were 124 ± 22 and 122 ± 11. In both cases the ouabain-resistant uptake of K^+($^{86}Rb^+$) seemed to be larger when calcium was present in the medium. The differences were not statistically significant in the absence of monensin, but the significance level was reached in presence of monensin ($P < 0.05$).

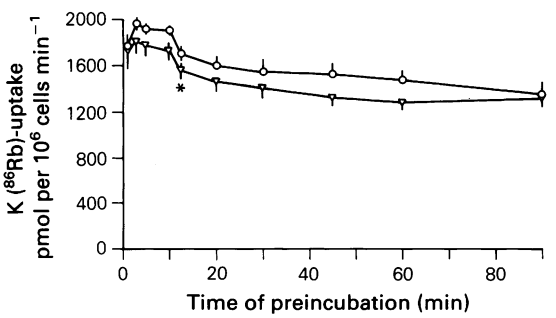

Figure 5 Cellular uptake of K^+($^{86}Rb^+$) after preincubation for 1 to 90 min at 37°C in a potassium-free medium with (▽) or without calcium (○), 0.5 mM, present in the medium. The cells were incubated for five min with 4.7 mM potassium and the radioactive tracer in order to measure the uptake. Mean values from 5 experiments are shown and vertical lines indicate s.e.mean. * $P < 0.05$.

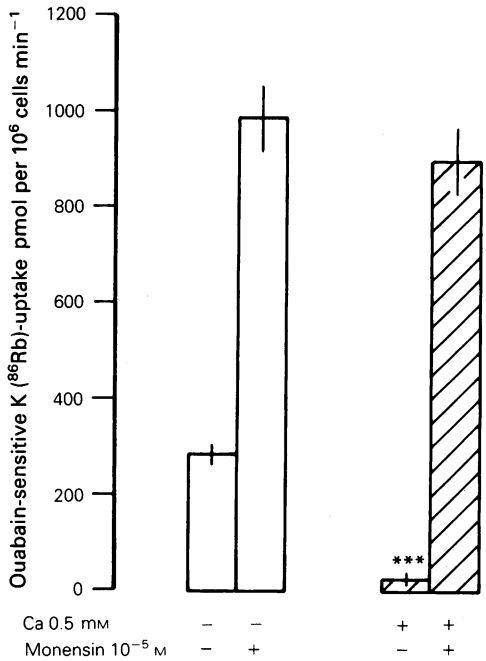

Figure 6 Effect of monensin on the pump activity of mast cells incubated in the presence and absence (control samples) of 0.5 mM calcium. The cells were preincubated for 30 min at 37°C with 4.7 mM potassium in a calcium-free medium. After exposure to monensin, 10 μM, in an additional preincubation period of 30 min, in which the cells were incubated in presence and absence of calcium, the cells were incubated with K^+($^{86}Rb^+$). The control samples were treated identically but without the addition of monensin to the medium. Open columns show uptake in the absence of calcium and hatched columns show uptake in the presence of calcium. Mean values and s.e.mean (vertical lines) from 5 experiments. *** $P < 0.001$ indicates the level of significance for the comparison between the pump activity of cells incubated without monensin and with or without calcium.

Discussion

The time course of the development of inhibition of the Na^+-K^+ pump activity by calcium was dependent on the concentration of calcium in the medium, and the inhibition is likely to be exerted within a narrow concentration range of calcium. It is noticeable that the marked effect of 1 mM calcium was attained within a few min.

In the absence of extracellular potassium Na^+-K^+ pump exhibits no activity, since this requires the simultaneous stimulation of the pump from inside the cell by Na^+ and from outside the cell by K^+. Apparently, inhibition of the pump by calcium does not develop if the pump is inactive (Figure 5). The

presence of potassium in the millimolar range, on the other hand, had only a minor influence on the degree of pump inhibition; the inhibition gradually increased (45%, 60%, and 65%, respectively) in the presence of potassium 1.5, 5.0 and 8.0 mM. This increase in pump inhibition proportional to the increase in potassium argues against the possibility that pump inhibition occurs through an interaction between potassium and calcium. Thus, it seems unlikely that the decrease in $K^+(^{86}Rb^+)$-uptake by calcium reflects a change in the potassium binding site or could be due to partial substitution by calcium for potassium in the sense that calcium takes part in the ion transport by the pump (Lindenmayer & Schwartz, 1975). It has been found that calcium can occupy the binding centre for potassium on the isolated sodium and potassium ion-transport adenosine triphosphatase, but there seems to be a selective barrier against calcium between the potassium binding centre and the solution in contact with the external face of the pump, and calcium would not be expected to be a substrate for transport by the pump (Vasallo & Post, 1986).

The decrease in pump activity measured in the presence of increasing concentrations of potassium (Figure 2) could be due to different affinities of the Na^+-K^+ ATPase for K^+ and Rb^+. K_m values were found by Skou (1960) to be 1.8 mM (K^+) and 2.1 mM (Rb^+) for the isolated enzyme from crab nerve. However, a more likely explanation is changes in pump stimulation from inside the cell due to a variation in the concentration of sodium. Equilibration of the cells is known to decrease the pump activity to a steady state level in 30 min (Knudsen & Johansen, 1989). By equilibration of the cells with increasing concentrations of potassium the intracellular concentration of sodium decreases inversely and becomes rate limiting for the pump activity (Skou, 1986).

In order to study whether the inhibition by calcium (Figure 1, 1 mM calcium) is reversible, the effect of addition of EGTA to calcium-treated cells was examined. Removal of extracellular calcium for up to 15 min before determination of the cellular uptake of $K^+(^{86}Rb^+)$ did not counteract the inhibition (Figure 3). Furthermore, increasing the concentration of EGTA up to a value of 3 mM above the extracellular concentration of calcium had only a minor effect on the $K^+(^{86}Rb^+)$-uptake (Figure 4). These observations indicate that the inhibition of the pump activity by calcium is not readily reversible. Control experiments with EGTA exclude the possibility that EGTA by itself inhibits the pump activity (Figure 4). The somewhat higher control values of cellular $K^+(^{86}Rb^+)$-uptake found in the absence of calcium (1 and 5 min in Figure 3, compared with those in Figure 4) explain the lack of a significant effect of EGTA. The reason for these high values could be a delay in the development of the equilibration of the pump activity (Knudsen & Johansen, 1989).

The very fast development of pump inhibition by calcium (Figure 1) supports the view that this effect may occur through an interaction between extracellular calcium and a surface constituent of the plasma membrane. The enhancement of the uptake of $K^+(^{86}Rb^+)$ observed after exposure of the cells to even a low concentration of EGTA for 1 min (Figure 4) is consistent with this, assuming that chelation of calcium is involved in the mechanism of action of EGTA. It is not likely that a calcium chelator even at a high concentration would cause serious disturbances of the mast cells as was demonstrated in the Trypan Blue exclusion test. In agreement with this, incubation of mast cells in 2 mM EDTA for 3 h failed to produce any toxic effects (Kagayama & Douglas, 1974; Cochrane & Douglas, 1974), and this treatment had no effect on the spontaneous histamine secretion from rat mast cells (Johansen, 1980).

The inhibition by calcium of the activity of the isolated Na^+, K^+-ATPase (Skou, 1957) occurs by binding to the sites for Mg^{2+} and Na^+ (Tobin *et al.*, 1973; Lindenmayer & Schwartz, 1975; Apell & Marcus, 1986), located on the inside of the plasma membrane. However, the pump inhibition in mast cells cannot be explained by an effect of calcium on these sites, since this would require a large increase in cytosol calcium (Lindenmayer & Schwartz, 1975) which is incompatible with an uninhibited aerobic energy metabolism as indicated by the stable level of adenosine triphosphate in these cells in the presence of calcium (references in Johansen, 1987).

Recently, we proposed that changes in the activity of the Na^+-K^+ pump may reflect changes in the sodium permeability of the plasma membrane (Knudsen & Johansen, 1989). An increased transport of Na^+ into the cells may explain the high rate of ouabain-sensitive $K^+(^{86}Rb^+)$-uptake of the mast cells incubated in a calcium-free medium, and the addition of calcium then decreases the Na^+-permeability of the plasma membrane and hence the stimulation of the pump mechanism from inside the cell. This hypothesis is supported by the present observation of the dependence of the inhibition on extracellular potassium. In the presence of extracellular potassium in the millimolar range the pump activity is mainly dependent on the intracellular concentration of sodium, and below that level the extracellular potassium concentration may be rate limiting for the outward transport of Na^+ (Aviv *et al.*, 1983; Skou, 1986). In the last case a decrease in the permeability of the plasma membrane and the subsequent decrease in the intracellular stimulation may not become apparent (Figure 2). Furthermore, increasing the permeability of the plasma membrane

to Na^+ by the Na^+-ionophore, monensin, would mimic the effect of cellular calcium-deprivation and, therefore, abolish the effect of calcium (Figure 6). The effect of ethanol is generally assumed to be due to an increase in the fluidity of the plasma membrane, either the bulk lipid or protein-associated lipid domains of the membrane (Wood & Schroeder, 1988). Although this may change the activity of membrane-bound proteins, more specific effects of ethanol on membrane-bound protein have also been demonstrated (Bode & Molinoff, 1988). In addition, Knutsson & Katz (1967) showed that ethanol increases the permeability of the plasma membrane to Na^+, and this may be the most likely explanation of the increased pump activity caused by ethanol in the present investigation.

The possibility may be considered that in a calcium-free medium a high rate of the sodium-calcium exchange mechanism in the plasma membrane could explain the increased permeability of the plasma membrane to sodium and the increased stimulation of the Na^+-K^+ pump from inside the cell. This is, however, not a likely explanation in view of the low cytosol concentration in resting cells ($< 1\,\mu M$) compared to the K_m value for the exchange mechanism ($> 10\,\mu M$) (Philipson, 1985; Reeves, 1985). In addition, a calcium-free medium may contain micromolar concentrations of calcium in the absence of a calcium chelator, and this would limit the rate of exchange to some extent.

The total uptake of $K^+(^{86}Rb^+)$ after preincubation for 30 min in a potassium-free medium (Figure 5) was about 1.5 nmol per 10^6 cells min^{-1}. In the presence of monensin the pump activity was about 0.94 nmol per 10^6 cells min^{-1}. By addition of the ouabain-resistant uptake in these experiments, 0.09 nmol per 10^6 cells min^{-1}, a total uptake of 1.03 nmol per 10^6 cells min^{-1} was found. The lower value observed with monensin may be explained by the fact that the intracellular concentration of Na^+ in monensin-treated cells is determined by the combined effect of a high rate of inward, passive transport of Na^+, and a high rate of outward, active transport of Na^+ by the pump. In the experiments with potassium-free medium Na^+ accumulates during the cell preparation, since it was performed at 4°C, and this is known to block the pump activity.

The high rate of cellular $K^+(^{86}Rb^+)$-uptake that is maintained for up to 90 min incubation of the cells at 37°C (Figure 5) indicates a minimal net extrusion of Na^+ during this time period. Since in the absence of potassium and of calcium the $K^+(^{86}Rb^+)$-uptake did not increase during a prolonged preincubation period at 37°C, it is likely that the maximum attainable stimulation of the mast cells from inside the cells is obtained after 5–10 min of preincubation at 37°C. Then a total uptake of $K^+(^{86}Rb^+)$ of about 2.00 nmol per 10^6 cells min^{-1} was measured in the presence of 4.7 mM potassium in the medium.

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The mechanism of tetrahydroaminoacridine-evoked release of endogenous 5-hydroxytryptamine and dopamine from rat brain tissue prisms

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1 Tetrahydroaminoacridine (THA) is an acetylcholinesterase (AChE) inhibitor which may have a greater therapeutic effect in Alzheimer-type dementia (ATD) than other cholinergic agents. This suggests possible non-cholinergic properties. We have therefore studied the effects of THA on the release of endogenous 5-hydroxytryptamine (5-HT) from rat cortical prisms and dopamine from striatal prisms.

2 In the presence of K^+ (1 mM), THA stimulated release of both 5-HT and dopamine. THA (100 μM)-evoked monoamine release was comparable, but not additive with the release produced by K^+ (35 mM). The effect was not maximal at 1 mM THA. THA-evoked release of 5-HT was independent of the presence of Ca^{2+} in the external medium.

3 Drugs acting on the cholinergic system, nicotine, mecamylamine, atropine, oxotremorine, physostigmine and neostigmine (all 10 μM) had no effect on 5-HT and dopamine release. 4-Aminopyridine (4-AP), a potent acetylcholine-releasing agent, had no effect on 5-HT release and was approximately 100 fold less active than THA on dopamine release.

4 Both THA and reserpine enhanced the release of 5-HT in the presence of the monoamine oxidase inhibitor, pargyline. Reserpine- but not THA-evoked release was abolished in the absence of pargyline. Reserpine (5 mg kg^{-1}, i.p.) markedly depleted brain monoamine concentrations 3 h after injection, while THA (15 mg kg^{-1}, i.p.) had no effect.

5 Chloroamphetamine and fenfluramine both released 5-HT in a Ca^{2+}-independent manner and with a similar potency to THA, while (+)-amphetamine released dopamine with a similar potency to THA. The effects of the amphetamines were not maximal at 1 mM. However, unlike THA, chloroamphetamine-evoked release of 5-HT was additive with release evoked by K^+ (35 mM).

6 Clomipramine (IC_{50} = 0.036 μM), imipramine (IC_{50} = 0.20 μM) and THA (IC_{50} = 19.9 μM) all inhibited the uptake of [^{3}H]-5-HT into a P_2 membrane preparation. However, none of these compounds inhibited [^{3}H]-5-HT uptake into tissue prisms during the release experiments in which the reuptake inhibitor fluoxetine (5 μM) was present.

7 We conclude that THA does not release endogenous 5-HT through a cholinergic, reserpine- or amphetamine-like mechanism or through inhibition of reuptake. The possibility exists that the release may occur via blockade of 4-AP-insensitive K^+ channels.

Introduction

Tetrahydroaminoacridine (THA) is a potent, reversible inhibitor of acetylcholinesterase which has been reported to be of therapeutic potential in some patients with Alzheimer-type dementia (ATD) (Summers *et al.*, 1986). The effectiveness of THA in comparison with other agents acting on the cholinergic system has led to the suggestion that THA may exert at least part of its action through some as yet undefined mechanism. The structural similarity between THA and 4-aminopyridine led Summers *et al.* (1986) to suggest that THA may block neuronal potassium channels in a manner similar to 4-aminopyridine and thus enhance acetylcholine release. Subsequent electrophysiological experiments (Drukarch *et al.*, 1987; Osterrieder, 1987; Rogawski, 1987; Schauf & Sattin, 1987; Stevens & Cotman, 1987) have confirmed that THA blocks potassium channels in a variety of preparations, although, in

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general, high concentrations of THA (0.1–1 mM) are required.

More recently Drukarch *et al.* (1988) have found that THA releases radiolabelled noradrenaline, 5-hydroxytryptamine (5-HT) and dopamine from preloaded slices, and they suggested that this activity was through a reserpine-like action by interfering with the uptake mechanism of these transmitters into storage vesicles. We describe here the effects of THA on the release of endogenous 5-HT from cortical prisms and dopamine from striatal prisms. The mechanism of action of THA is compared with cholinergic ligands, K^+-channel blockers, and compounds known to influence monoamine release.

A preliminary account of some of this work has been presented to the British Pharmacological Society (De Souza *et al.*, 1989).

Methods

Animals

Adult male Lister Hooded rats (Harlan Olac, Bicester) were used for all experiments. Animals weighed 200–300 g and were housed in groups of 6 in conditions of constant temperature (21°C) and controlled lighting (light period 07 h 00 min–19 h 00 min) and fed an *ad libitum* diet of RMI expanded pellets and tap water.

Monoamine release from tissue prisms

The release of endogenous 5-HT and dopamine from tissue prisms was measured by a modification of the method of Auerbach & Lipton (1985).

Rats were stunned and killed by decapitation and the brains were rapidly removed. The olfactory tracts were separated from the inferior surface of the frontal cortex and a single coronal cut was made to separate the frontal cortex from the rest of the brain. Frontal cortex was used to measure endogenous 5-HT release. Striata were removed for the measurement of endogenous dopamine release. Prisms of tissue (300 × 300 μm, 45°) were prepared with a McIlwain tissue chopper. The slices were suspended in 30 ml Krebs-Ringer bicarbonate buffer (pH 7.4, containing (mM): NaCl 118.5, KH_2PO_4 1.18 mM, $MgSO_4$ 1.18 mM, $NaHCO_3$ 25 mM, $CaCl_2$ 2.54 mM and glucose 10). For the measurement of 5-HT release the 5-HT uptake inhibitor, fluoxetine (5 μM) was present and for the measurement of dopamine release, nomifensine (10 μM), a dopamine uptake inhibitor, was present in the buffer throughout unless otherwise stated. Both 5-HT and dopamine release were routinely measured in the presence of 10 μM pargyline.

Tissue prisms were preincubated at 37°C for 1 h, the buffer being changed every 15 min. The buffer was discarded leaving a packed slice suspension. Aliquots of the slice suspension (60 μl, 1 mg protein) were distributed evenly into baskets made from plastic test tubes with nylon mesh bases. The baskets of slices were preincubated in 1 ml of buffer for 10 min at 37°C. The baskets were blotted and immersed in a second set of vials containing 1 ml fresh, pregassed buffer (containing drug where appropriate) and incubated for 5 min (basal release). The baskets were blotted and transferred into vials containing buffer plus 35 mM KCl (containing drug where appropriate) for a further 5 min (K^+-evoked release). The baskets were then blotted and placed in vials containing 1 ml of 0.1 M NaOH solution containing sodium dodecyl sulphate (0.7% w/v) and $NaCO_3$ (2% w/v) to solubilize the slices prior to analysis of the protein content by a phenol reagent technique (Dawson & Heatlie, 1984). Supernatants (800 μl) from the basal and K^+-evoked incubations were mixed with perchloric acid (2 M, containing cysteine, 1% w/v; sodium metabisulphite, 1% w/v; EDTA, 0.1% w/v), centrifuged at 1000 *g* for 5 min and the supernatants analysed for their 5-HT or dopamine content.

[^{3}H]-5-HT uptake under basal release conditions

Tissue prisms were prepared from rat frontal cortex and incubated as described for the basal release of 5-HT, except that [^{3}H]-5-HT (10 nCi) was present during the 5 min incubation period. The baskets were then rapidly washed twice in ice-cold Krebs buffer before being immersed in solubilising solution as described above. Duplicate aliquots of the solubilised slices were taken for liquid scintillation counting, and protein was assayed in the remainder as described above.

Analysis of 5-HT, dopamine and their metabolites by h.p.l.c.

The chromatographic system comprised a Spectroflow SF 400 pump and a Promis automatic sample injector (Spark-Holland). Residual microparticulate material was removed by a guard column (30 × 4.6 mm, Spheri-5-RP-18). A reversed phase analytical cartridge column (100 × 4.6, Spheri-5-OD-MP, Anachem) was used at a constant temperature of 30°C. Analytes were detected with a model 5010 electrochemical cell (ESA) and the potential across the electrodes (+0.35 V) was applied from an ESA Coulochem controller (model 5100A). The mobile phase (flow rate 1 ml min^{-1}) comprised KH_2PO_4 (0.1 M), EDTA (0.0035% w/v), sodium octane sulphonate (0.016% w/v) and methanol (10%

v/v) and was adjusted to pH 3.0 with concentrated phosphoric acid and degassed before use. Chromatograms were analysed using the peak area ratio of standards to analyte.

Animal treatment

Groups of rats were pretreated with either vehicle (0.9% w/v NaCl), THA (15 mg kg^{-1}), reserpine (5 mg kg^{-1}), fenfluramine (10 mg kg^{-1}) or amphetamine (3 mg kg^{-1}) and the animals killed 3 h later. All drugs were injected i.p. The brains were dissected, and the frontal cortices, striata and hippocampi were removed onto solid CO_2. Monoamines were extracted from these samples and estimated by h.p.l.c. Drugs were dissolved in saline with the exception of reserpine which was dissolved in an acetic acid/water mixture adjusted to pH 5 with 1 M NaOH.

Tissue extraction

Brain regions were homogenized in ice-cold 0.2 M perchloric acid containing 0.1% (w/v) cysteine, 0.1% (w/v) sodium metabisulphite and 0.01% (w/v) EDTA using a 2 ml glass/glass Potter-Elvejham homogenizer. The homogenates were centrifuged at 15,000 *g* for 5 min and the supernatants analysed for 5-HT, dopamine, and their metabolites by h.p.l.c.

Uptake of [^{3}H]-5-HT by a washed P_2 preparation

Fresh whole rat cortex was homogenized in 10 volumes of 50 mM Tris-HCl, pH 7.4 containing 0.32 M sucrose with a Potter-Elvejham homogenizer, and the homogenate centrifuged at 1000 *g* for 5 min at 4°C. The supernatant was removed, centrifuged at 20,000 *g* for 20 min at 4°C and the resulting pellet resuspended, by use of a glass/teflon homogenizer in 20 volumes oxygenated Krebs-Ringer phosphate-buffered saline (KRS (mM): NaCl 118, NaH_2PO_4 32, KCl 4.7, $CaCl_2$ 1.8, $MgSO_4$ 1.2, EDTA 1.3, glucose 5.6 and ascorbic acid 1.7; pargyline 100 μM, pH 7.4 with 2 M NaOH). The resuspended preparation was further diluted with KRS to 40 volumes and incubated at 37°C for 15 min before use at this dilution in the [^{3}H]-5-HT uptake assay.

P_2 preparations were incubated in 96-well microtitre plates in the presence of [^{3}H]-5-HT (0.1 μCi per well) and drugs where appropriate. Following 5 min incubation at 37°C the membranes were filtered through GF/B glassfibre filters using a Skatron semi-automatic cell-harvester and the filters washed with 0.9% NaCl (2.5 ml) in 4 s at room temperature. The filters were dried and the radioactivity counted by liquid scintillation counting. Specific [^{3}H]-5-HT uptake was defined as the difference in uptake in the absence and presence of 100 μM imipramine.

Statistics

Where appropriate, data were analysed by a one-way analysis of variance followed by Dunnett's test for differences from control.

Drugs

Sodium-1-octane sulphonic acid was obtained from Eastman-Kodak (Liverpool) and 'h.p.l.c. grade' methanol and H_2O from Rathburn Chemicals (Walkerburn, Scotland). [^{3}H]-5-HT (27.8 Ci mmol^{-1}) was supplied by New England Nuclear Du Pont (U.K.) and fluoxetine and nomifensine were gifts from Eli Lilly Co. (Indiana, U.S.A.) and Hoechst Pharmaceuticals (Hounslow), respectively. 9-Amino-1,2,3,4-tetrahydroacridine HCl hydrate (THA) was supplied by Aldrich Chemical Co. (Dorset) and all other chemicals were obtained from Sigma Chemical Co. (Poole).

Results

Effects of drugs on the release of endogenous 5-HT

The release of endogenous 5-HT from tissue prisms of rat frontal cortex was dependent on the K^+ ion concentration with a plateau at 50–70 mM K^+ (data not shown). A K^+ concentration of 35 mM was used for the standard K^+-evoked release in all subsequent experiments. THA stimulated the basal release of 5-HT and the release produced by THA (100 μM) was comparable to that evoked by K^+ (35 mM) (Figure 1). Chloroamphetamine and fenfluramine (100 μM) also caused release of 5-HT (78% and 68% of the K^+-evoked signal respectively). Reserpine caused less release of 5-HT than the above compounds (33% of the K^+-evoked release) while 4-aminopyridine (4-AP) did not produce any significant release of 5-HT (Figure 1).

In the presence of THA (100 μM) no additional release of 5-HT was observed in the presence of 35 mM K^+. However, in the presence of chloroamphetamine (100 μM) or fenfluramine (100 μM) additional 5-HT release was observed with K^+ (35 mM) (Figure 1).

When 5-HT release was investigated in the absence of added Ca^{2+}, the K^+-evoked signal was abolished (Figure 1), but the release caused by THA was unaffected. K^+ did not evoke any further release when combined with the drugs in the absence of Ca^{2+} (Figure 1).

THA was more potent than fenfluramine in stimulating basal 5-HT release, but neither of these compounds had produced a maximal effect by 1 mM, at which concentration the release of 5-HT by THA

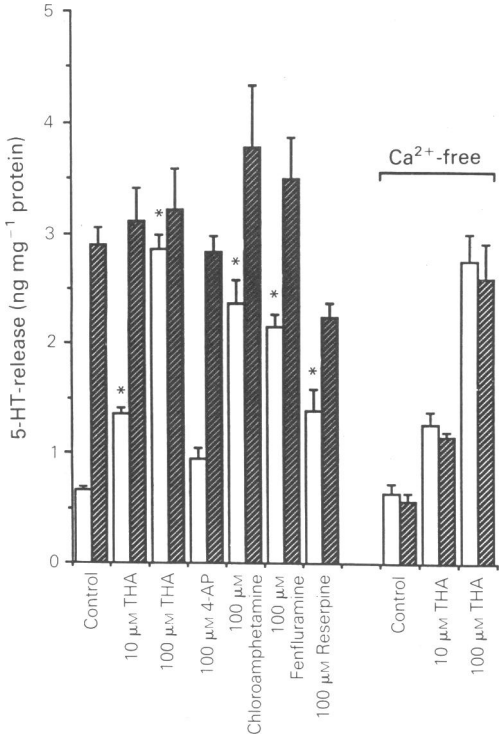

Figure 1 Effects of drugs on the release of endogenous 5-HT in the presence and absence of Ca^{2+}. Drugs were tested at 10 μM and 100 μM as indicated. Results are the mean of 3–27 experiments, each conducted in triplicate; vertical bars show s.e.mean. In the presence of Ca^{2+}, basal release (open columns) in the presence of drug was tested for significant difference from control basal release. K^+-evoked release (35 mM, hatched column) in the presence of drug was tested against control K^+-evoked release. $^*P < 0.01$. Experiments conducted in the absence of Ca^{2+} lacked 2.5 mM $CaCl_2$ in the buffers throughout the experiment. In the absence of Ca^{2+} there was no significant difference between basal and K^+-evoked release in each condition ($P < 0.05$).

was comparable to that evoked by K^+ at 70 mM (Figure 2).

Effects of drugs on the release of endogenous dopamine

THA and (+)-amphetamine stimulated the basal release of dopamine and the release produced by these compounds at 100 μM was comparable to that evoked by K^+ (35 mM) (Figure 3). Reserpine and 4-AP (100 μM) caused less release than THA (22% and 25% of the K^+-evoked signal respectively). THA and (+)-amphetamine were of similar potency in releasing dopamine (Figure 4) and these compounds

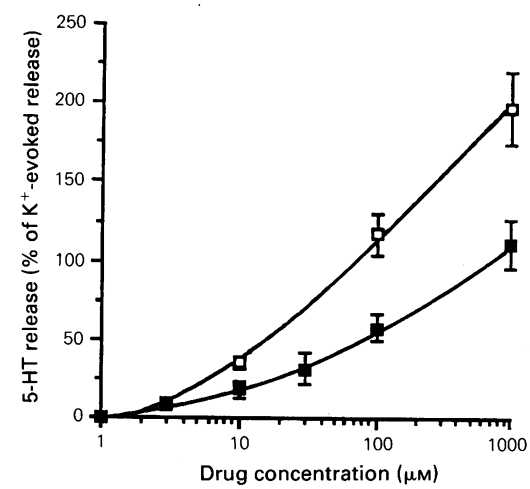

Figure 2 Concentration-dependency of drug evoked 5-HT release. Results are mean of 3 experiments each conducted in triplicate; vertical bars show s.e.mean. Lack of error bar means it is within the point. Data are expressed as a percentage of K^+-evoked release (35 mM) with basal release subtracted. Tetrahydroaminoacridine (□); fenfluramine (■).

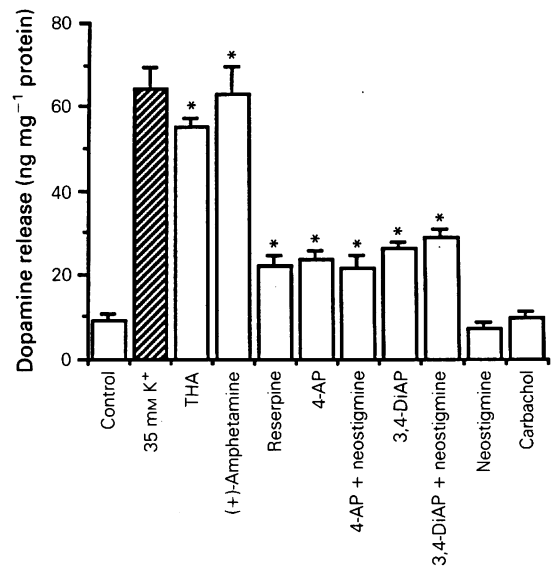

Figure 3 Effects of drugs on the release of endogenous dopamine. All drugs were tested at 100 μM except neostigmine (10 μM). The release in the presence of 35 mM K^+ (hatched column) is also shown. 4-AP, 4-aminopyridine; 3,4-diAP, 3,4-diaminopyridine. Results are the mean of 3–8 experiments, each conducted in triplicate; vertical bars show s.e.mean. Release in the presence of drug was tested for significant difference from control basal release. $^*P < 0.01$.

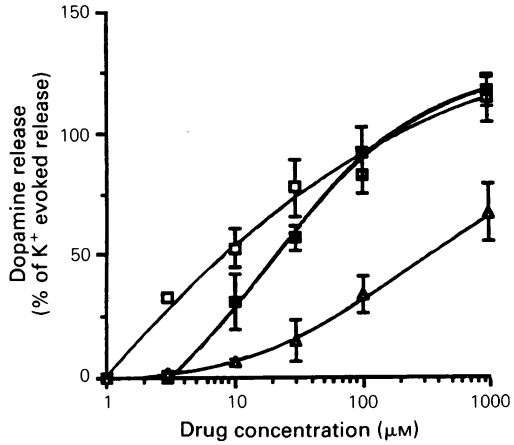

Figure 4 Concentration-dependency of drug evoked dopamine release. Results are mean of 3 experiments, each conducted in triplicate; vertical bars show s.e.mean. Lack of error bar indicates that it is within the point. Data are expressed as a percentage of K^+-evoked release (35 mM) with basal release subtracted. Tetrahydroaminoacridine (□); (+)-amphetamine, (■); 4-aminopyridine (△).

had produced a near maximal effect by 1 mM. 4-AP was nearly 100 fold less potent than THA in releasing dopamine (Figure 4).

Omission of pargyline from the medium

When the 5-HT release experiments were repeated in the absence of pargyline (Figure 5; compare with Figure 1) the observed 5-HT release was significantly decreased compared with experiments conducted in the presence of pargyline. The basal release of 5-HT produced by THA (100 μM) was still comparable, but not additive, with the K^+-evoked release. In contrast, chloroamphetamine-evoked and K^+-evoked release of 5-HT were additive. The reserpine-evoked release was abolished in the absence of pargyline and the K^+-evoked signal was significantly reduced ($P < 0.01$) in the presence of reserpine. In the absence of pargyline, fenfluramine did not significantly stimulate basal 5-HT release but the compound did reduce the K^+-evoked signal ($P < 0.05$).

The effect of drugs acting on the cholinergic system on THA- and 4-AP-induced release of 5-HT and dopamine

Carbachol, nicotine, oxotremorine, physostigmine, atropine and mecamylamine (all at 10 μM) were without effect on basal 5-HT release, and atropine and mecamylamine had no effect on THA (10 μM)-evoked 5-HT release (data not shown). Neostigmine

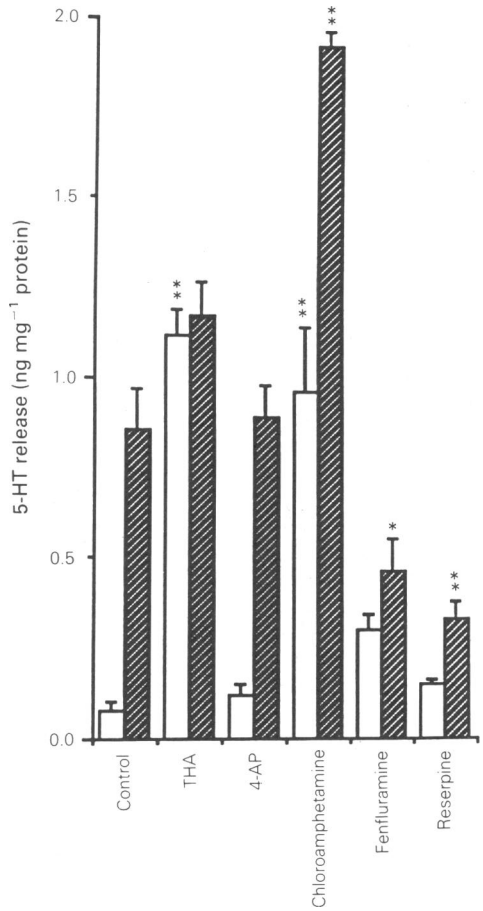

Figure 5 Effect of drugs on basal (open columns) and K^+-evoked (35 mM, hatched columns) 5-HT release in the absence of pargyline. Release was measured as described in the methods except that buffers lacked 50 μM pargyline. All drugs were test at 100 μM. Results are the mean of 3 experiments each conducted in triplicate; vertical bars show s.e.mean. Basal release in the presence of drug was tested for significant difference from control basal release. K^+-evoked release in the presence of drug was tested for significant difference from control K^+-evoked release. $^{**}P < 0.01$; $^{*}P < 0.05$.

(10 μM) and carbachol (100 μM) had no effect on basal dopamine release (Figure 3). 4-AP and 3,4-diaminopyridine, stimulated basal dopamine release (Figure 3), an effect not increased in the presence of neostigmine (10 μM).

Effect of THA on 5-HT reuptake

To examine whether any of the apparent effects on 5-HT release by THA were due to an alteration of reuptake several experiments were performed.

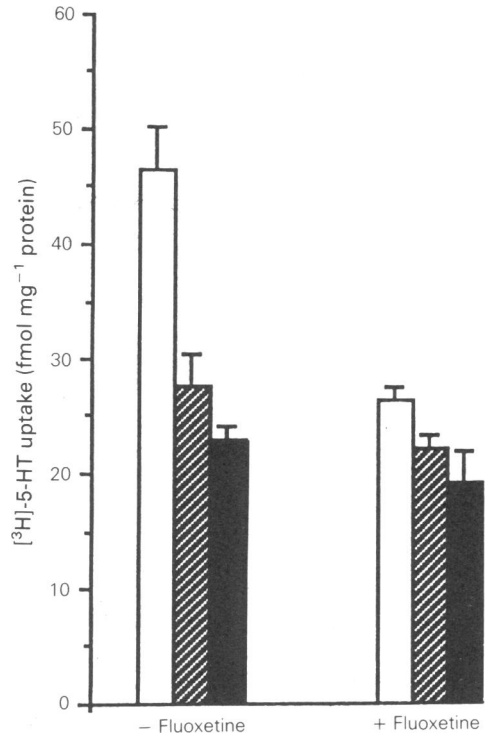

Figure 6 Uptake of [^{3}H]-5-HT by frontal cortical prisms under basal release conditions. Tissue prisms were incubated with [^{3}H]-5-HT under release conditions as described in the methods. Slices were prepared and incubated in the absence and presence of 5 μM fluoxetine as indicated. Results are the mean of 3 experiments, each conducted in triplicate; vertical bars show s.e.mean. Control (open columns); tetrahydroaminoacridine (100 μM, hatched columns); imipramine (100 μM, solid columns).

In the absence of fluoxetine, THA (100 μM) produced the same degree of uptake inhibition of 5-HT into cortical prisms as that seen with imipramine (100 μM). However in the presence of fluoxetine (5 μM), a condition under which all earlier release studies were performed, no further inhibition of uptake was observed following addition of THA or impramine (Figure 6). Furthermore, when inhibition of [^{3}H]-5-HT uptake was measured in a washed P_2 preparation, THA was over 200 fold less potent than imipramine or clomipramine (Table 1).

Effects of drugs on monoamine and metabolite concentrations in rat brain

Administration to rats of THA (15 mg kg^{-1}) or (+)-amphetamine (3 mg kg^{-1}), did not alter the concentration of 5-HT, dopamine or their respective metabolites in the striatum or frontal cortex 3 h later (Table 2). However reserpine (5 mg kg^{-1}) caused pronounced depletion of dopamine and 5-HT with concomitant increases in the concentration of homovanillic acid (HVA) and 5-hydroxyindoleacetic acid (5-HIAA); the fall in dihydroxyphenylacetic acid (DOPAC) was modest but statistically significant. A similar pattern of change was seen for 5-HT and 5-HIAA in the striatum and hippocampus (data not shown). Injection of fenfluramine (10 mg kg^{-1}) caused depletion of 5-HT, but not dopamine, in all three brain areas studied. However fenfluramine differed from reserpine in that there was not a concomitant rise in 5-HIAA concentration.

Table 1 Inhibition of [^{3}H]-5-hydroxytryptamine ([^{3}H]-5-H) uptake in a rat brain P_2 preparation

Compound	IC_{50} (nM)
Clomipramine	36 ± 11
Imipramine	200 ± 9
THA	19,900 ± 7200

The inhibition of [^{3}H]-5-HT uptake by tetrahydroaminoacridine (THA), clomipramine and imipramine was measured as described in the methods. IC_{50} values (nM) are the mean ± s.e.mean of three to six experiments.

Discussion

THA stimulated both basal 5-HT and dopamine release from brain slices in a concentration-dependent way, the stimulation of 5-HT release being calcium-independent. This agrees with a recent report that THA releases prelabelled [^{3}H]-5-HT, [^{3}H]-dopamine and [^{3}H]-noradrenaline from brain tissue prisms (Drukarch *et al.*, 1988).

The effect was clearly seen at a THA concentration of 10^{-5} M. It is however unlikely that the concentration of THA when given therapeutically would rise above 10^{-6} M (Hsieh *et al.*, 1983). While it is difficult to extrapolate from concentrations required *in vitro* to those necessary *in vivo*, the potency of THA was similar to that of the amphetamines, so an effect of THA *in vivo* cannot be ruled out. The effect of THA on monoamine release could occur through a variety of mechanisms, including blockade of reuptake, depletion of monoamine stores or through a cholinergic mechanism. It is well established that both nicotinic and muscarinic presynaptic heteroreceptors can regulate the release of [^{3}H]-dopamine from striatal slices (Giorguieff *et al.*, 1977;

Table 2 Concentrations of monoamines in rat striatum and frontal cortex 3 h following the i.p. injection of saline, tetrahydroaminoacridine (THA, 15 mg kg^{-1}), (+)-amphetamine (3 mg kg^{-1}), fenfluramine (10 mg kg^{-1}) and reserpine (5 mg kg^{-1})

	Striatum			*Frontal cortex*		
	DA	*DOPAC*	*HVA*	*5-HT*	*5-HIAA*	n
Control	4645 ± 230	3086 ± 124	683 ± 47	705 ± 52	347 ± 52	5
THA	3934 ± 480	2958 ± 394	707 ± 76	819 ± 54	419 ± 20	3
(+)-Amphetamine	5012 ± 138	2864 ± 178	930 ± 33	665 ± 24	470 ± 41	6
Fenfluramine	4717 ± 212	2730 ± 196	868 ± 45	**204 ± 26	305 ± 12	6
Reserpine	**259 ± 28	*2183 ± 120	**2432 ± 148	**159 ± 16	**679 ± 40	6

Results are the mean ± s.e.mean from 3–6 animals as indicated (*n*), and are expressed as ng g^{-1} wet weight. Drug treatments are compared with control for each monoamine to test for significant differences in each brain region. **$P < 0.01$; *$P < 0.05$.

DA, dopamine; DOPAC, dihydroxyphenylacetic acid; HVA, homovanillic acid; 5-HIAA, 5-hydroxyindoleacetic acid.

Giorguieff-Chesselet *et al.*, 1979; Lehman & Langer, 1982) and synaptosomes (Sakurai *et al.*, 1982; Rapier *et al.*, 1988). It has also been reported that nicotine will stimulate striatal 5-HT release (Westfall *et al.*, 1983).

In addition to its inhibitory activity against AChE (Shaw & Bentley, 1953; Steinberg *et al.*, 1975) THA is centrally active at both muscarinic and, more weakly, at nicotinic receptors (Nilsson *et al.*, 1987; Hunter *et al.*, 1989; Perry *et al.*, 1988; Pearce & Potter, 1988). It was feasible therefore that THA might modulate monoamine release via ACh receptors, either through a direct action or by increasing the concentration of ACh. However, the cholinoceptor agonist carbachol had no effect on 5-HT or dopamine release (Figure 3) and the selective nicotinic and muscarinic drugs, nicotine, mecamylamine, atropine and oxotremorine, were without effect on 5-HT release under our experimental conditions. Therefore it is unlikely that THA is causing monoamine release through a direct interaction with ACh receptors. Similarly, inhibition of AChE by THA is unlikely to explain the stimulation of monoamine release, as the AChE inhibitors neostigmine and physostigmine were without effect. An alternative explanation might be that THA stimulates the release of ACh and simultaneously potentiates its action on monoamine release through inhibition of AChE (Summers *et al.*, 1986). This is also unlikely however as 4-AP and 3,4-diaminopyridine (3,4-diAP), which are both potent ACh releasing agents (Dolezal & Tucek, 1983; Jones *et al.*, 1988) had markedly less effect than THA on dopamine release (Figure 3) and their action was not potentiated by neostigmine. Furthermore, 4AP did not stimulate 5-HT release. We conclude therefore that THA does not stimulate monoamine release through a cholinergic mechanism.

Reserpine depletes brain monoamine levels through interference with their storage vesicles (Carlsson, 1965) and under our experimental conditions this drug increased basal 5-HT release (Figure 1). THA was more potent than reserpine. However in the absence of pargyline (Figure 5) there was a marked reduction of K^+-evoked release by reserpine, and no stimulation of basal release. In contrast, in the absence of pargyline THA stimulated basal 5-HT release. Following administration to rats, reserpine depleted 5-HT and dopamine in the striatum with a concomitant rise in their respective metabolites, 5-HIAA and HVA, as previously reported (Kuhn *et al.*, 1985), while THA had no significant effect on the tissue concentrations of monoamines (see Table 2). We conclude therefore that THA does not cause monoamine release by a mechanism analogous to that of reserpine. This conclusion conflicts with that of Drukarch *et al.* (1988) who suggested that THA induced monoamine release through interference with the accumulation of monoamines in storage vesicles. In the study of Druckarch *et al.* (1988) the uptake of radiolabelled monoamines was examined and may therefore have involved a different transmitter pool.

Amphetamine releases a reserpine-resistant pool of endogenous dopamine from slices of rat striatum (Niddam *et al.*, 1985). In our experiments THA and amphetamines caused comparable release of dopamine and 5-HT (Figures 2 and 4). This effect was not maximal at 100 μM THA or 100 μM amphetamine and both drugs at a concentration of 1 mM produced markedly greater release than that caused by 35 mM K^+. The non-saturating effect of amphetamine on dopamine release is in agreement with previous observations (Parker & Cubeddu, 1988). The similarities between THA- and amphetamine-evoked monoamine release continue when one looks at the

Ca^{2+}-dependency of their effects (Figure 1) as the release of 5-HT caused by THA (this paper), fenfluramine and chloroamphetamine (Robinson, unpublished observations) was Ca^{2+}-independent. However, the effects of THA and chloroamphetamine on K^+-evoked 5-HT release as well as basal release, either in the presence (Figure 1) or absence (Figure 5) of pargyline were different. THA (100 μM) released a comparable amount of 5-HT to 35 mM K^+, but no additional release was achieved when the drug was combined with 35 mM K^+ while the effects of chloroamphetamine and K^+ (35 mM) were additive implying that amphetamines cause release from a different pool from K^+. As the effects of THA are not additive with K^+, it would seem likely that THA releases 5-HT from a similar pool to K^+. We therefore conclude that THA does not release monoamines through an amphetamine-like mechanism although, *in vitro*, THA is as potent a releaser of monoamines as amphetamines.

A further mechanism which might result in an apparent increase in release is the blockade of reuptake by THA. While THA at high concentrations can inhibit reuptake (Table 1) this effect would not have been seen in the release studies since the presence of fluoxetine (5 μM) apparently blocks the uptake of [^{3}H]-5-HT (Figure 6). While these data are consistent with the observations of Drukarch *et al.* (1988) that THA can inhibit monoamine uptake, they demonstrate that such inhibition is not responsible for the stimulation of 5-HT release.

Summers *et al.* (1986), when reporting on the use of THA in the long-term treatment of ATD patients, suggested that THA might have pharmacological activity in addition to AChE inhibition, possibly resulting from its structural similarity to 4-AP. The latter blocks K^+ channels (Llinas *et al.*, 1976; Yeh *et al.*, 1976) and stimulates the basal release of radiolabelled ACh, GABA and glutamate from mouse brain synaptosomes (Tapia & Sitges, 1982) and radiolabelled dopamine from striatal slices (Arzate *et al.*, 1986). THA also blocks K^+ channels in a number of tissues, although in general high (1–0.1 mM) concentrations are required (Drukarch *et al.*, 1987; Schauf & Sattin, 1987; Rogawski, 1987; Osterrieder, 1987; Stevens & Cotman, 1987) thus raising the possibility that THA might cause monoamine release through modulation of K^+ channels. The K^+ channel modulators, 4-AP and 3,4-diAP, can stimulate the release of endogenous ACh from slices of rat cortex (Vizi *et al.*, 1977; Dolezal & Tucek, 1983; Jones *et al.*, 1988) but THA was much less potent than 4-AP in this system (unpublished observations). However, 4-AP did not cause release of 5-HT, and although 4-AP and 3,4-diAP released dopamine (Figure 3), 4-AP was almost 100 fold less potent than THA (Figure 4). Additionally, THA-evoked release of 5-HT differs from 4-AP-induced ACh release in being insensitive to the removal of Ca^{2+} from the external medium. Therefore if THA affects monoamine release via K^+ channels it must be through a different population of channels from the 4-AP-sensitive channels which modulate ACh release and indeed it has recently been postulated that THA acts on different K^+ currents from 4-AP (Osterrieder, 1987; Stevens & Cotman, 1987). It should be noted that K^+ channel blockers can cause neurotransmitter release in an apparently Ca^{2+}-independent manner (Tapia & Sitges, 1982; Barnes *et al.*, 1987). Recently, Reiner & McGeer (1988) reported that THA increased the action potential duration of central histamine neurones maintained *in vitro* at concentrations as low as 1 μM and this is probably through blockade of K^+ channels. As the authors point out, the membrane properties of histaminergic neurones are very similar to those of 5-HT neurones. These electrophysiological observations therefore complement our data and taken together, suggest that blockade of K^+ channels may explain the stimulation of 5-HT release by THA.

When describing the benefits of THA in the treatment of ATD, Summers *et al.* (1986) suggested that it was probable that THA acts like 4-AP in blocking K^+ channels, and that this was the basis for THA having a more impressive therapeutic activity than classical AChE inhibitors such as physostigmine. We have shown that THA releases endogenous monoamines with an *in vitro* potency similar to that of amphetamine, and that this is not through 4-AP-sensitive K^+ channels. Bearing in mind the established monoamine deficits in ATD (Cross *et al.*, 1986; Palmer *et al.*, 1988) we consider that the ability of THA to stimulate monoamine release may be of therapeutic value.

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The effect of adrenalectomy on interleukin-1 release *in vitro* and *in vivo*

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1 Peritoneal macrophages (Mø) collected from adrenalectomized (ADX) rats released more interleukin-1 (IL-1) activity and prostaglandin E_2 (PGE_2) than macrophages from sham-operated (SHO) rats.

2 The increase in IL-1 activity in the supernatants was confirmed by the increase of the cell-associated 33 kD IL-1α precursor in ADX macrophages stimulated by lipopolysaccharide (LPS).

3 After the injection of Complete Freund's Adjuvant (CFA) to induce adjuvant arthritis, 60% of the ADX rats died, while no deaths occurred in the SHO group.

4 The *in vivo* administration of dexamethasone inhibited both IL-1 and PGE_2 release by macrophages as well as protecting ADX animals from CFA-induced death. Indomethacin and BW 755C partially protected the animals from this lethal effect.

5 These results suggest that adrenalectomy induces an increased release of IL-1 both *in vitro* and *in vivo*, and are consistent with a feedback mechanism between IL-1 and glucocorticoid hormones.

Introduction

The acute inflammatory response is an important part of the body's defence mechanisms against injury or infection. This response has as its main objectives the neutralization and removal of pathogens, the rebuilding of injured tissues and ultimately the restoration of normal structure and function. Sometimes the activation of defence reactions lasts well after the removal of the injurious agents triggering the transition from acute to chronic inflammation. It has been proposed that the rise in adrenocortical steroid hormones occurring during inflammation might turn off the activated defence reactions in order to restore homeostasis (Munck *et al.*, 1984). Accordingly, we have shown previously that the extent of the acute inflammatory response in adrenalectomized rats is much greater than in sham-operated animals (Flower *et al.*, 1986). This phenomenon was sustained by the activation of phospholipase A_2 (PLA_2) with consequent formation of pro-inflammatory eicosanoids. The administration of dexamethasone decreased the severity of the inflammatory process probably via the induction of anti-phospholipase proteins (lipocortins) (Parente & Flower, 1985; Flower *et al.*, 1986).

In recent years the important role of interleukin-1 (IL-1) in inflammation has been well documented (Dinarello, 1988; Cybulsky *et al.*, 1988). It has been shown that IL-1 is able to stimulate prostaglandin formation by both increasing cellular cyclo-oxygenase biosynthesis (Raz *et al.*, 1988) and activating PLA_2 (Burch *et al.*, 1988; Solito & Parente, 1989). On the other hand, IL-1 can directly stimulate the adrenal cortex (Roh *et al.*, 1987) and its synthesis and release is inhibited by glucocorticoids (Snyder & Unanue, 1982; Knudsen *et al.*, 1987; Lew *et al.*, 1988). In order to investigate the relationship between IL-1 and endogenous steroid hormones we have studied the effect of adrenalectomy on IL-1 release and we report that macrophages from ADX rats (hereafter referred to as ADX macrophages) release significantly more IL-1 than macrophages from SHO animals (hereafter referred to as SHO macrophages). This enhanced release may occur also *in vivo* following the challenge with Complete Freund's Adjuvant (CFA).

Methods

Animals

Adrenalectomized and sham-operated outbred Wistar rats (200 ± 10 g body wt) were obtained from

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Charles River, Calco, Italy. ADX rats were given 0.9% NaCl solution to drink, while SHO rats were given tap water. Animals were fed on standard chow pellets *ad libitum* and used for experiments two weeks after surgery.

Cell culture

Resident peritoneal cells were collected by lavage with phosphate-buffered saline (PBS) containing heparin 5 units ml^{-1}. Cells from 5–8 animals were pooled and plated at 2×10^6 cells per well in multiwell plates 24 × 10 mm (Costar, Cambridge, MA, USA) and incubated in 0.5 ml of RPMI 1640 (Gibco, Paisley, U.K.) containing L-glutamine 2 mM, HEPES buffer 25 mM, and gentamicin sulphate 50 $\mu g\,ml^{-1}$ (Sigma Chemical Co., St. Louis, MO, U.S.A.) and supplemented with 20% Foetal Calf Serum (FCS, Seromed, Berlin, FRG). After 2 h incubation at 37°C in 5% CO_2 atmosphere, nonadherent cells were removed by washing thoroughly with serum-free RPMI and the adherent population was further incubated in RPMI 1640 + 5% FCS, either with or without 10 $\mu g\,ml^{-1}$ lipopolysaccharide (LPS) from *E. coli* (055:B5, Difco, Detroit, MI, U.S.A.). Adherent cells were shown to be >98% macrophages by positive esterase staining. After 24 h incubation, supernatant aliquots were collected for PGE_2 assay, the remaining supernatants were dialyzed against PBS (1:200 v/v) twice and sterile filtered.

Prostaglandin E_2 assay

Concentrations of PGE_2 in the supernatants were determined by specific radioimmunoassay with minimal (3.7%) cross-reactivity with PGE_1 (N.E.N., Dreieich, FRG).

Interleukin-1 assay

IL-1 activity in dialyzed supernatants was assessed by the murine thymocyte co-stimulation assay according to Massone *et al.* (1988). Briefly, 6×10^5 thymocytes from C3H/HeJ mice were incubated in multiwell plates (Costar) in 0.2 ml of RPMI 1640 5% FCS with 1.5 $\mu g\ ml^{-1}$ phytohaemoagglutinin (PHA, The Wellcome Foundation, Beckenham, U.K.) and serial twofold dilutions of the supernatants. After 72 h cells were pulsed for further 15 h with 1 μCi per well [^{3}H]-thymidine (185 GBq $mmol^{-1}$, Amersham International, U.K.).

Data are presented as units ml^{-1} calculated for sample dilutions giving 50% of the maximal thymidine incorporation compared to standard curves obtained with endotoxin-free human recombinant IL-1β (specific activity 1.10^7 units mg^{-1}, Sclavo, Siena, Italy).

Immunoprecipitation

ADX and SHO peritoneal macrophages (1×10^7 cells in 2 ml) were incubated for 20 h in RPMI 1640 + 10% FCS with or without LPS (10 $\mu g\,ml^{-1}$) in the presence of 1 $\mu g\,ml^{-1}$ cold methionine (Sigma Chemical Co.) and 50 $\mu Ci\,ml^{-1}$ [^{35}S]-methionine (N.E.N.). At the end of the incubation the cells were washed twice with PBS and lysed for 10 min at 25°C in 0.5 ml PBS containing 0.5% Nonidet P-40 (LKB, Bromma, Sweden and 1 mM phenylmethylsulphonylfluoride (PMSF). The cell lysates were spun at 10,000 *g* for 10 min and cleared with 5 μl of normal rabbit serum followed by 100 μl of protein A coupled to Sepharose 4B (Pharmacia, Uppsala, Sweden). To the cleared lysates were added 1:100 dilutions of either rabbit polyclonal anti-human recombinant IL-1β (Massone *et al.*, 1988) or rabbit polyclonal anti-human recombinant IL-1α (Genzyme Biochemicals, Maidstone, U.K.). After incubation for 18 h at 4°C, 100 μl of protein A Sepharose 4B was added, and the immunoprecipitates were washed 3 times in Tris-HCl 10 mM pH 7.5 containing NaCl 150 mM and PMSF 4 $\mu g\,ml^{-1}$, followed by three washes with the same buffer containing NaCl 500 mM and finally by 3 washes with the same buffer without NaCl. The immunoprecipitated material was subjected to electrophoresis on 15% SDS polyacrylamide gels (LKB) according to Laemmli (1970). The gels were fixed with 30% methanol, enhanced with Enlightning (N.E.N.), vacuum dried and exposed to photographic film (Kodak X-Omat) at −40°C for 48 h.

In vivo *experiments*

In order to induce adjuvant arthritis ADX and SHO rats were injected s.c. with Complete Freund's Adjuvant (0.2 ml, 10 mg ml^{-1}, *M. tuberculosis*, Difco) into the right hind paw.

Drugs

Dexamethasone 21-acetate and indomethacin were obtained from Sigma Chemical Co. BW 775C (3-amino-1-[*m*-(trifluoromethyl)-phenyl]-2 pyrazoline) was kindly supplied by The Wellcome Foundation. On the basis of preliminary results, in the *ex vivo* experiments dexamethasone (0.1 mg kg^{-1}, s.c.) was administered for 9 days and macrophages collected 24 h after the last administration (see Figure 1).

Statistics

Statistical analysis was performed using Student's *t* test for unpaired samples with $P < 0.05$ regarded as significant.

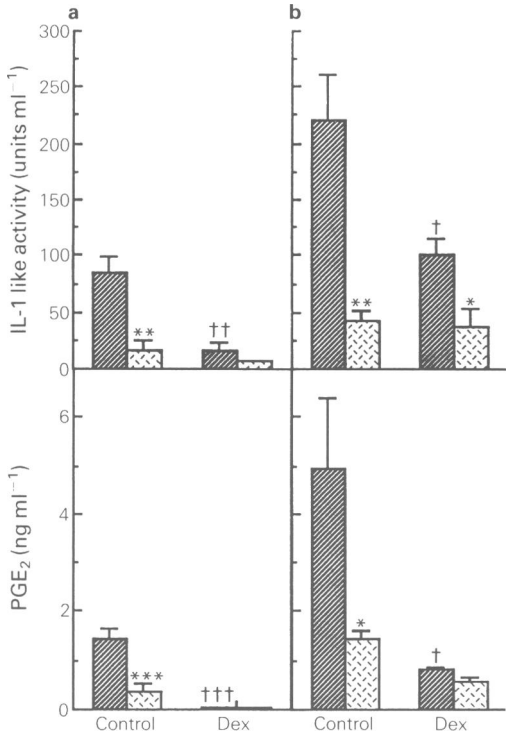

Figure 1 Interleukin-1 (IL-1)-like activity (upper panel) and prostaglandin E_2 (PGE_2) concentration (lower panel) in supernatants of peritoneal macrophages collected from adrenalectomized (hatched columns) and sham-operated (stippled columns) rats. Animals were treated either with vehicle $1\,ml\,kg^{-1}$ s.c. (Control) or with dexamethasone $0.1\,mg\,kg^{-1}$ s.c. (Dex) for 9 days and macrophages were collected 24 h after last administration. (a) Unstimulated macrophages; (b) Lipopolysaccharide-stimulated macrophages. Columns represent mean (with s.e.mean shown by vertical bars) of one experiment performed in triplicate. Four other experiments gave similar results. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$ vs corresponding ADX group. † $P < 0.05$; †† $P < 0.01$; ††† $P < 0.005$ vs control ADX group.

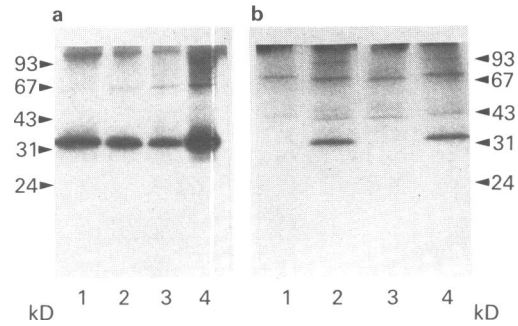

Figure 2 Immunoprecipitation pattern of macrophage lysates from sham-operated (lanes 1 and 2) and adrenalectomized rats (lanes 3 and 4). Macrophages were incubated for 20 h either in absence (lanes 1 and 3) or in presence of $10\,\mu g\,ml^{-1}$ lipopolysaccharide (lanes 2 and 4). Antibodies used were rabbit polyclonal anti-hr interleukin-1α (IL-1α, a) and rabbit polyclonal anti-hr IL-1β (b).

Results

Interleukin-1 and prostaglandin E_2 release by macrophages

Control peritoneal macrophages from ADX rats secreted into the culture medium significantly more IL-1 activity than those from SHO animals (Figure 1a, upper panel). LPS stimulation increased the absolute amount of IL-1 released by either type of macrophage, leaving the proportional difference between ADX and SHO macrophages virtually unchanged (Figure 1b, upper panel). The *in vivo* administration of dexamethasone significantly reduced IL-1 release by ADX and SHO macrophages whether stimulated or not by LPS. Moreover, control peritoneal macrophages from ADX rats released significantly more PGE_2 than those from SHO animals (Figure 1a, lower panel). As with IL-1, PGE_2 biosynthesis was greatly enhanced by LPS stimulation (Figure 1b, lower panel) and was reduced by *in vivo* administration of dexamethasone.

Interleukin-1 immunoprecipitation

In order to investigate the involvement of the two molecular species of IL-1 in the biological activity observed in the macrophage supernatants, we carried out immunoprecipitation experiments using specific polyclonal antibodies raised against human recombinant IL-1α and IL-1β. The results show that the anti-IL-1α antibody detected the presence of cell-associated 33 kD IL-1α in macrophages whether stimulated or not by LPS (Figure 2a). The largest amount of pro-IL-1α was observed in ADX macrophages stimulated by LPS (Figure 2a, lane 4). On the other hand, the anti-IL-1β antibody was able to recognize the cell-associated pro-IL-1β only upon stimulation with LPS (Figure 2b). No evident difference in the band corresponding to the 33 kD pro-IL-1β could be observed between SHO and ADX macrophages. It is also clear that rat macrophages express more pro-IL-1α than pro-IL-1β. Finally, we were unable to observe immunoprecipitation of soluble IL-1 in the macrophage supernatant (data not shown).

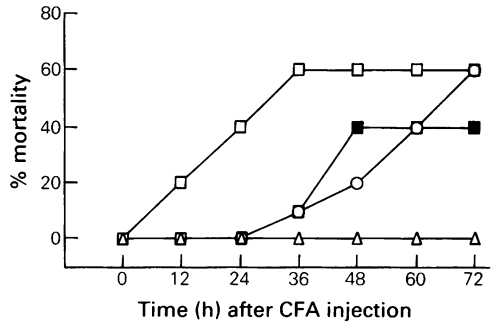

Figure 3 Cumulative mortality in adrenalectomized rats ($n = 10$) injected with Complete Freund's Adjuvant (CFA) at time 0. Drugs were administered at 0, 4 and 8 h. Control group (□) received vehicle 1 ml kg^{-1} orally; (○) indomethacin 3 mg kg^{-1} orally; (■) BW755C 20 mg kg^{-1} orally; (△) dexamethasone 1 mg kg^{-1} s.c.

In vivo *experiments*

We have shown elsewhere that macrophages collected from rats with adjuvant arthritis release more IL-1 than those from control animals (Becherucci *et al.*, 1989). In order to investigate the effect of adrenalectomy on IL-1 release in arthritic animals CFA was injected in ADX and SHO rats. Thirty-six hours after the injection, 60% of ADX rats died (Figure 3). No rats died either in the SHO group or in ADX animals injected with Incomplete Freund's Adjuvant (not shown). The lethal effect of CFA injection was completely reversed by dexamethasone given three times on the first day. Indomethacin and BW755C, given according to the same dosing schedule as the steroid, protected only up to 24 h after CFA injection (Figure 3).

Discussion

We have previously proposed that the enhancement of the inflammatory response in ADX animals is at least partially due to the activation of PLA_2 not counteracted by an increase in circulating corticosteroid hormones. This hypothesis was based on the observation that inflammatory exudates from ADX rats contain significantly higher levels of eicosanoids and macrophages collected from ADX rats release more lipid mediators than SHO animals (Parente & Flower, 1985; Flower *et al.*, 1986). Our present results show that macrophages collected from ADX animals release more IL-1 and PGE_2 than control cells from SHO animals. As IL-1 stimulates PLA_2 (Burch *et al.*, 1988; Solito & Parente, 1989) it is conceivable that the enzyme activation is secondary to the increase of IL-1 synthesis. In order to investigate the IL-1 release during an inflammatory process we decided to induce adjuvant arthritis in ADX and SHO rats, since we have shown elsewhere that macrophages from arthritic animals release more IL-1 than control cells (Becherucci *et al.*, 1989). Unexpectedly, 36 h after CFA injection 60% of the ADX rats died, while no death occurred in the SHO animals. This is consistent with the recent observation that IL-1 and tumour necrosis factor (TNF) are lethal for adrenalectomized mice but not for sham-operated ones (Bertini *et al.*, 1988). It is tempting to hypothesize that the CFA injection prompted the release of IL-1 by macrophages and other IL-1-forming cells *in vivo* causing the death of ADX rats. The involvement of TNF in this phenomenon is currently under investigation.

In summary our results may suggest that adrenalectomy increases the release of IL-1 both *in vitro* and *in vivo*. Several investigators have shown that glucocorticoids suppress IL-1 synthesis by blocking the transcription of IL-1 mRNA (Knudsen *et al.*, 1987; Lew *et al.*, 1988; Lee *et al.*, 1988). Therefore, the synthesis of IL-1 in ADX animals may be deregulated because of the absence of endogenous corticosteroid hormones. The *in vivo* administration of dexamethasone was indeed able to inhibit both IL-1 and PGE_2 release by macrophages, as well as exhibiting a long-lasting protective effect from CFA-induced death in ADX rats. Indomethacin, a cyclo-oxygenase inhibitor, and BW755C, a dual cyclo-oxygenase/lipoxygenase inhibitor, given three times on the first day, only protected up to 24 h following CFA injection (see Figure 3). We have subsequently observed that three daily administrations of the non-steroid drugs for three days completely protected the ADX rats from death (data not shown). This suggests that CFA injection caused a sustained biosynthesis of toxic eicosanoids. Therefore, we would like to propose that in ADX rats injected with CFA, a continuous release of IL-1 occurs with subsequent activation of PLA_2, which may cause directly a circulatory collapse (Vadas *et al.*, 1988) as well as induce the formation of toxic metabolites like thromboxanes and platelet activating factor (PAF). It has been shown that a shock-like state caused by IL-1 in rabbits was abolished by cyclo-oxygenase inhibitors (Okusawa *et al.*, 1988). Moreover, IL-1 stimulated PAF formation (Bussolino *et al.*, 1986) and a PAF antagonist blocked IL-1-induced inflammatory effects (Rubin & Rosenbaum, 1988). In this light the long-lasting protective effect of dexamethasone can be ascribed to the combined inhibition of IL-1 synthesis and induction of anti-PLA_2 proteins. Indeed, we have recently shown that in human fibroblasts, hydrocortisone blocks IL-1-induced PLA_2 activation through the induction of lipocortins (Solito & Parente, 1989). The enhanced synthesis

and release of IL-1 following adrenalectomy and the inhibitory effect of glucocorticoids are consistent with the hypothesis that endogenous steroid hormones control homeostasis by lowering the intensity of the body's defence reactions (Munck *et al.*, 1984).

It has been shown that IL-1 stimulates the secretion of both corticotropin-releasing factor (Salposki *et al.*, 1987; Berkenbosch *et al.*, 1987) and adrenocorticotrophic hormone (ACTH, Besedowsky *et al.*, 1986). Moreover, IL-1 increases rat serum corticosterone levels by direct stimulation of the adrenal cortex (Roh *et al.*, 1987). In view of these observations, an immunoregulatory feedback mechanism between IL-1 and glucocorticoids has been proposed by Besedowski *et al.* (1986). Our results provide a physiological basis for this feedback mechanism, showing, for the first time, the modulation of IL-1 release by endogenous steroid hormones.

Immunoprecipitation of IL-1 synthesized in peritoneal macrophages shows that IL-1α is constitutively produced and in larger amounts than IL-1β, which is synthesized only after stimulation by LPS. Interestingly, LPS-stimulated macrophages from ADX rats appeared to have the highest quantity of the IL-1α precursor. Since it has been shown that only the pro-IL-1α is biologically active (Mosley *et al.*, 1987) it is possible that the enhanced IL-1-like activity released by ADX macrophages is due to this form of the interleukin. It has been also reported that rat macrophages release more IL-1α than IL-1β (Kampschmidt & Franks, 1985). No extracellular forms of IL-1 were observed in the immunoprecipitation gels. It is likely that the bioassay is able to detect IL-1 amounts not revealed by immunoprecipitation because of different sensitivity, as previously reported (Hazuda *et al.*, 1988). However, the involvement of other macrophage products in the observed biological activity cannot, at the moment, be excluded.

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[^{3}H]-idazoxan binds with high affinity to two sites on hamster adipocytes: an α_2-adrenoceptor and a non-adrenoceptor site

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1 [^{3}H]-idazoxan labels a single population of high affinity sites (K_d 2.26 ± 0.02 nM; B_{max} 372 ± 25 fmol mg^{-1} protein) in hamster adipocyte membranes. In the presence of 1 μM yohimbine to preclude binding to α_2-adrenoceptors, the density of [^{3}H]-idazoxan binding sites was reduced (287 ± 18 fmol mg^{-1} protein) without an apparent decrease in the affinity (K_d 2.19 ± 0.24 nM) of the radioligand.

2 Displacement studies indicate that α-adrenoceptor ligands with an imidazoline side chain completely inhibit [^{3}H]-idazoxan binding to hamster adipocyte membranes; in contrast, the α_2-adrenoceptor antagonists yohimbine, rauwolscine, BDF 6143 and phentolamine inhibited only 20–30% of the specific binding with affinity values consistent with an interaction at α_2-adrenoceptors.

3 The low potency of noradrenaline and adrenaline in displacing [^{3}H]-idazoxan binding to the second site on hamster adipocyte membranes indicates that it is unlikely that this site is a type of adrenoceptor.

4 These results suggests that [^{3}H]-idazoxan binds with high affinity to two sites in hamster adipocytes: an α_2-adrenoceptor and a non-adrenoceptor imidazoline site.

Introduction

Catecholamine-induced lipolysis from adipocytes isolated from white fat in man (Burns *et al.*, 1971), dog (Berlan & Dang Tran, 1978) and hamster (Hittelman *et al.*, 1973; Giudicelli *et al.*, 1977) is mediated through a stimulatory β-adrenoceptor and an inhibitory α-adrenoceptor. Studies indicate that α_1- and α_2-adrenoceptors exist in hamster (Garcia-Sainz *et al.*, 1980; Pecquery & Giudicelli, 1980) and human (Fain & García-Sainz, 1983) fat cells. The antilipolytic effects of α-adrenoceptor agonists in hamster (Curtis-Prior & Tan, 1984; Tan & Curtis-Prior, 1984) and man (Berlan & Lafontan, 1980; Lafontan & Berlan, 1980; Curtis-Prior & Tan, 1984) are through the predominant α_2-adrenoceptor.

The adipocyte is considered a suitable tissue to study the potency and selectivity of drugs that interact with α_2-adrenoceptors (Huerta-Bahena *et al.*, 1983). The α_2-adrenoceptor present on human adipocytes is labelled with high affinity by [^{3}H]-clonidine, [^{3}H]-yohimbine and [^{3}H]-rauwolscine (Tharp *et al.*, 1981; Berlan & Lafontan, 1982; Lafontan *et al.*, 1983; Richelsen & Pedersen, 1985). [^{3}H]-clonidine binds with high affinity to hamster adipocyte membranes (Villeneuve *et al.*, 1985; Taouis *et al.*, 1986) whereas the labelling of α_2-adrenoceptors by [^{3}H]-yohimbine has proved difficult to demonstrate (Villeneuve *et al.*, 1985; Lafontan *et al.*, 1985). Yohimbine, rauwolscine and idazoxan reverse clonidine-induced inhibition of lipolysis in hamster fat cells (Tan & Curtis-Prior, 1983). The α_2-adrenoceptor nature of the reversal is based upon rank order potency and not on receptor affinity.

In this study, we have examined the binding of [^{3}H]-idazoxan to hamster fat cells and compared its

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displacement by adrenaline, noradrenaline and a number of imidazoline and non-imidazoline α-adrenoceptor ligands. We show that [^{3}H]-idazoxan, in addition to being a potent α_2-adrenoceptor antagonist (Chapleo *et al.*, 1981), labels an imidazoline binding site on hamster adipocytes, with high affinity.

Methods

Preparation of adipocyte membranes

Golden male hamsters (2–3 months old; weight range, 100–150 g) were killed by cervical dislocation and the white adipose tissue carefully removed. Hamster adipocytes from epididymal fat were isolated essentially as described by Rodbell (1964) with minor modification with regard to the level of collagenase used in the digestion process. Adipocyte tissue (8–10 g per animal) was minced finely and digested with crude collagenase (1 mg ml^{-1}) in Krebs-Ringer bicarbonate buffer (5% w/v) containing 3.5% (w/v) fatty acid deficient bovine serum albumin and 1 mM $CaCl_2$ for 20 min at 37°C. During the digestion, the minced tissue was shaken vigorously approximately every 5 min by hand. The suspension containing the adipocytes was filtered through three layers of muslin and centrifuged at 400 *g* for 1 min at room temperature. The infranatant was removed by aspiration and the cells washed three times with Krebs-Ringer bicarbonate buffer to remove the collagenase. Isolated adipocytes were suspended in 10 vol. lysing medium (5 mM Tris HCl, pH 7.4 containing 0.5 mM EDTA) and centrifuged at 400 *g* for 1 min at room temperature. The membrane fraction was collected and the unbroken cells lysed by a further two washes in the 5 mM Tris HCl buffer. The membrane fractions were pooled and centrifuged at 30,000 *g* for 15 min at 4°C. The final pellet was suspended in 50 mM Tris HCl buffer pH 7.4 containing 10 mM $MgCl_2$ and used immediately for ligand binding or stored for not more than 7 days under liquid nitrogen.

Male Sprague-Dawley rats (180–200 *g*) were stunned, decapitated and the cerebral cortex dissected from the other brain regions over ice. Tissues were homogenized in 20 volumes of 50 mM Tris HCl, 5 mM EDTA, pH 7.4 with a polytron PT 10 tissue disruptor (setting 10; 2 × 10 s bursts). The homogenate was centrifuged at 48,000 *g* for 15 min at 4°C. The supernatant was discarded and the pellet resuspended in the original volume of assay buffer (50 mM Tris HCl, 0.5 mM EDTA, pH 7.4). Membranes were washed twice by repeated centrifugation at 48,000 *g* for 15 min at 4°C. The final pellet was resuspended in assay buffer at an approximate protein level of 1 mg ml^{-1}. Membranes were stored under liquid nitrogen until used in the binding assay.

[^{3}H]-*idazoxan binding*

Saturation experiments Adipocyte membranes (250–300 μg protein) and nine concentrations of [^{3}H]-idazoxan over the range 0.1–12 nM were incubated to equilibrium in 50 mM Tris HCl buffer, pH 7.4 containing 10 mM $MgCl_2$. Saturation studies were performed in the absence of yohimbine to determine the total density of binding sites or in the presence of 1 μM yohimbine to preclude [^{3}H]-idazoxan binding to α_2-adrenoceptors. Incubations were carried out for 30 min at 25°C in a final volume of 0.5 ml. Bound ligand was separated from free by vacuum filtration over GF/B filters on a Brandell cell harvester. The filters were washed twice for 10 s with incubation buffer and the bound radioactivity determined by liquid scintillation counting. Non-specific binding at each free ligand concentration was determined in the presence of 1 mM phentolamine. Preliminary studies indicated that the imidazolines, cirazoline and naphazoline displaced non-specific [^{3}H]-idazoxan binding when defined by 10 μM phentolamine, a concentration often used in α_2-adrenoceptor binding studies. The level of non-specific binding obtained with cirazoline and naphazoline was similar to the level obtained with 1 mM phentolamine.

Equilibrium binding parameters (K_d and B_{max}) were obtained by the iterative non-linear least square curve fitting programme 'ligand' (Munson & Rodbard, 1980).

Competition experiments

[^{3}H]-*idazoxan binding to hamster adipocytes* Competition experiments in hamster adipocytes, were carried out in 50 mM Tris HCl buffer, pH 7.4 containing 10 mM $MgCl_2$, 1.5 nM [^{3}H]-idazoxan and various drugs over the concentration range 10^{-3} to 10^{-12} M in a final volume of 0.5 ml. Conditions for the competition assays were identical to those described for the saturation experiments. Non-specific binding was defined by 1 mM phentolamine and represented approximately 25% of total binding. Each determination was carried out in duplicate. Inhibition of specific binding of [^{3}H]-idazoxan by the drug was analysed to estimate the IC_{50} (concentration of drug displacing 50% of specific binding). The inhibitory constant (K_i) was calculated from the IC_{50} by the equation of Cheng & Prusoff (1973).

[^{3}H]-*idazoxan binding to rat cortical* α_2-*adrenoceptors* The displacement of [^{3}H]-idazoxan

from α_2-adrenoceptors in rat cortical membranes (100–200 μg protein) was carried out in 50 mM Tris HCl buffer, pH 7.4 containing 0.5 mM EDTA. Binding conditions were as previously described for hamster adipocyte membranes. Under these conditions, no evidence was obtained for [^{3}H]-idazoxan at a free ligand concentration of 1.5 nM, binding to an imidazoline site in rat cortex. Non-specific binding was defined by 10 μM phentolamine and represented approximately 35% of total binding.

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

Computer analysis of displacement curves

Binding isotherms from displacement studies were analysed by a non-linear least square parametric curve fitting programme capable of fitting to a single or two site model. The programme provided a sum of square error for a single or two site model. The single site and two site models for each isotherm were compared using the differential F value defined by the following equation:

$$F = \frac{(SS_1 - SS_2)/(df_1 - df_2)}{SS_2/(df_2)}$$

where SS_1 is the sum of squares error for the single site, SS_2 is the sum of squares error for the two site model, df_1 is the degrees of freedom for the single site model and df_2 the degrees of freedom for the two site model (Munson & Rodbard, 1980; Petrash & Bylund, 1986). A two site fit was assumed to be significantly better than a single site fit if the determined F value had a $P < 0.05$.

Chemicals and drugs

[^{3}H]-idazoxan (40 Ci mmol^{-1}) was purchased from Amersham, U.K. Drugs were obtained from the following sources; noradrenaline, naphazoline, phenylephrine, clonidine and adrenaline from Sigma; cirazoline from Synthelabo; rauwolscine from Inverbeff; idazoxan from Reckitt & Colman; UK 14304 (5-bromo-6(2-imidazolin-2-ylamino)-quinaxoline tartrate) from Pfizer; phentolamine from Ciba-Geigy; BHT 920 (6-allyl-2-amino-5,6,7,8-tetrahydro-4H-thiazolo[4,5-d]azepine dihydrochloride) from Boehringer Ingelheim; WB 4101 (2-(2,6-dimethoxyphenoxyethyl)-aminomethyl-1,4-benzodioxan) from Ward Blenkinsop; cirazoline and BDF 6143 (4-chloro-2-[2-imidazoline-2-ylamino]-isoindoline hydrochloride) were synthesized by Syntex. All other chemicals were of the highest purity commercially available.

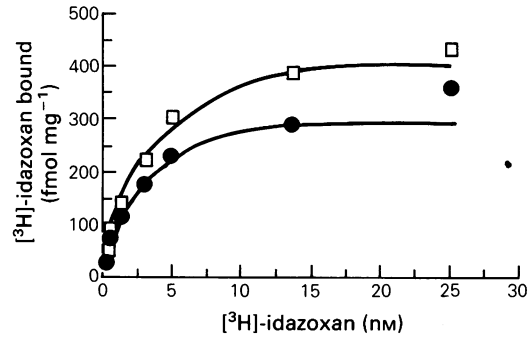

Figure 1 [^{3}H]-idazoxan saturation binding was carried out under control conditions (□) K_d 1.7 nM, B_{max} 396 fmol mg^{-1} protein, or in the presence of 1 μM yohimbine (●), K_d 2.2 nM, B_{max} 260 fmol mg^{-1} protein. The results presented are of a single experiment.

Results

The binding of [^{3}H]-idazoxan to membranes of hamster adipocytes was saturable and of high affinity (Figure 1). Iterative non-linear analysis of the binding isotherms demonstrated that [^{3}H]-idazoxan labelled a single population (K_d 2.26 ± 0.02 nM, B_{max} 373 ± 25 fmol mg^{-1} protein) of binding sites (Table 1). The saturation data was also fitted to a two-site model (double rectangular hyperbola). The two-site fit was not significantly better than the one-site fit. Saturation studies were repeated in the presence of 1 μM yohimbine to preclude [^{3}H]-idazoxan binding to α_2-adrenoceptors (Figure 1). The number of [^{3}H]-idazoxan binding sites (B_{max} 287 ± 18 fmol mg^{-1} protein) was reduced, in the presence of 1 μM yohimbine, without an apparent decrease in the affinity (K_d 2.19 ± 0.24 nM) of the radioligand (Table 1). Thus α_2-adrenoceptors only

Table 1 [^{3}H]-idazoxan binding to hamster adipocyte membranes

	K_d (nM)	B_{max} (fmol mg^{-1} protein)
Control	2.26 ± 0.02	373 ± 25
Yohimbine (1 μM)	2.19 ± 0.24	287 ± 18*

Saturation experiments were carried out with 9 concentrations of [^{3}H]-idazoxan over the range 0.1–12 nM. In addition, saturation studies were repeated in the presence of 1 μM yohimbine to preclude [^{3}H]-idazoxan binding to α_2-adrenoceptors. Each value represents the mean ± s.e.mean of three separate determinations. Statistical significance relative to control density. * $P < 0.05$.

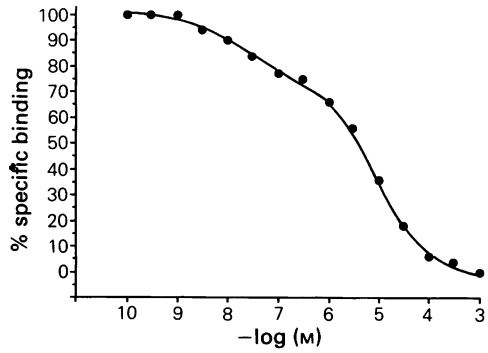

Figure 2 Displacement of [^{3}H]-idazoxan binding to hamster adipocyte membranes by phentolamine. Membranes were incubated with 1.5 nM [^{3}H]-idazoxan and increasing concentrations of phentolamine for 30 min at 25°C. The result shown is a single experiment performed in duplicate. Computer-assisted curve fitting demonstrated that a two site fit was significantly better than a one site fit, $P < 0.001$. (one site, ssmin 334010, d.f. 9; two site, ssmin 1473, d.f. 7, F value 76).

Table 2 Displacement of [^{3}H]-idazoxan binding to hamster adipocyte and rat cerebral cortical membranes

	pKi		
	Hamster adipocytes		
	Site$_1$	*Site$_2$*	*Rat cortex*
Yohimbine	7.28 ± 0.02	4.48 ± 0.16	7.25 ± 0.08
Rauwolscine	7.23 ± 0.04	<4.00	7.10 ± 0.06
BDF 6143	9.10 ± 0.06	5.92 ± 0.04	8.66 ± 0.22
Phentolamine	7.92 ± 0.14	5.21 ± 0.02	8.33 ± 0.21
Noradrenaline	6.21 ± 0.10	<4.00	6.70 ± 0.13*
Adrenaline	6.92 ± 0.12	<4.00	7.49 ± 0.13*
Imiloxan	5.67 ± 0.08		6.68 ± 0.09
Cimetidine	4.30 ± 0.05		4.53 ± 0.07
Histamine	4.78 ± 0.07		5.13 ± 0.07

The affinity values were obtained for the displacement of specific binding of 1.5 nM [^{3}H]-idazoxan from hamster adipocyte and rat cortical membranes. Site$_1$ represents the site that displays high affinity towards the α_2-adrenoceptor antagonists but only represents 20–30% of specific binding. Site$_2$, comprising the remaining specific binding, displays low affinity towards the α_2-adrenoceptors and adrenaline and noradrenaline. Each value represents the mean ± s.e.mean of three separate determinations. * denotes Hill slope less than unity.

contribute approximately 23% of the total [^{3}H]-idazoxan binding to hamster adipocytes. The failure of 1 μM yohimbine to alter the K_d of [^{3}H]-idazoxan for the remaining sites implies that [^{3}H]-idazoxan has similar affinity for both the α-adrenoceptor and the non α_2-adrenoceptor site.

The displacement of [^{3}H]-idazoxan binding to adipocyte membranes by phentolamine was complex with Hill slopes considerably less than unity. Computer analysis of the displacement curves indicated that phentolamine displaced [^{3}H]-idazoxan binding with high (Site$_1$, pKi 7.92) and low (Site$_2$, pKi 5.21) affinity (Figure 2). Complex displacement curves for [^{3}H]-idazoxan binding to adipocytes were not confined to phentolamine. Noradrenaline and the α_2-adrenoceptor antagonists, yohimbine, rauwolscine and BDF 6143 displaced [^{3}H]-idazoxan with low Hill slopes that could be resolved into two sites (Table 2).

We therefore compared the ability of a number of drugs with affinity for α_2-adrenoceptors to displace [^{3}H]-idazoxan from site$_1$ in hamster adipocytes and from α_2-adrenoceptors in rat cerebral cortex (Table 2). The displacement of [^{3}H]-idazoxan binding by the α_2-adrenoceptor antagonists was monophasic in rat cerebral cortex, with Hill slopes close to unity. The antagonist affinity rank order of BDF 6143 > phentolamine > rauwolscine > yohimbine > imiloxan, is consistent with [^{3}H]-idazoxan labelling an α_2-adrenoceptor in rat cortex. The affinity values at the α_2-adrenoceptor in rat cortex were similar to the values obtained for the displacement of [^{3}H]-idazoxan binding to Site$_1$ in adipocytes, suggesting that Site$_1$ represents the α_2-adrenoceptor. Inhibition of [^{3}H]-idazoxan binding to hamster adipocyte membranes by the α_2-adrenoceptor antagonist imiloxan, the α_1-adrenoceptor antagonist prazosin and the H_2-histamine receptor antagonist cimetidine were monophasic, with Hill slopes close to unity. Each drug displaced [^{3}H]-idazoxan binding with low affinity. All drugs, however were capable of displacing 100% specific binding.

The nature of the second [^{3}H]-idazoxan binding site on hamster adipocytes was examined by use of a number of α-adrenoceptor ligands. In the presence of 1 μM yohimbine to block [^{3}H]-idazoxan binding to the α_2-adrenoceptor, the α-adrenoceptor ligands with the imidazoline side chain displaced [^{3}H]-idazoxan to the remaining sites with a potency order of cirazoline > naphazoline > UK 14304 > clonidine (Table 3). Cirazoline displaced [^{3}H]-idazoxan with a Hill slope of unity. In contrast, naphazoline, UK 14304 and clonidine displaced [^{3}H]-idazoxan binding with Hill slopes significantly different from unity. Noradrenaline and the non-imidazoline α_2-adrenoceptor agonist BHT 920 failed to displace [^{3}H]-idazoxan binding from this site on hamster adipocytes. The low affinity of the α_1-adrenoceptor antagonist WB 4101 and agonist phenylephrine

Table 3 Displacement of [^{3}H]-idazoxan binding to hamster adipocytes in the presence of yohimbine (1 μM)

	pIC_{50}	nH
Cirazoline	9.02 ± 0.13	1.00
Naphazoline	7.90 ± 0.32	0.50 ± 0.08
UK 14304	7.52 ± 0.22	0.70 ± 0.05
Clonidine	5.71 ± 0.21	0.72 ± 0.06
BHT 920	<4.00	—
Phenylephrine	<4.00	—
WB 4101	<5.00	—
Noradrenaline	<4.00	—

Inhibition of [^{3}H]-idazoxan (1.5 nM) binding to hamster adipocyte membranes in the presence of yohimbine (1 μM) to preclude binding to α_2-adrenoceptors. Each value represents the mean ± s.e.mean of four separate determinations.

against [^{3}H]-idazoxan binding suggests that the potency of cirazoline and naphazoline is due to their imidazoline side chain and not to an interaction with α_1-adrenoceptors.

Discussion

This study indicates that [^{3}H]-idazoxan labels with high affinity two sites in hamster adipocytes; an α_2-adrenoceptor and a non-adrenoceptor site. Saturation studies indicate that [^{3}H]-idazoxan has similar affinity towards the two sites and that α_2-adrenoceptors only represent a relatively small proportion of the binding of [^{3}H]-idazoxan. The results of displacement studies provide additional evidence for [^{3}H]-idazoxan binding to α_2-adrenoceptors and a non-adrenoceptor site. The first binding site ($Site_1$) representing approximately 20–30% of specific binding has a pharmacological profile consistent with binding to an α_2-adrenoceptor. In contrast, the other site ($Site_2$) representing 70–80% specific binding, is unlikely to be an α_2-adrenoceptor since the antagonists yohimbine, rauwolscine, and BDF 6143 failed to displace [^{3}H]-idazoxan binding at pharmacologically relevant concentrations. Furthermore, the low affinity of noradrenaline and adrenaline towards this binding site makes it unlikely that it is an adrenoceptor.

Competition experiments indicate that compounds containing the imidazoline side chain are potent displacers of [^{3}H]-idazoxan binding to $Site_2$ in adipocytes. The rank order potency of: α_1-adrenoceptor agonists, (cirazoline and naphazoline) > the imidazoline α_2-adrenoceptor agonists, UK 14304 > clonidine > the non-imidazoline α_2-adrenoceptor agonist, BHT 920 is consistent with [^{3}H]-idazoxan labelling an imidazoline site. The failure of the α_1-adrenoceptor agonist phenylephrine and the antagonist WB 4101 to displace [^{3}H]-idazoxan from $Site_2$ in adipocytes, suggests that the high affinity of cirazoline and naphazoline is due to the imidazoline side chain and not to an interaction at α_1-adrenoceptors. Cirazoline has been reported to display higher affinity towards [^{3}H]-clonidine binding than [^{3}H]-prazosin binding to rat brain (Van Meel *et al.*, 1981). In field stimulated guinea-pig ileum, cirazoline competitively antagonized the α_2-adrenoceptor mediated inhibition of twitch height with a pA_2 of 7.56 (Ruffolo & Waddell, 1982). Thus cirazoline can act as an α_1-adrenoceptor agonist and an α_2-adrenoceptor antagonist. The finding of this study that cirazoline displaces [^{3}H]-idazoxan binding, in the presence of yohimbine, with high affinity indicates that this compound also binds to the imidazoline site in hamster adipocytes.

Imidazoline binding sites, labelled by [^{3}H]-idazoxan, have been described previously. The imidazoline related compounds, cirazoline and UK 14304 displace [^{3}H]-idazoxan from rabbit urethral membranes with high affinity, whereas yohimbine, rauwolscine and the phenylethanolamines, adrenaline, noradrenaline and α-methyl-(±)-noradrenaline are essentially devoid of affinity (Yablonsky *et al.*, 1988). Imidazoline binding sites have been identified in rabbit cerebral cortex and kidney (Coupry *et al.*, 1987; Hamilton *et al.*, 1988) and rabbit adipocytes (Langin & Lafontan, 1989). The affinity rank order of cirazoline (pKi 8.50) > naphazoline (pKi 8.3) UK 14304 (pKi 6.52) > clonidine (pKi 5.90) for the displacement of [^{3}H]-idazoxan from rabbit urethral membranes is in agreement, with the exception of UK 14304, with the values obtained in this study. The affinities reported by Langin & Lafontan (1989) for naphazoline (pKi 8.00) and clonidine (pKi 6.01) suggests that the imidazoline binding site in hamster adipocytes is likely to be similar to the one that exists in rabbit adipocytes. However, the affinity of UK 14304 in both rabbit urethral membranes and adipocytes (pKi 6.02) is less than its affinity (pKi 7.52) for the imidazoline site in hamster adipocytes. In both rabbit urethral and adipocyte membranes, the affinity of idazoxan was higher at the imidazoline binding site than at the α_2-adrenoceptor in the corresponding tissue. This contrasts with the findings of this study in which [^{3}H]-idazoxan has a similar affinity at the α_2-adrenoceptor and the imidazoline binding site. Whether this represents a species difference between the imidazoline sites or a difference in sensitivity of [^{3}H]-idazoxan and UK 14304 in the different buffer systems used has still to be resolved. The α_2-adrenoceptor agonist, *p*-aminoclonidine

binds to two sites in bovine ventrolateral medulla: an α_2-adrenoceptor and a non-adrenoceptor site, insensitive to catecholamines (Meeley *et al.*, 1986; Ernsberger *et al.*, 1987). This second site has also been identified in human brainstem (Bricca *et al.*, 1988) where it is labelled by [^{3}H]-clonidine (Bricca *et al.*, 1989). In displacement studies, cirazoline displaced [^{3}H]-clonidine with high affinity whereas μmolar concentrations of yohimbine were required to induce the same effect. At present, insufficient evidence is available to determine whether the non-adrenoceptor [^{3}H]-clonidine binding site in human brainstem is identical with the non-adrenoceptor [^{3}H]-idazoxan binding site on hamster adipocytes. The low affinity of clonidine towards the non-adrenoceptor site on hamster adipocytes would argue against these sites being similar.

The displacement of [^{3}H]-idazoxan binding to Site$_2$ in adipocyte membranes is apparently selective for the imidazoline structure. Imiloxan, an imidazole α_2-adrenoceptor antagonist was considerably less potent in displacing [^{3}H]-idazoxan from this site in hamster adipocytes than at the α_2-adrenoceptor in rat brain. Imidazole binding sites have also been described. Displacement of the binding of the imidazole H_2-histamine receptor antagonist [^{3}H]-cimetidine to membranes of cerebral cortical and medulla pons indicated that this ligand was not binding to an H_2-receptor but to an imidazole site (Rising *et al.*, 1980; Subramanian & Slotkin, 1981). Clonidine and phentolamine potently inhibited [^{3}H]-cimetidine binding to the imidazole site in rat brain (Burkard, 1978; Subramanian & Slotkin, 1981). In this study, the imidazoles histamine and cimetidine although displacing [^{3}H]-idazoxan binding completely, were only of low potency.

[^{3}H]-idazoxan is used frequently as a radioligand to label α_2-adrenoceptors in both central and peripheral tissue. A recent autoradiographical and binding study carried out in a sodium phosphate buffer, suggests that [^{3}H]-idazoxan labels a heterogeneous population of binding sites in rat brain and that one population of these sites is selectively labelled by [^{3}H]-raulwolscine (Boyajian *et al.*, 1987; Boyajian & Leslie, 1987). There were approximately 4 times the number of 'idazoxan' sites as for rauwolscine. A possible explanation for the apparent discrepancy between the density of sites identified by the two radioligands is that [^{3}H]-idazoxan labels an imidazoline binding site in addition to α_2-adrenoceptors. [^{3}H]-idazoxan binding to rat cortical membranes was carried out in this study under conditions that define α_2-adrenoceptors. The existence of imidazoline binding sites in rat cortex, must await specifically designed studies.

Evidence from studies on isolated adipocytes suggest that it is the α_2-adrenoceptor that is responsible for modifying α-adrenergic stimulation of lipolysis in human (Berlan & Lafontan, 1982), rat (Sundin & Fain, 1983) and hamster adipocytes (Carpene *et al.*, 1980). Cirazoline, a compound shown in this study to possess high affinity for the imidazoline binding site on hamster adipocytes, had no effect on noradrenaline- or isoprenaline-stimulated lipolysis, at concentrations up to 1×10^{-5} M (Curtis-Prior & Tan, 1983). Under identical conditions, a concentration-dependent inhibition of lipolysis was observed with clonidine. Thus at present the functional significance of the imidazoline binding site in hamster adipocytes remains uncertain.

These results provide additional evidence that [^{3}H]-idazoxan labels in addition to α_2-adrenoceptors, a non-adrenoceptor imidazoline site in hamster adipocytes that is similar to the site recently described in rabbit adipocytes (Langin & Lafontan, 1989). The hamster adipocyte may provide a useful test system for exploring the relationship between α_2-adrenoceptors and imidazoline binding sites.

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Studies on the adrenomedullary dependence of κ-opioid agonist-induced diuresis in conscious rats

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1 The dependence of κ-opioid agonist-induced diuresis, upon an intact and functional adrenal medulla in conscious rats, was investigated in order to test the hypothesis that the diuresis is mediated by a blood-borne 'diuretic factor', of adrenomedullary origin, released by κ-opioid receptor stimulation.

2 Confirming previous observations, adrenal demedullation significantly attenuated diuretic responses to the κ-opioid agonists U50488H, ethylketocyclazocine (EKC) and tifluadom, but did not affect basal urine output, furosemide-induced diuresis or the antidiuretic response to the μ-opioid agonist, buprenorphine. Naloxone abolished U50488H-induced diuresis, confirming an involvement of opioid receptors.

3 Transfusion studies established that blood, from intact rats treated with U50488H, induced diuresis in intact and demedullated recipient rats, whether or not the recipients had been pretreated with naloxone. However, blood from demedullated rats treated with U50448H was unable to induce diuresis when administered to intact or demedullated recipients.

4 It is concluded that κ-opioid agonist-induced diuresis is dependent upon an intact and functional adrenal medulla and appears to be mediated by a blood-borne 'diuretic factor' of adrenomedullary origin.

Introduction

An opioid receptor-mediated suppression of the neurohypophysial release of vasopressin was considered to be responsible for the water diuresis induced by κ-opioid agonists in normally hydrated rats (Leander, 1983a,b), although Miller (1975) had shown vasopressin release to be inhibited by non-selective narcotic antagonists. The identification of opioid receptors (Castanas *et al.*, 1984) and immunoreactivity to vasopressin (Ang & Jenkins, 1984) in the adrenal medulla, raised the possibility that the diuretic response to κ-opioid agonists might involve a peripheral component, dependent upon an intact and functional adrenal medulla (Blackburn *et al.*, 1985; 1986). Indeed, adrenal demedullation was found to abolish selectively the diuretic response to the κ-opioid agonists, U50488H, ethylketocyclazocine (EKC) and tifluadom (Blackburn *et al.*, 1986).

This adrenomedullary dependence of κ-opioid agonist-induced diuresis, led to the suggestion that κ-opioids may be capable of stimulating the release of an, as yet unidentified, 'diuretic factor' of adrenomedullary origin. The present transfusion experiments were performed in an attempt to establish the existence of the postulated 'diuretic factor'.

Methods

Animals

Male Wistar rats (175–200 g body weight), obtained from Charles River, Montreal, were used throughout. Under Saffan (alphaxolone/alphadolone; 12 mg kg^{-1}, i.v.) anaesthesia, bilateral adrenal demedullation was performed by methods described previously (Borkowski & Quinn, 1983). Seven days later, a carotid artery and a jugular vein were catheterized, under Saffan anaesthesia, to facilitate arterial blood sampling and intravenous drug administration respectively. The rats were used in experiments not less than 48 h after catheter implantation. To minimize post-operative discomfort, all animals were treated with Talwin (pentazocine; 10 mg kg^{-1}, s.c.) in the immediate post-operative period. In accord with the previous study (Blackburn *et al.*, 1986), preliminary observations established that sham adrenal

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demedullated rats exhibited plasma catecholamine levels and urinary responses to U50488H, tifluadom, EKC, furosemide and buprenorphine, which were similar to those obtained in catheterized but otherwise unoperated control animals. Thus, all further experiments were performed on catheterized rats with (intact) and without (demedullated) adrenal medullae.

Diuretic studies

Intact and demedullated animals were given a saline load (0.9% w/v NaCl; 10 ml kg^{-1}, p.o.) and placed in individual Nalge metabolism cages (Fisher Scientific) for rodents. The arterial and venous catheters were connected to 1 ml syringes via polyethylene tubing (PE50) filled with heparinized saline. These enabled arterial blood sampling and intravenous drug administration without disturbing the rats during the experiment.

The urine collected in the first 30 min after saline-loading was measured and discarded. At this time, test drugs or saline were administered intravenously, in a volume of 1 ml kg^{-1}, and flushed-in with 0.2 ml of heparinized saline. Antagonists were administered in a similar manner 5 min before the agonists. The volume of urine collected in the subsequent 4 h was expressed in terms of:- ml h^{-1} 100 g^{-1} body weight.

Transfusion studies

Donor rats were saline-loaded and the urine collected in the first 30 min discarded. The donors were then given the test drug intravenously and the recipient rats saline-loaded. Thirty minutes later, the urine collected from recipients was discarded, 1 ml of arterial blood was drawn from the donor rats (over 30 s) and immediately given intravenously to the recipients (again over 30 s). The blood withdrawn from the donors was replaced with 1 ml of saline and this, and the blood infused into the recipients, was flushed-in with 0.2 ml of heparinized saline. Antagonists were injected 5 min before the agonists in donor rats and 5 min before the blood transfusion in recipient animals. The rats remained in the metabolism cages until 4 h had elapsed from the time of drug/donor blood administration.

Catecholamine assays

The effectiveness of adrenal demedullation was determined by measuring plasma and adrenal gland catecholamine content in 6 intact and 6 demedullated rats, chosen at random from the experimental groups, before they had been subjected to any drug treatment. Forty eight hours after catheterization (i.e. 9 days after demedullation), the rats were placed in metabolism cages. One hour later, 1 ml of blood was collected, by free-flow, into ice-cold centrifuge tubes containing heparin (20 units in 20 μl). The red cells were spun down (3000 g × 10 min at 4°C) and the plasma catecholamines extracted onto activated alumina and eluted with 0.1 M perchloric acid, according to a modification of the method of Anton & Sayre (1962). Catecholamine concentration was measured by reverse-phase high performance liquid chromatography with electrochemical detection (h.p.l.c.-e.d.). Two days after blood sampling, the rats were anaesthetized with Saffan, the adrenal glands removed and homogenized in 1 ml aliquots of 0.1 M perchloric acid containing a trace (400 $\mu l\,l^{-1}$) of 1 M $NaHSO_3$. The homogenates were centrifuged (3000 g × 10 min), the supernatant filtered through 0.22 μm pore-size filters to remove colloidal protein, and assayed by h.p.l.c.-e.d.

Drugs

Generous donations of the following drugs are gratefully acknowledged:- U50488H (trans-3,4-dichloro-N-methyl-N-[2-(pyrrolidinyl)-cyclohexyl]-benzeneacetamide methane sulphonate) (Upjohn), tifluadom (Sandoz), EKC (Winthrop) and buprenorphine (Schering). Furosemide and naloxone were obtained from Sigma. All drugs were dissolved freshly in saline, with the aid of minimal amounts of dilute HCl or gentle warming if necessary, given intravenously in a volume of 1 ml kg^{-1} and flushed-in with 0.2 ml of heparinized saline. Saline was used as the control. Experimental groups consisted of a minimum of 6 animals and data are presented as the mean ± standard error of the mean (s.e.mean), where n is the number of observations. Statistical analysis was by Student's unpaired t test and significance was accepted when $P < 0.05$.

Results

Diuretic studies in intact and adrenal demedullated rats

Nine days after bilateral adrenal demedullation, plasma adrenaline levels were reduced by 93% compared to intact rats and by 92% compared to sham-operated animals (intact: 492 ± 86 pg ml^{-1}; sham-operated: 451 ± 99 pg ml^{-1}; demedullated: 34 ± 7 pg ml^{-1}, $n = 6$ in all cases). Demedullation-induced changes in plasma noradrenaline and dopamine levels were small and did not achieve statistical significance. Eleven days after demedullation, adrenal gland noradrenaline (64.5 ± 6.3 ng) and adrenaline (90.5 ± 22.3 ng) content, per pair of glands, was reduced by more than 99% compared to levels in intact (9.9 ± 1.4 μg and 25.5 ± 3.8 μg, respectively) or sham-operated (10.4 ± 2.1 μg and 24.9 ± 3.0 μg) rats.

In keeping with earlier observations (Blackburn *et al.*, 1986) in the Alderley Park Wistar rat, there was

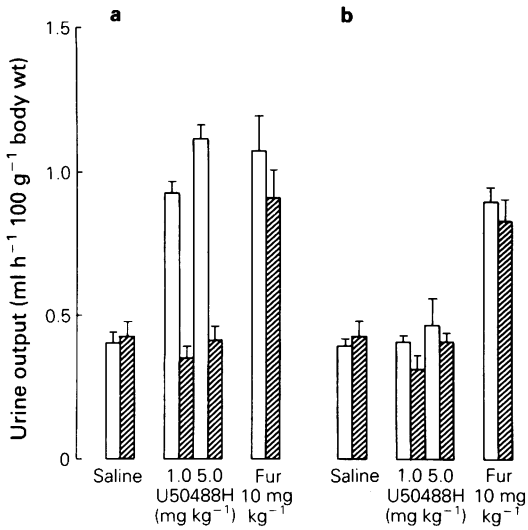

Figure 1 Effect of pretreatment with naloxone (10 mg kg^{-1}, i.v.) on the cumulative urine output over 4 h, expressed in terms of:- ml h^{-1} 100 g^{-1} body weight, induced by saline (1 ml kg^{-1}, i.v.), furosemide (Fur; 10 mg kg^{-1}, i.v.) and U50488H (1–5 mg kg^{-1}, i.v.) in conscious saline (10 ml kg^{-1}, p.o.)-loaded intact (a) and adrenal demedullated (b) rats. Effects in saline (1 ml kg^{-1}, i.v.) pretreated controls are indicated by the open columns. The hatched columns indicate urine output in naloxone pretreated rats. Each column represents the mean of a group of 8 rats and the vertical lines indicate s.e.mean.

no significant difference in basal urine output between intact (0.407 ± 0.038 ml h^{-1} 100 g^{-1} body weight, $n = 16$), sham-operated (0.449 ± 0.058 ml h^{-1} 100 g^{-1} body weight, $n = 8$) and adrenal demedullated (0.393 ± 0.026 ml h^{-1} 100 g^{-1} body weight, $n = 16$) conscious, saline-loaded Wistar rats. Nor did adrenal demedullation significantly affect the diuretic response (1.080 ± 0.120 ml h^{-1} 100 g^{-1} body weight, $n = 8$) to furosemide (10 mg kg^{-1}, i.v.) or the antidiuretic response (0.165 ± 0.035 ml h^{-1} 100 g^{-1} body weight, $n = 8$) to buprenorphine (1 mg kg^{-1}, i.v.). However, as was the case in the previous study (Blackburn *et al.*, 1986), the diuretic responses to U50488H (1 mg kg^{-1}, i.v.), tifluadom (5 mg kg^{-1}, i.v.) and EKC (1 mg kg^{-1}, i.v.) were attenuated in demedullated rats (0.430 ± 0.030 ml h^{-1} 100 g^{-1} body weight, 0.600 ± 0.055 ml h^{-1} 100 g^{-1} body weight and 0.510 ± 0.050 ml h^{-1} 100 g^{-1} body weight, respectively; $n = 8$ in all cases) when compared to those obtained in intact animals (0.930 ± 0.040 ml h^{-1} 100 g^{-1} body weight, 0.855 ± 0.065 ml h^{-1} 100 g^{-1} body weight and 1.000 ± 0.115 ml h^{-1} 100 g^{-1} body weight, respectively; $n = 8$ in all cases).

Table 1 Urine output over 4 h, (ml h^{-1} 100 g^{-1} body weight) in conscious intact donor and recipient rats

Group	*Donors*	*Recipients*
1 ($n = 7$)	Intact + saline	Intact
	0.374 ± 0.093	0.464 ± 0.114
2 ($n = 7$)	Intact + U50488H	Intact
	0.871 ± 0.079[a]	0.757 ± 0.062[b]
3 ($n = 6$)	Intact + U50488H	Intact + Nal
	0.897 ± 0.099[a]	0.810 ± 0.102[b]
4 ($n = 6$)	Intact + Nal + U50488H	Intact
	0.413 ± 0.054[c]	0.450 ± 0.050[d]
5 ($n = 6$)	Intact + Nal + U50488H	Intact + Nal
	0.455 ± 0.054[c]	0.473 ± 0.041[e]

Thirty min after saline loading, recipient animals received a 1 ml blood transfusion from donor rats which had been given test drugs 30 min previously. The test drugs administered were:- saline (1 ml kg^{-1}, i.v.); U50488H (5 mg kg^{-1}, i.v.) and naloxone (Nal; 10 mg kg^{-1}, i.v.). U50488H and naloxone were administered in a volume of 1 ml kg^{-1} and all injections were flushed in with 0.2 ml of heparinized saline.

[a] $P < 0.01$, comparing donor groups 2 or 3 to group 1.

[b] $P < 0.05$, comparing recipient groups 2 or 3 to group 1.

[c] $P < 0.01$, comparing donor groups 4 or 5 to groups 2 or 3.

[d] $P < 0.01$, comparing recipient group 4 to group 2.

[e] $P < 0.05$, comparing recipient group 5 to group 3.

There were no significant differences between donors and recipients in any one experimental group. All analyses were performed by Student's unpaired *t* test.

Transfusion studies

The agonist U50488H, which is highly selective for κ-opioid receptors (James & Goldstein, 1984), was chosen for use in the transfusion studies. A dose of 5 mg kg^{-1} U50488H (i.v.) was selected since it induced a marked but submaximal diuresis, in intact rats, which could be abolished by naloxone (10 mg kg^{-1}, i.v.) pretreatment and was attenuated significantly in adrenal demedullated rats (Figure 1; Tables 1 and 2).

Naloxone (10 mg kg^{-1}, i.v.) pretreatment did not affect basal urine output nor furosemide (10 mg kg^{-1}, i.v.)-induced diuresis (Figure 1), indicating a selective action against U50488H. The ability of naloxone to block the diuretic effects of U50488H, together with the lack of a diuretic response to control injections of saline (1 ml kg^{-1}, i.v.), also indicates that U50488H-induced diuresis is not a volume effect.

Transfusing 1 ml of blood, from an intact donor rat which had received saline (1 ml kg^{-1}, i.v.) 30 min previously, into an intact recipient, did not affect urine output in the recipient animal (Table 1), again indicating a lack of volume effect of the transfused blood. However, transfusing 1 ml of blood, from an intact donor which had received U50488H (5 mg kg^{-1}, i.v.) 30 min previously, into an intact recipient, induced a diuretic response which was smaller but not significantly different from that induced by U50488H in the donor rat (Table 1). This diuretic response in the recipient was not due to U50488H in the donor blood, since pretreating the recipient with naloxone (10 mg kg^{-1}, i.v.), a dose which abolished U50488H (5 mg kg^{-1}, i.v.) induced diuresis, did not attenuate the diuretic response induced by the transfused blood in the recipient rat (Table 1).

Pretreating intact donor rats with naloxone (10 mg kg^{-1}, i.v.) not only abolished the diuretic response to the subsequent injection of U50488H (5 mg kg^{-1}, i.v.) in these animals (Table 1), but the blood from these donors no longer induced diuresis in intact recipients, whether or not the recipient rats had been pretreated with naloxone (Table 1). This observation supports the view that the contents, and not the volume, of the transfused blood induce diuresis in the recipient animals.

Despite there being no difference between demedulated and intact rats, when comparing their basal urine output and urinary responses to intravenous saline, furosemide (Figure 1; Tables 1 and 2) and buprenorphine, U50488H (5 mg kg^{-1}, i.v.) was no longer able to induce a diuretic response in conscious rats which had undergone bilateral adrenal demedullation 9 to 14 days previously (Figure 1; Table 2).

Transfusing 1 ml of blood from demedullated donor rats, which had received either saline (1 ml kg^{-1}, i.v.) or U50488H (5 mg kg^{-1}, i.v.) 30 min previously, failed to induce a diuretic response in either intact or demedullated recipients (Table 2) and offers further support to the contention that the volume of transfused blood is without diuretic effect. This also indicates that 1 ml of blood transfused, from a donor rat treated with U50488H, contains insufficient U50488H to induce diuresis in a recipient.

Blood transfused from intact donors treated with saline (1 ml kg^{-1}, i.v.) did not affect urine output in demedullated recipients (Table 2). However, blood from intact donors treated with U50488H (5 mg kg^{-1}, i.v.) induced diuresis in demedullated recipients (Table 2). This diuretic response was significantly smaller than that induced by a similar transfusion or the direct injection of U50488H (5 mg kg^{-1}, i.v.) in intact rats, but was significantly greater than the urine output observed in demedullated rats treated with U50488H (5 mg kg^{-1}, i.v.) and in demedullated recipients of blood from either intact donors treated with saline or demedullated donors treated with U50488H (Tables 1 and 2).

Table 2 Urine output over 4 h (ml h^{-1} 100 g^{-1} body weight) in conscious intact and adrenal demedullated (Demed) donor and recipient rats

Group	*Donors*	*Recipients*
1 ($n = 7$)	Demed + saline	Demed
	0.389 ± 0.068	0.440 ± 0.065
2 ($n = 6$)	Demed + U50488H	Demed
	0.466 ± 0.093	0.444 ± 0.068
3 ($n = 6$)	Demed + U50488H	Demed + Nal
	0.444 ± 0.030	0.391 ± 0.031
4 ($n = 6$)	Demed + saline	Intact
	0.396 ± 0.034	0.455 ± 0.041
5 ($n = 6$)	Demed + U50488H	Intact
	0.420 ± 0.058	0.506 ± 0.053
6 ($n = 6$)	Intact + saline	Demed
	0.440 ± 0.059	0.416 ± 0.075
7 ($n = 6$)	Intact + U50488H	Demed
	0.962 ± 0.147[a,d]	0.628 ± 0.052[b]
8 ($n = 6$)	Intact + U50488H	Demed + Nal
	0.886 ± 0.092[a,d]	0.604 ± 0.080[c]

Thirty min after saline loading, recipient rats received a 1 ml blood transfusion from donor animals which had been given test drugs 30 min previously.
Test drugs and their administration was the same as in Table 1.
[a] $P < 0.01$, comparing donor groups 7 or 8 to groups 2, 3, 5 or 6.
[b] $P < 0.05$, comparing recipient group 7 to groups 2 or 6.
[c] $P < 0.05$, comparing recipient group 8 to group 3.
[d] $P < 0.05$, comparing donor groups 7 and 8 to recipient groups 7 and 8 respectively.
All analyses were performed by Student's unpaired *t* test.

Discussion

A previous study (Blackburn *et al.*, 1986) established that bilateral adrenal demedullation selectively abolished the diuretic responses to subcutaneous injections of equieffective doses of the κ-opioid agonists U50488H, tifluadom and EKC in conscious saline-loaded rats. Basal urine output, responses to furosemide, clonidine and the antidiuretic responses to the μ-opioid agonists buprenorphine and morphine were unaffected, indicating the selective nature of adrenal demedullation against κ-opioid agonists.

The present studies employed intravenous drug administration and paralleled these findings, supporting the view that κ-opioid agonist-induced

diuresis involves the adrenal medulla and cannot be explained purely in terms of a central action at the neurohypophysis, since adrenal demedullation would not be expected to have a direct effect at this site. This adrenomedullary dependence lead to the hypothesis that κ-opioid agonist-induced diuresis may involve the release of a 'diuretic factor' of adrenomedullary origin (Blackburn *et al.*, 1986). The present studies sought to confirm earlier observations and attempt to establish the possible existence of the factor. The isolation of the factor, its identification and determination of its mechanism/s of action (e.g. a direct effect on the kidney or an indirect effect via the inhibition of neurohypophysial vasopressin release) have yet to be attempted.

The adrenal medulla contains numerous endogenous transmitters and hormones (Ang & Jenkins, 1984; Lemaire *et al.*, 1984), yet catecholamines comprise the major portion of the adrenomedullary contents (Kirshner, 1972). Thus, the effectiveness of adrenal demedullation was assessed by measuring plasma adrenaline and adrenal gland catecholamine content. In agreement with previous studies (Borkowski & Quinn, 1983; 1985; Blackburn *et al.*, 1986), surgical enucleation significantly reduced plasma adrenaline and adrenal gland catecholamine levels, indicating effective removal of the adrenal medulla. Whilst not measured in the present study, plasma corticosterone is unaffected by demedullation (Borkowski & Quinn, 1983; Blackburn *et al.*, 1986) indicating that the adrenal cortex remains functional and appears not to be involved in the diuretic response to κ-opioid agonists.

The adrenomedullary dependence of κ-opioid agonist-induced diuresis, observed in this and the previous studies (Blackburn *et al.*, 1985; 1986), might be explicable as follows:-

(a) κ-opioid agonists induce diuresis by stimulating opioid receptors, suppressing vasopressin release, in both the neurohypophysis and the adrenal medulla. The medullary opioid receptors are more sensitive than those in the neurohypophysis, so that higher doses of agonist are required to induce diuresis in the absence of the adrenal medulla, or (b) κ-opioid agonists act at the neurohypophysis to suppress the release of vasopressin. Their access to this central site of action is aided by a factor released from the adrenal medulla in response to κ-opioid receptor stimulation. The factor is absent in adrenal demedullated rats which, therefore, require higher systemic doses of the agonists in order to achieve concentrations at the neurohypophysis similar to those in intact rats.

Unfortunately, neither of these possibilities is consistent with the results of the studies which established that the diuretic effect of U50488H was transferable, between rats, by transfusing 1 ml of blood. That this was not a volume effect is indicated by observations that blood, transfused from saline-treated intact or demedullated donor rats, had no effect on urine output in intact or demedullated recipients. Moreover, blood from demedullated donors treated with U50488H failed to induce a diuretic response in either intact or demedullated recipients, supporting the contention that the amount of U50488H, in the blood transfused from a U50488H-treated donor, is insufficient to induce diuresis.

Of still greater interest was the observation that blood, from a U50488H-treated intact donor, induced a diuretic response in both intact and demedullated recipients. The inability of U50488H to induce diuresis in demedullated rats and the inability of naloxone pretreatment of the recipients to attenuate the transfusion-induced diuresis, indicates that the diuretic response in the recipients was not due to any U50488H in the donor blood. The lack of effect of naloxone in the recipients, also indicates that opioid receptors are not involved in mediating the diuretic effects of the transfused blood and suggests that the postulated adrenomedullary 'diuretic factor' is not an opioid.

The data indicate that κ-opioid agonist-induced diuresis is, indeed, dependent upon an intact and functional adrenal medulla.

The transferability of U50488H-induced diuresis between rats, by blood transfusion, indicates that this dependence may be due to the presence of a blood-borne 'diuretic factor' of adrenomedullary origin. That the release of the factor is due to κ-opioid receptor stimulation, is supported by observations that naloxone pretreatment of intact rats not only abolished U50488H-induced diuresis in these animals, but also deprived the blood transfused from them of its ability to induce diuresis in recipient rats. The identity and mechanism/s of action of the 'factor' remain to be elucidated.

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Evidence that different regional sympathetic outflows vary in their sensitivity to the sympathoinhibitory actions of putative 5-HT_{1A} and α_2-adrenoceptor agonists in anaesthetized cats

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1 An investigation was carried out to determine whether the centrally acting hypotensive drugs whose mechanisms of action are due either to activation of 5-HT_{1A} receptors (flesinoxan, 8-hydroxy-2-(di-*n*-propylamino)tetralin (8-OH-DPAT) and urapidil — also an α_1-adrenoceptor antagonist) or to activation of α_2-adrenoceptors (clonidine and moxonidine) cause differential sympathoinhibition.

2 Cats were anaesthetized with α-chloralose and simultaneous recordings were made of whole cardiac, splanchnic and renal nerve activity, blood pressure and heart rate. Cumulative dose-response (i.v.) curves were constructed in separate experiments for the above hypotensive agents on these parameters.

3 Renal nerve activity was found to be more sensitive to the sympathoinhibitory action of flesinoxan and 8-OH-DPAT when compared with cardiac nerve activity, whereas the reverse was observed for clonidine and moxonidine, cardiac being more sensitive than renal nerve activity. Splanchnic nerve activity was similarly affected by all drugs. Furthermore at the highest dose, all drugs tended to cause complete inhibition in all regional sympathetic nerve outflows.

4 Urapidil differed from all the above hypotensive drugs in that it caused a similar degree of sympathoinhibition in all sympathetic outflows at all doses. It is suggested that this may be due to the ability of urapidil to block central α_1-adrenoceptors in addition to stimulation of 5-HT_{1A} receptors.

Introduction

There is a large amount of evidence to suggest that 8-hydroxy-2-(di-*n*-propylamino)tetralin (8-OH-DPAT) and flesinoxan activate 5-HT_{1A} receptors to cause their hypotensive action (Fozard *et al.*, 1987; McCall *et al.*, 1987; Ramage & Fozard, 1987; Doods *et al.*, 1988; Wouters *et al.*, 1988; Laubie *et al.*, 1989) and that such an action is entirely within the central nervous system. However, only moderate thoracic preganglionic sympathoinhibition is observed in anaesthetized cats (Ramage & Fozard, 1987; Ramage *et al.*, 1988). One explanation for this lack of correlation between the thoracic sympathoinhibitory action and the hypotensive action of 8-OH-DPAT and flesinoxan is that these drugs could be having a more potent sympathoinhibitory action at a different level or levels of sympathetic outflow. In this respect the sympathoinhibitory action of flesinoxan on renal outflow, when compared with other sympathetic outflows has been shown to be greater (Ramage *et al.*, 1988). However a weakness of this latter investigation was that the recordings made from different sympathetic outflows were carried out in separate animals and further, that some of the outflows were recorded from preganglionic, while others were recorded from postganglionic nerves. In order to overcome these weaknesses the present experiments were carried out in which the sympathoinhibitory actions of flesinoxan and 8-OH-DPAT were studied on cardiac, splanchnic and renal nerve activity recorded simultaneously in the same animal. Further, the effects of other hypotensive agents, urapidil (which has also been shown to bind to 5-HT_{1A} receptors; Fozard & Mir, 1987; Groβ *et al.*, 1987), along with clonidine and the newer α_2-adrenoceptor agonist moxonidine (Armah, 1987) were also studied in the above experimental model. The latter two

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drugs were chosen because it has been suggested (Schmitt, 1975) that the sympathoinhibitory action of clonidine, and therefore other α_2-adrenoceptor agonists, may also vary from one regional outflow to another.

Preliminary accounts of these observations have been published (Ramage & Wilkinson, 1988a,b).

Methods

Experiments were performed on 27 male adult cats (2.5–4.2 kg) anaesthetized with a mixture of α-chloralose (70 mg kg^{-1}, i.v.) and pentobarbitone sodium (12 mg per animal). Supplementary doses of α-chloralose (10–15 mg kg^{-1}) anaesthetic were given as required. The animals were artificially ventilated using positive pressure after neuromuscular blockade with vecuronium bromide (200 μg kg^{-1}). Arterial blood gases, pH, body temperature, blood pressure and heart rate were monitored as previously described (Ramage, 1984). Drugs were given by bolus injection into the jugular vein.

Simultaneous recordings were made of sympathetic nerve activity from the inferior cardiac (postganglionic), splanchnic (mixed pre- and postganglionic) and renal (postganglionic) nerves. To record cardiac nerve activity the animal was laid on its right side and the second and third ribs removed retropleurally to expose the inferior cardiac nerve. A pneumothorax was then created between ribs 5 and 6 to reduce mechanical movement of the rib cage. At this stage the respiration pump was connected to an expiratory resistance of 0.5 cmH_2O to prevent atelectasis. The splanchnic and renal nerves were exposed by a retroperitoneal approach. Whole nerve activity was recorded by use of bipolar silver hook electrodes from the central cut or crushed ends of these nerves. Nerve activity was quantified by rectifying and integrating the signal above background noise over 5 s with solid state electronic integrators (constructed by Mr A. Charles, Electronic Workshop, RFHSM). The validity of the threshold setting was verified at the end of the experiment after administration of pentobarbitone sodium (60 mg per animal) or by crushing the nerve to block all activity. The output of the integrator was displayed on a Grass polygraph. Sympathetic nerve activity was tested to ensure that it was under baroreceptor modulation, by checking that activity in these nerves was increased by a fall in blood pressure caused by sodium nitroprusside (2 μg kg^{-1}) and decreased by a rise in blood pressure caused by noradrenaline (0.25 μg per animal).

In all experiments all parameters were recorded over a 20 min period before injection of the test solutions. In separate experiments cumulative dose-response curves were constructed for flesinoxan (3–300 μg kg^{-1}), 8-OH-DPAT (0.5–128 μg kg^{-1}), clonidine (0.3–10 μg kg^{-1}), moxonidine (1–100 μg kg^{-1}) and urapidil (0.01–3.0 mg kg^{-1}). Intervals between injections times were: for flesinoxan 5, 10, 15 and 15 min, for 8-OH-DPAT 5, 5, 10 and 10 min, for clonidine 5, 10 and 15 min, for moxonidine 5, 7, 10 and 15 min and for urapidil 5, 5, 10, 10 and 10 min. The time between the injections represents the time taken for the change in blood pressure caused by these drugs to stabilize. All drugs except urapidil were dissolved in saline; urapidil was dissolved in 0.04 M lactic acid. All doses except noradrenaline, urapidil and 8-OH-DPAT refer to the salts of the drugs.

Analysis of data

Baseline values were measured just before the first injection. Responses for all parameters were measured just prior to the next injection. The changes that occurred after the final dose of each drug were measured after 15 min for flesinoxan, 10 min for 8-OH-DPAT and urapidil, and finally 30 min for clonidine and moxonidine. Nerve activity was taken as the mean level over 1 min in arbitrary units. All results are expressed as changes from baseline values. Activity over the complete dose range for all the nerves for each drug was analysed with a two way analysis of variance with replicates. Comparison of nerve activity at each dose was carried out using a least significant difference test (Sokal & Rohlf, 1969). Differences were considered significant when $P < 0.05$.

Drugs

The following drugs were purchased from Sigma, α-chloralose, atropine methonitrate and sodium nitroprusside; 8-OH-DPAT HBr was obtained from RBI: Semat Technical Ltd and pentobarbitone sodium from May & Baker Ltd. The following drugs were kindly donated by the companies indicated, flesinoxan HCl (Duphar B.V.), clonidine HCl (Boehringer Ingelheim), moxonidine HCl·H_2O (Beiersdorf AG) and urapidil (Byk Gulden Pharmazeutika).

Results

Effect of cumulative doses of flesinoxan (3–300 μg kg^{-1}) over 60 min

Cumulative doses of flesinoxan ($n = 5$) caused dose-related falls in mean blood pressure, reaching a maximum of -63 ± 6 mmHg (mean ± s.e.mean) from a mean baseline value of 126 ± 7 mmHg, and

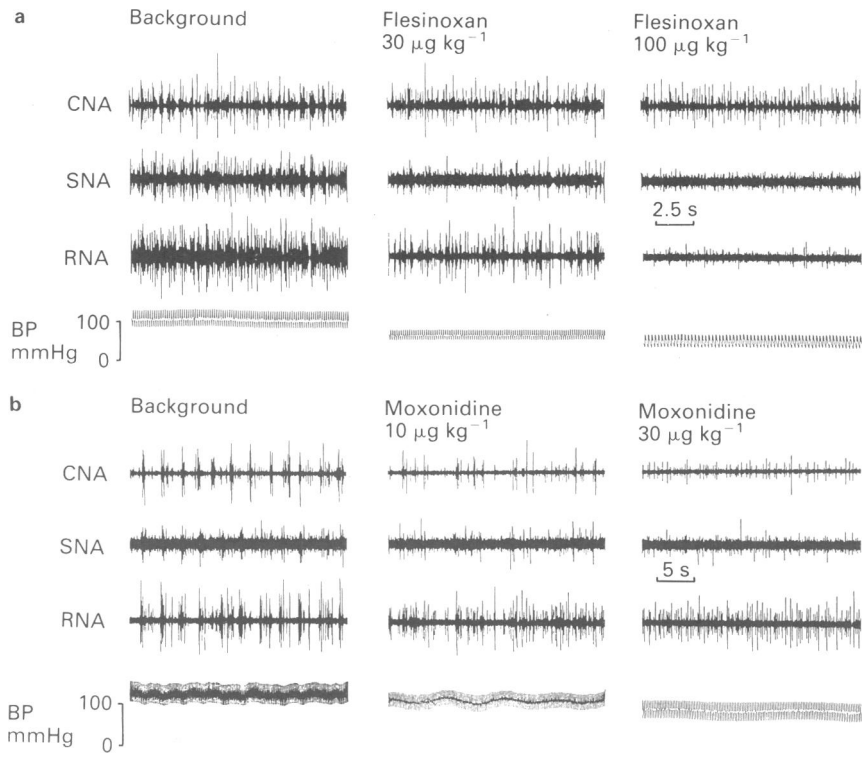

Figure 1 Traces comparing the effect of cumulative i.v. injections of flesinoxan (a) and moxonidine (b) in two separate anaesthetized cats on cardiac nerve activity (CNA), splanchnic nerve activity (SNA), renal nerve activity (RNA) and blood pressure (BP).

in heart rate, reaching -130 ± 8 beats min^{-1} from a mean baseline value of 215 ± 9 beats min^{-1} at the highest dose. These falls in mean blood pressure and heart rate were associated with a reduction in cardiac, splanchnic and renal nerve activity of $80 \pm 10\%$, $93 \pm 3\%$ and 100% respectively caused by the highest dose. However, the different sympathetic outflows showed varying sensitivity to the sympathoinhibitory action of flesinoxan (Figure 1). Sympathetic nerve activity first began to decline in the renal and splanchnic nerves followed by the cardiac nerve (Figure 2). At doses of 30 and 100 μg kg^{-1} sympathoinhibition in the renal nerve was significantly greater than that in the cardiac nerve ($P < 0.05$: least significant difference two way analysis of variance; Figure 2).

Effect of cumulative doses of 8-OH-DPAT (0.5–128 μg kg^{-1}) over 40 min

Cumulative doses of 8-OH-DPAT ($n = 6$) caused dose-related falls in mean blood pressure, reaching a maximum of -76 ± 5 mmHg at the highest two doses (32 and 128 μg kg^{-1}) from a mean baseline value of 137 ± 4 mmHg, and a fall in heart rate of -128 ± 12 beats min^{-1} from a mean baseline value of 220 ± 11 beats min^{-1}. These falls in mean blood pressure and heart rate were associated with a reduction in cardiac, splanchnic and renal nerve activity of $86 \pm 5\%$, $87 \pm 5\%$ and 100% respectively by the highest dose (Figure 2). 8-OH-DPAT also caused a similar pattern of differential sympathoinhibition to that seen with flesinoxan, sympathetic nerve activity first declining in the renal, followed by splanchnic and then the cardiac nerve. At doses of 8 and 32 μg kg^{-1}, sympathoinhibition in the renal nerve was significantly greater than that in the cardiac nerve while also at the 32 μg kg^{-1} dose sympathoinhibition was greater in renal than in splanchnic nerve activity.

Effect of cumulative doses of urapidil (0.01–3 mg kg^{-1}) over 50 min

From the dose of 0.1 mg kg^{-1} upwards urapidil caused decreases ($n = 6$; Figure 3) in a dose-

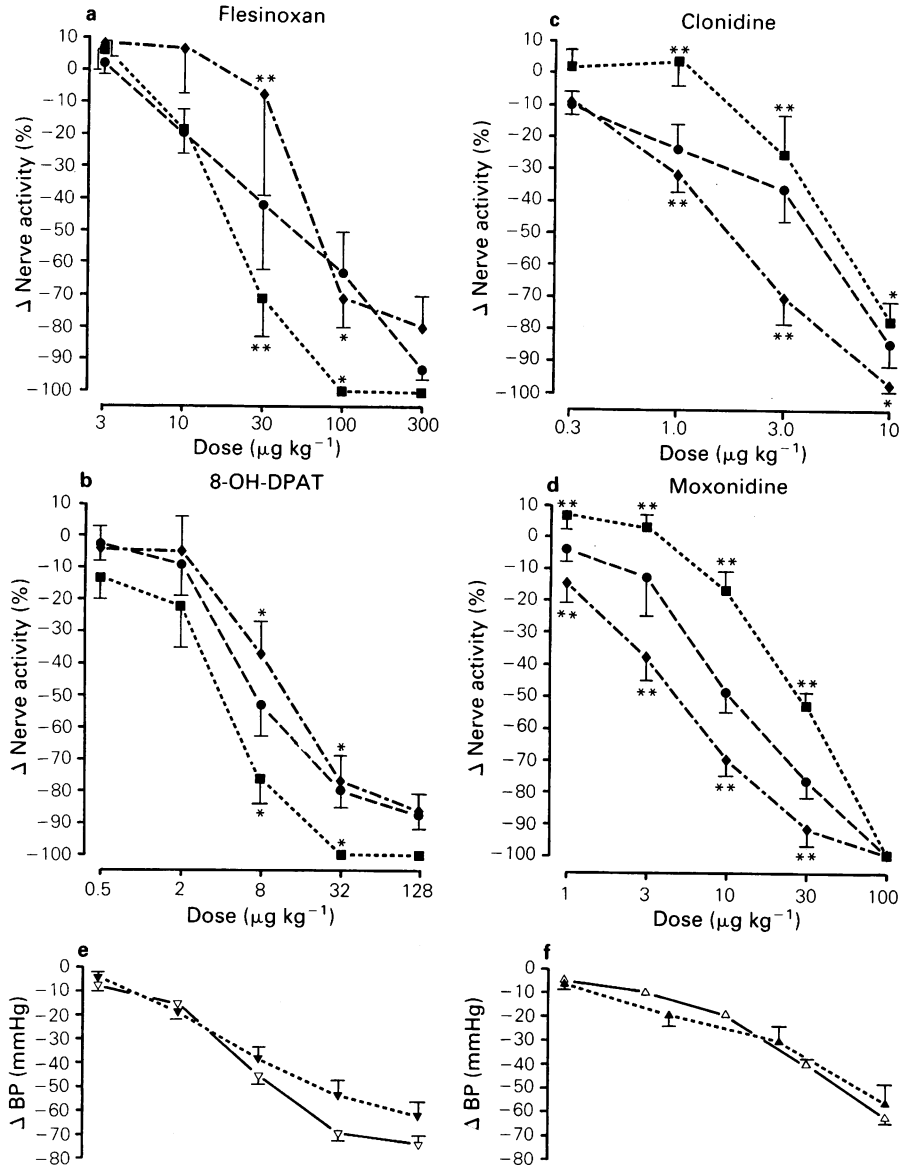

Figure 2 Anaesthetized cats: the effect of cumulative i.v. doses of flesinoxan (a; $n = 5$) 8-OH-DPAT (*b*; $n = 6$) clonidine (c; $n = 5$) and moxonidine (d; $n = 5$) on changes (Δ) in cardiac nerve activity (◆), splanchnic nerve activity (●), renal nerve activity (■). The changes in blood pressure caused by these drugs are also shown: in (e) 8-OH-DPAT (▽) and flesinoxan (▼); and in (f) moxonidine (△) and clonidine (▲). The ordinates are the same as in (a)–(d). Each point represents the mean value and the vertical bars show s.e.mean. $*P < 0.05$; $**P < 0.01$, comparison between cardiac and renal nerve activity least significant difference two analysis of variance. For clarity, comparison between splanchnic and the other nerves has not been included in the graph; see text for details.

dependant manner in all the parameters being recorded. At the highest dose, mean blood pressure had decreased by 47 ± 4 mmHg from a baseline value of 120 ± 7 mmHg, heart rate by 60 ± 8 beats min^{-1} from a baseline value of 198 ± 10 beats min^{-1} and cardiac, splanchnic and renal nerve activity by $89 \pm 5\%$, $93 \pm 3\%$ and $95 \pm 1\%$ respectively. However, the different sympa-

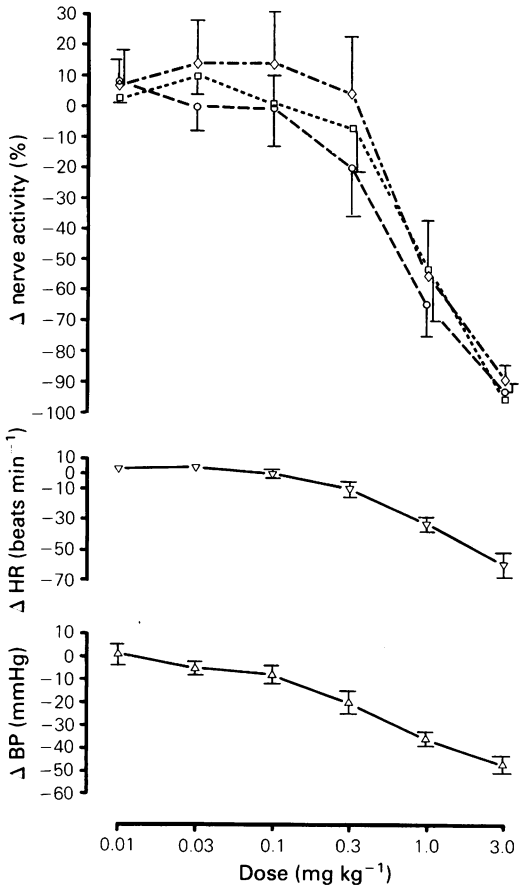

Figure 3 Anaesthetized cats: the effect of cumulative i.v. doses of urapidil ($n = 6$) on changes (Δ) in cardiac nerve activity (◇), splanchnic nerve activity (○), renal nerve activity (□), heart rate (HR) and blood pressure (BP). Each point represents the mean value and the vertical bars show s.e.mean.

thetic outflows did not show any varying sensitivity to the sympathoinhibitory action of urapidil. An injection of atropine methonitrate (0.1 mg kg^{-1}) at the end of the experiments caused a rise in heart rate of 26 ± 4 beats min^{-1}.

Effect of cumulative doses of clonidine (0.3–10 μg kg^{-1}) over 60 min

Cumulative doses of clonidine ($n = 5$) caused dose-related falls in mean blood pressure, reaching a maximum of −56 ± 8 mmHg from a mean baseline value of 149 ± 12 mmHg and in heart rate, reaching −68 ± 11 beats min^{-1} from a mean baseline value of 234 ± 10 beats min^{-1}, at the highest dose. These falls in mean blood pressure and heart rate were associated with a reduction in cardiac, splanchnic and renal nerve activity of 97 ± 2%, 84 ± 5% and 77 ± 6% respectively by the highest dose (Figure 2). The different sympathetic outflows did show varying sensitivity to the sympathoinhibitory action of clonidine but this was in the reverse order to that which had been observed for flesinoxan and 8-OH-DPAT. Overall, cardiac nerve activity was the most sensitive while renal nerve activity was the least. At the doses of 1, 3 and 10 μg kg^{-1} clonidine caused a significantly greater degree of inhibition of cardiac nerve activity than of renal nerve activity. Also at a dose of 3 μg kg^{-1}, sympathoinhibition was significantly greater in the cardiac nerve than in the splanchnic nerve. At doses of 0.3 and 1 μg kg^{-1}, sympathoinhibition was significantly greater in the splanchnic than in the renal nerve.

Effect of cumulative doses of monoxonidine (1–100 μg kg^{-1}) over 67 min

Cumulative doses of moxonidine ($n = 5$) caused dose-related falls in mean blood pressure, reaching a maximum of −62 ± 3 mmHg, from a mean baseline value of 142 ± 2 mmHg, and in heart rate, reaching −132 ± 12 beats min^{-1} from a mean baseline value of 260 ± 3 beats min^{-1} at the highest dose. These falls in mean blood pressure and heart rate were associated with a 100% reduction in cardiac, splanchnic and renal nerve activity by the highest dose (Figure 1). Moxonidine also caused a similar pattern of differential sympathoinhibition to that seen with clonidine, sympathetic nerve activity declining first in the cardiac, followed by splanchnic and then the renal nerve (Figure 2). Over the dose-range 1–30 μg kg^{-1} moxonidine had a significantly greater sympathoinhibitory action on cardiac than on renal nerve activity (Figure 1). Moxonidine also had a significantly greater sympathoinhibitory action on splanchnic than on renal nerve activity over the dose-range 1–10 μg kg^{-1}. Only at a dose of 3 μg kg^{-1} was the sympathoinhibitory action of moxonidine significantly greater on cardiac than on splanchnic nerve activity.

Discussion

The present results demonstrate that different sympathetic outflows vary in their sensitivity to the sympathoinhibitory actions of flesinoxan, 8-OH-DPAT, clonidine and moxonidine, when administered intravenously to anaesthetized cats. Renal nerve activity was found to be more sensitive to the sympathoinhibitory action of flesinoxan and 8-OH-DPAT than cardiac nerve activity, whereas the reverse was observed for clonidine and moxonidine, cardiac

being more sensitive than renal nerve activity. However, all these four drugs caused a similar depression of splanchnic nerve activity. Urapidil differed from these four drugs in that it caused a uniform reduction in sympathetic nerve activity at all the levels being recorded. These observations combined with the large amount of evidence that descending projections can selectively influence certain sympathetic outflows (Futuro-Neto & Coote, 1982b; Barman *et al.*, 1984; Meckler & Weaver, 1985; Stein *et al.*, 1989) suggest that such patterning may be dependent on the chemical transmitter that is released by a particular descending pathway.

5-HT-containing neurones, which are found in the raphe and the ventrolateral medulla, have been shown to project to preganglionic sympathetic motoneurones in the intermediolateral cell column (Bowker *et al.*, 1981; Loewy & Neil, 1981; Jacobs *et al.*, 1984; Marson & Loewy 1985; Ciriello *et al.*, 1986; 1988). These areas in the cat brain have also been shown to contain 5-HT_{1A} binding sites (Dashwood *et al.*, 1988a,b). As flesinoxan and 8-OH-DPAT are agonists at 5-HT_{1A} receptors (see Introduction) the present results would suggest that the above regions may be more important in the control of renal sympathetic outflow than other sympathetic outflows. In this respect electrical stimulation of the caudal part of the nucleus raphe obscurus can cause reduction or abolition of renal nerve activity with simultaneous facilitation of sympathetic nerve activity to the skeletal muscle vascular bed (Futuro-Neto & Coote, 1982b). The effect of flesinoxan and 8-OH-DPAT on skeletal muscle sympathetic nerve activity is unknown, however, both drugs cause profound hypotension associated with little change in femoral arterial conductance (Ramage & Fozard, 1987; Ramage *et al.*, 1988) suggesting, indirectly, that these drugs do not cause inhibition of skeletal muscle sympathetic nerve activity. Furthermore, Barman *et al.* (1984) showed that 50% of neurones in the medullary raphe and 30% in the ventrolateral medulla showed activity which was differential, related either to renal or cardiac nerve activity. In the rostral ventrolateral medulla, application of glycine was shown to cause a much greater reduction in renal than in mesenteric nerve activity and a small fall in heart rate (Stein *et al.*, 1989). In this respect, in the anaesthetized dog, microinjection of 8-OH-DPAT into the ventrolateral medulla caused a large fall in blood pressure and renal nerve activity but only a small fall in heart rate (Laubie *et al.*, 1989). A similar pattern of sympathoinhibition to that observed for 8-OH-DPAT and flesinoxan in the present experiments, has been reported during periods of desynchronized-like sleep in cats (Futuro-Neto & Coote, 1982b) and depletion of 5-HT significantly reduced the inhibition of renal nerve activity occurring during desynchronized sleep (Coote *et al.*, 1985).

The present results also show that cardiac nerve activity is more sensitive to the sympathoinhibitory action of clonidine and moxonidine, both of which cause their hypotensive action by stimulating α_2-adrenoceptors (see Schmidt, 1977; Unnerstall *et al.*, 1984; Gillis *et al.*, 1985; Armah, 1987), than the 5-HT_{1A} agonists, flesinoxan and 8-OH-DPAT. With respect to differential control of cardiac nerve activity there are few observations concerning any particular brainstem area. However, the most sensitive site in terms of the hypotension occurring after microinjections of clonidine, has been shown to be the lateral reticular nucleus which is near to the ventral lateral surface of the brainstem (Gillis *et al.*, 1985). Interestingly α_2-adrenoceptor binding sites have been shown to be in similar areas of the brain stem (Unnerstall *et al.*, 1984; Dashwood *et al.*, 1985) as observed for 5-HT_{1A} binding sites (see above).

Of the five drugs used in this study, urapidil failed to show any differential pattern of sympathoinhibition although it is known to bind to 5-HT_{1A} receptors. However, the present experiments support the suggestion that urapidil does stimulate central 5-HT_{1A} receptors as urapidil caused an increase in vagal drive to the heart, a characteristic feature of 5-HT_{1A} agonists (Ramage & Fozard, 1987). It should be noted that α-chloralose anaesthetized cats have little or no vagal tone (unpublished observations). However, in prazosin pretreated animals a differential sympathoinhibitory action was not observed for the 5-HT_{1A} agonists 8-OH-DPAT (Ramage, 1988). This may explain why urapidil failed to cause a differential pattern of sympathoinhibition as it is also an α_1-adrenoceptor antagonist (see Ramage, 1986). In this respect it has been shown that α_1-adrenoceptor antagonists can block 5-HT excitation of raphe neurones (Baraban & Aghajanian, 1980).

The finding that splanchnic nerve activity seems to be affected equally by all the drugs studied may be explained by the observation that activity in this nerve is only slightly influenced by supra-spinal pathways, as transection of the spinal cord at C1 only slightly reduced the activity in this nerve (Qu *et al.*, 1988). However, the differential sensitivity of different sympathetic outflows observed in this study may just reflect the accessibility of various receptor sites within the CNS to these drugs, when given i.v.

In conclusion, the present results demonstrate that the degree of sympathoinhibition caused by centrally acting antihypertensive drugs when given i.v. depends on the dose of the drug, the particular sympathetic nerve being recorded from and whether they act by selectively stimulating 5-HT_{1A} or α_2-adrenoceptors. From these observations it is sug-

gested that renal nerve activity is under a greater influence from central 5-hydroxytryptaminergic than noradrenergic pathways, while the opposite is true for cardiac nerve activity.

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The antiarrhythmic efficacy of intravenous anipamil against occlusion and reperfusion arrhythmias

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1 Anipamil, a long acting analogue of verapamil, was tested for its actions against arrhythmias induced by ischaemia and reperfusion in conscious and anaesthetized rats, as well as for effects on epicardial intracellular action potentials.

2 When given 15 min or 4 h before coronary occlusion, 1 and 5 mg kg^{-1} anipamil reduced ischaemia-induced arrhythmias in conscious rats. The same doses also reduced arrhythmias when given 15 min before occlusion in acutely-prepared anaesthetized rats. ED_{50} values were between 1 and 5 mg kg^{-1}.

3 The incidence of reperfusion arrhythmias depended upon the period of regional ischaemia prior to reperfusion such that the peak incidence occurred after 5–7 min of ischaemia. Anipamil (2.5 mg kg^{-1}, i.v.) selectively abolished the reperfusion arrhythmias induced by short periods of ischaemia, although some antiarrhythmic effects were seen for all periods of ischaemia.

4 Anipamil slowed the rate of development of R-wave increases and S-T segment elevations induced by ischaemia, but did not reduce the maximum values they attained.

5 Anipamil (2.5 mg kg^{-1} i.v.) lacked Class I or III electrophysiological actions on intracellular action potentials recorded *in vivo* from the epicardium of rat hearts.

6 In conclusion, the antiarrhythmic actions of anipamil appeared to depend upon calcium antagonism which may have reduced arrhythmias by a combination of anti-ischaemic and direct anti-arrhythmic actions. Presumed anti-ischaemic actions changed the relationship between the duration of preceding ischaemia and resulting reperfusion arrhythmias.

Introduction

In a series of experiments (Curtis *et al.*, 1984; 1985; Curtis & Walker, 1986; 1988) we demonstrated that calcium antagonists have antiarrhythmic actions against arrhythmias induced in conscious rats by occlusion of a coronary artery. Both the racemate and enantiomers of verapamil were active with a potency order of (−) > (±) > (+) (Curtis & Walker, 1986; Au *et al.*, 1987). Two analogues of verapamil were also tested in the same model. Only anipamil, a long acting calcium antagonist (Dillon & Nayler, 1988) with cardiovascular actions, had anti-arrhythmic activity when given orally (Curtis & Walker, 1986). In addition to antiarrhythmic actions, calcium antagonists also slowed the rate at which ischaemia-induced changes appeared on the ECG. In view of the above, further experiments were performed to investigate the antiarrhythmic efficacy of anipamil administered i.v., and to determine whether the putative anti-ischaemic actions (Gries & Raschack, 1984; Raschack, 1984; Curtis & Walker, 1986) of anipamil influenced either ischaemia-induced or reperfusion arrhythmias.

In rat hearts, abrupt resumption of coronary flow after regional ischaemia lasting 2–20 min is associated with a burst of arrhythmias (Manning & Hearse, 1984; Curtis *et al.*, 1987; Curtis & Hearse, 1989). Such arrhythmias have been investigated in many species (Balke *et al.*, 1981; Dennis *et al.*, 1983; Benfey *et al.*, 1984) and in each species a characteristic period of ischaemia results in the maximum number of reperfusion arrhythmias. The period of ischaemia necessary for maximum reperfusion arrhythmias is longer in larger species (Bergey *et al.*, 1982). The actions of drugs against reperfusion arrhythmias have been investigated despite difficulties in differentiating between drugs which are antiarrhythmic by virtue of anti-ischaemic actions, and those which act directly.

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In this study anipamil, a drug which appears to have anti-ischaemic as well as direct antiarrhythmic actions, was used to try to differentiate between primary and secondary antiarrhythmic actions. A secondary antiarrhythmic effect due to anti-ischaemic actions would be expected to reduce ischaemia-induced arrhythmias in the early period of irreversible ischaemia, but not in later periods. Thus the total number of arrhythmias would not be reduced by anti-ischaemic actions while the rate at which they appeared following the onset of ischaemia would be delayed. On the other hand, a direct antiarrhythmic action would be expected to reduce arrhythmias in all time periods. When tested against reperfusion arrhythmias, an anti-ischaemic drug would be expected to extend the period of occlusion required for maximization of reperfusion arrhythmias, i.e. it would shift the time-effect curve for reperfusion arrhythmias in the direction of longer times.

In vivo electrophysiological analyses were also performed in this study to determine whether anipamil had Class I or III antiarrhythmic effects on intracellular potentials which would explain its antiarrhythmic actions.

Methods

Chronically prepared conscious rats subject to irreversible ischaemia

Anipamil (1 or 5 mg kg^{-1} i.v.) was administered 15 min or 4 h before occlusion of the left anterior descending (LAD) coronary artery in conscious chronically prepared rats. The model has been previously described in detail (Johnston *et al.*, 1981; 1983) and uses male rats (250–300 g) surgically-prepared 7 days before occlusion. On the day of occlusion blood pressure and ECG were continuously recorded for 30 min before, and for 4 h after occlusion of the LAD coronary artery which was accomplished by traction on an implanted occluder. After 24 h of occlusion, animals were monitored for a further 30 min before they were killed and hearts perfused for estimation of occluded zone size by dye exclusion (Johnston *et al.*, 1983).

Acutely prepared anaesthetized rats subject to irreversible ischaemia

Anipamil (1 and 5 mg kg^{-1} i.v.) was also given 15 min prior to occlusion in a series of rats acutely prepared and occluded in the manner we have previously described (Au *et al.*, 1979; 1983). These rats were anaesthetized with pentobarbitone (50 mg kg^{-1}) i.p.

Chronically-prepared conscious rats subject to reperfusion

In a separate series of experiments in which rats were given 2.5 mg kg^{-1} i.v. anipamil, the coronary artery occluder was rapidly released following a carefully timed period of ischaemia. The dose of 2.5 mg kg^{-1} was chosen as the probable ED_{50} for the antiarrhythmic actions of anipamil against ischaemia-induced arrhythmias, and because its use was not associated with excessive cardiovascular depression which might be expected to impair an animal's ability to withstand reperfusion. Ischaemia was maintained for 3, 5, 7, 10 or 20 min prior to the abrupt release of the occluder. The method used was somewhat similar to that described by Kinoshita *et al.* (1988), but with technical modifications (MacGeough, MacLeod, Moult & Walker, unpublished observations). To ensure parity of reperfusion, a set of exclusion criteria was developed on the basis of initial experiments. These were: (1) Following initiation of ischaemia the characteristic ECG pattern of occlusion had to occur within 1 min. (2) Physical release of the occluder had to be rapid and complete such that initiation of reperfusion could be presumed to have occurred within 10 s. (3) Reperfusion had to induce a rapid return of the ECG toward normal, i.e., both elevated S-T segment and R-wave had to return rapidly towards normal. (4) After reperfusion, each animal had to have an occluded zone, defined by dye exclusion *in vitro*, which was 25–30% of total ventricular mass.

Exclusion criteria were applied blindly.

As in the experiments in which occlusion was maintained and not released, all animals undergoing non-spontaneously reversing ventricular fibrillation were subjected to manual defibrillation. This has been shown to be effective in reducing mortality in control animals from 80% to 30% or less (Johnston *et al.*, 1983; Curtis & Walker, 1986).

Anipamil (Knoll, Ludwigshafen, W. Germany) was solubilized in HCl acidified (pH 3.0) water at a temperature of 50–60°C. The volume of injection never exceeded 10 ml kg^{-1} and all injections were made over a 3 min period. Control animals received an appropriate volume of acidified water. Altogether five different dose regimens of anipamil were used. These were: 1 mg kg^{-1} either 15 min or 4 h before occlusion, 5 mg kg^{-1} given in the same manner or 2.5 mg kg^{-1} given 15 min before occlusion.

Electrophysiological experiments

The effects of anipamil on intracellular potentials recorded from the epicardial surface of intact rat

hearts were determined by a recently developed procedure (MacLeod & Walker, unpublished observations). This involved exposing the heart by means of a mid-line incision in pentobarbitone (50 mg kg^{-1}, i.p.)-anaesthetized rats. Under positive pressure ventilation the heart was suspended in a pericardial cradle and a section of the exposed epicardial surface of the left ventricle firmly sutured to a specially shaped Ag/$AgCl_2$ electrode holder. Conventional 'floating' fibre-filled 3 *M* glass electrode tips were used to record epicardial potentials before and at various times after administration of 2.5 mg kg^{-1} i.v. anipamil.

Experimental design and statistics

The experimental design was blind and random with the rigid application of exclusion criteria. In each animal, blood pressure, and ECG were recorded together with occluded zone. Arrhythmias in the 0–30 min and 30–240 min post-occlusion periods were recorded in animals subjected to permanent occlusion (Johnston *et al.*, 1981; 1983). In animals subjected to temporary occlusion and subsequent reperfusion, ischaemia-induced arrhythmias were recorded in the usual manner, whereas only the occurrence and not number or duration of each type of reperfusion arrhythmia was recorded.

Data analyses were by previously described statistical procedures (Johnston *et al.*, 1983). Altogether, 6 groups ($n = 9$ rats) of chronically-prepared conscious rats were subjected to irreversible occlusion; 2 control groups, 2 groups treated (1 or 5 mg kg^{-1} i.v. anipamil) 15 min before occlusion and a similar 2 groups treated 4 h before occlusion. In acutely-prepared anaesthetized animals, 3 groups ($n = 9$) were studied; a control group and 2 groups treated with 1 or 5 mg kg^{-1} i.v. anipamil 15 min before occlusion.

Ten groups ($n = 9$) were subjected to reperfusion. They consisted of 5 control groups and 5 groups treated with 2.5 mg kg^{-1} i.v. anipamil 15 min before occlusion. One control and one treated group was subjected to either 3, 5, 7, 10 or 20 min of ischaemia prior to reperfusion.

The data for each separate group of conscious rats subjected to irreversible ischaemia were initially analysed separately, but, where no statistically significant ($P < 0.05$) differences were found, treatment groups receiving the same dose, but at different times prior to occlusion, were combined to give a single group of 18 rats. Statistically significant differences were never found between the two control groups and so these were combined ($n = 18$). With reperfusion, groups could not be combined because of the dependency of reperfusion arrhythmias on the duration of the preceding ischaemic period.

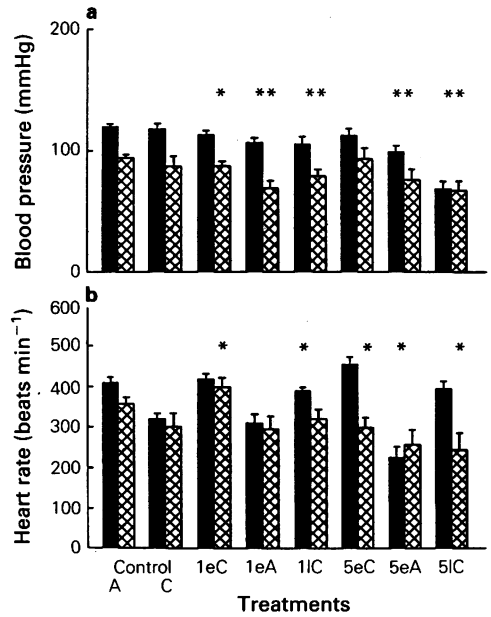

Figure 1 The effects of anipamil on blood pressure (a) and heart rate (b) before and after permanent occlusion of the LAD coronary artery in conscious and anaesthetized rats. Each column is mean (s.e.mean shown by vertical bars) ($n = 6$–9) of blood pressure (mmHg) in (a) and heart rate (beats min^{-1}) in (b). The time periods for measurements were 1 min before occlusion (filled columns) and 4 h after occlusion (cross-hatched columns). Data were divided on the basis of when anipamil was given, i.e. either 15 min (e) or 4 h (l) before occlusion, and whether rats were chronically-prepared and conscious (C) or acutely-prepared and anaesthetized (A). Doses of (1) or (5) mg kg^{-1} i.v. were given 15 min or 4 h before occlusion in conscious animals or 15 min before occlusion in anaesthetized rats. * $P < 0.05$ from appropriate controls.

Results

Cardiovascular effects of anipamil and occlusion

As shown in Figure 1 (a and b) anipamil tended to cause a fall in blood pressure and heart rate, especially at the higher dose and in the presence of anaesthesia. The hypotensive effects of anipamil were prolonged for up to 8 h. Occlusion of the LAD produced further falls in blood pressure in both the control and treated groups. The fall in blood pressure after occlusion was due to the functional loss of myocardium due to the formation of ischaemic zones (occluded zones). The size of the occluded zones (as percentage by weight of total ventricular weight) was not altered by any of the treatments or by conditions (i.e., maintained ischaemia or reperfusion). Values for the various control groups varied between 31.7 ± 2.2

Table 1 Summary of the antiarrhythmic effects of antipamil in rats subjected to permanent occlusion of the LAD coronary artery

n	*AS*	*VT*	*VF*	*Mortality*	*VPB*	*Treatment*
		A 0–30 min post-occlusion arrhythmias				
Conscious animals						
18	3.6 ± 0.5	17	15	0	1.9 ± 0.1	Control
18	3.4 ± 0.5	15	9*	1	1.9 ± 0.2	1 mg kg^{-1}
18	2.0 ± 0.5*	12	4*	0	2.0 ± 0.2	5 mg kg^{-1}
Anaesthetized animals						
9	4.1 ± 0.6	9	4	0	2.0 ± 0.2	Control
9	3.1 ± 0.3*	9	2	1	1.8 ± 0.2	1 mg kg^{-1}
9	2.1 ± 0.3*	6	0*	3†	1.7 ± 0.2	5 mg kg^{-1}
		B 30–240 min post-occlusion arrhythmias				
Conscious animals						
18	3.6 ± 0.6	14	14	1	2.6 ± 0.6	Control
17	2.4 ± 0.6	9	6*	0	2.6 ± 0.6	1 mg kg^{-1}
18	2.0 ± 0.6*	12	4*	0	2.5 ± 0.3	5 mg kg^{-1}
Anaesthetized animals						
9	4.1 ± 0.6	9	4	2	3.0 ± 0.3	Control
8	0.4 ± 0.4*	1*	0*	0	0.8 ± 0.3*	1 mg kg^{-1}
6	1.0 ± 0.6*	0*	0*	0	0.7 ± 0.2*	5 mg kg^{-1}

The arrhythmias induced by ischaemia in the 0–30 min and 30–240 min periods of occlusion were recorded as the incidence in the group of animals having one or more episodes of ventricular tachycardia (VT), or ventricular fibrillation (VF) and as the number of ventricular premature beats (VPB) (expressed as $\log_{10}$ of the number of VPBs in the observation period considered). The total arrhythmic history of each rat was summarized as an arrhythmia score (AS). Values are given either as number in group or $\bar{x} \pm$ s.e.mean, of size = n. $P < 0.05$ when compared with the appropriate control. † All deaths in this group were due to cardiac output failure.

and 41.2 ± 1.5 ($\bar{x} \pm$ s.e.mean). The values in the treated groups varied from 35.0 ± 2.2 to 42.6 ± 0.6. Analysis of variance failed to show treatment to be a source of variance.

Antiarrhythmic effects

The overall antiarrhythmic effects of anipamil treatment on arrhythmias induced by permanent occlusion are summarized in Table 1. Anipamil dose-dependently reduced the incidence of severe arrhythmias such as ventricular tachycardia and particularly ventricular fibrillation, as well as arrhythmia scores which are overall summaries of an animal's arrhythmic history. There were no statistically significant reductions in the number of ventricular premature beats (VPB), except in anaesthetized animals. Anti-arrhythmic effects of anipamil may have been more pronounced in such animals.

Since the ischaemic time-dependency of major tachy-arrhythmias gives insight as to possible mechanisms involved in antiarrhythmic drug action, the time-dependency curves for ischaemia-induced arrhythmias are shown in Figure 2. The data are plotted to show group incidences of major arrhythmias in each of the important time periods after occlusion (a) as well as their cumulative incidence (b). A selective reduction in incidence during the early period of occlusion, but no change in the overall cumulative incidence, would be assumed to reflect underlying anti-ischaemic actions. A non-selective decrease in all time periods would reflect direct anti-arrhythmic actions. Anipamil had two effects. It reduced the cumulative incidence of major arrhythmias in all time periods but in addition was most effective against the arrhythmias occurring early after occlusion. With ventricular tachycardia only, there was a tendency for its incidence to increase in later time periods but this was not statistically significant.

In Table 2 the occurrences of ventricular fibrillation and ventricular tachycardia with reperfusion are shown. The table illustrates the time-dependency of reperfusion arrhythmias which peaked after 5–7 min of ischaemia in controls, and were both less severe (VT, rather than VF) and less frequent after 20 min of ischaemia. Anipamil 2.5 mg kg^{-1} tended to reduce reperfusion arrhythmias in all time periods, but this tendency was most marked for the 3 min period.

Effects of anipamil on ECG changes induced by ischaemia

Although anipamil reduced arrhythmias, there were no corresponding effects on mortality since a

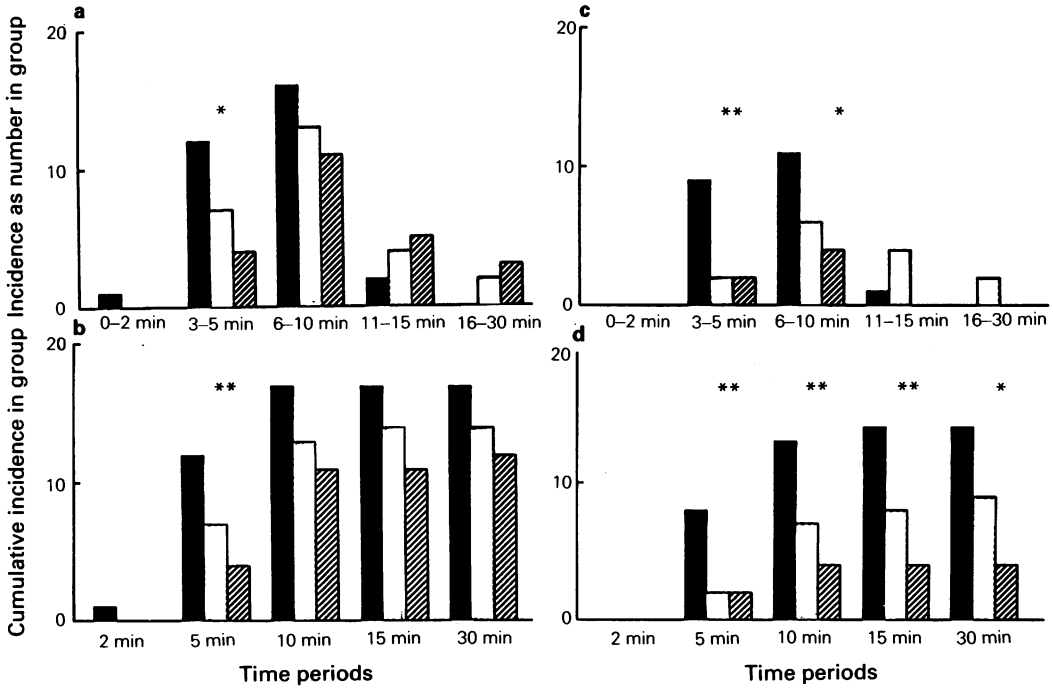

Figure 2 Incidence of major arrhythmias after various periods of ischaemia in conscious rats. The incidence of arrhythmias is given as number of animals having the major arrhythmias of ventricular tachycardia (a and b) or ventricular fibrillation (c and d) in groups of $n = 18$. In (a) and (c) incidences in the various separate time periods after occlusion are plotted while in (b) and (d) cumulative incidences at noted times after occlusion are given. In all panels, incidences are shown for controls (filled columns). 1 mg kg^{-1} i.v. anipamil (open columns) and 5 mg kg^{-1} i.v. anipamil (hatched). * $P < 0.05$ from control.

reduction in VF-induced mortality was associated with an increase in non-arrhythmic mortality (Table 3). The lack of effect of anipamil upon occluded zone size did not explain variations in mortality, although it was consistent with its failure to reduce the maximum value attained either by the R-wave or S-T segment elevation following permanent occlusion (Table 3). Anipamil treatment had no effect on the time to appearance of Q-waves (index of infarction), but it did, at 5 mg kg^{-1} i.v., reduce the number of animals that showed such a wave. Anipamil treatment also influenced the rate at which ECG changes appeared following the onset of ischaemia. This delaying effect is clearly illustrated in Figure 3 where anipamil, in a dose-related manner, reduced the rate at which S-T segment elevations developed. A similar delayed increase in R-wave was also seen (data not shown).

Table 2 Group incidence of major arrhythmias with reperfusion after various preceding periods of ischaemia in control (C) and anipamil (2.5 mg kg^{-1}) (A) treated rats

		Ischaemic period					
Arrhythmia	*Group*	*3 min*	*5 min*	*7 min*	*10 min*	*20 min*	*Total No.*
Ventricular fibrillation	C	6	9	8	6	0	29
	A	0*	5	7	2*	0	14*
Ventricular tachycardia &/or fibrillation	C	6	9	9	9	5	38
	A	1*	8	8	6	3	26*

The incidence in the group ($n = 9$) of major arrhythmias which occurred within 1 min of reperfusion after the specified period of preceding ischaemia are given together with the cumulative total for all time groups ($n = 45$).
* $P < 0.05$ compared with appropriate control.

Table 3 Mortality and ECG changes (maximum R-wave size and S-T segment elevation, Q-wave) in control (C) and anipamil (A) treatment groups

	Mortality		ECG changes – maximum values obtained			
			R-wave	S-T	Q-wave	
	Arr.	Non-Arr.	mV	% of R	Incidence	Time (h)
Control	1	0	1.2 ± 0.1	40 ± 3	14	1.9 ± 0.1
A 1 mg kg^{-1}	0	1	1.4 ± 0.2	40 ± 3	14	2.1 ± 0.1
A 5 mg kg^{-1}	0	3	1.3 ± 0.1	37 ± 3	8*	2.3 ± 0.2

Mortality in the 0–4 h post-occlusion period is expressed as the incidence in groups of 18, or arrhythmia-(Arr.) or non-arrhythmia-(Non-Arr.) related death. The maximum values for R-wave size and S-T segment elevation were found by inspection of each individual rat's ECG trace, and the mean values (± s.e.mean) calculated for group size of $n = 18$. Q-wave occurrence was recorded as both the incidence in the group, and as the time at which it was first detected in those animals which developed Q-waves.

Effects on epicardial intracellular potentials

Anipamil treatment did not reduce the major characteristics of resting or action potentials recorded *in vivo* (Table 4). Neither the depolarization and/or late repolarization phases were affected. However, anipamil produced a change in the configuration of the phase 2 portion of the action potential in the earliest phases of repolarization (Figure 4). The change proved very difficult to quantitate in a consistent manner and only a qualitative description can be given. Anipamil modified the shoulder of the action potential such that phase 1 appeared to become more pronounced while the 'plateau' portion appeared to be depressed.

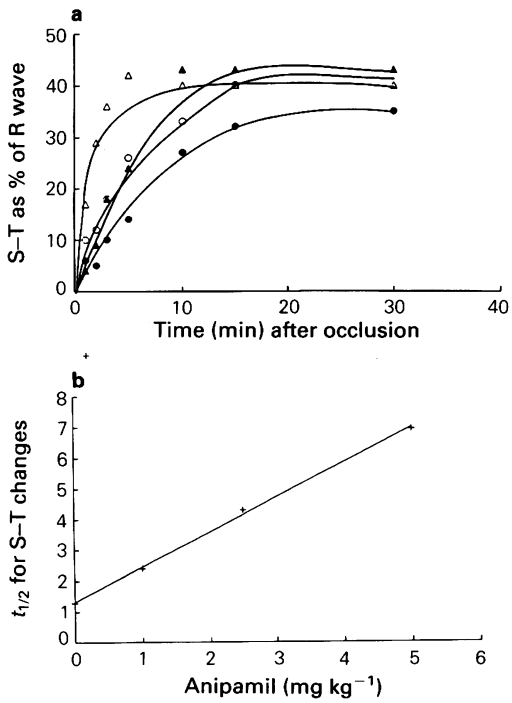

Figure 3 The rate of development of S-T segment changes following occlusion in control and anipamil-treated animals. In (a) the group mean size of the S-T segment elevation (as % of R-wave size) at the various time periods after occlusion are shown ($n = 18$). (△) control; (○) 1 mg kg^{-1}; (▲) 2.5 mg kg^{-1} and (●) 5 mg kg^{-1}. $t_{1/2}$ values were calculated from the data in (a) on the assumption that there was a saturation function adequately described by $t_{1/2}$ values. Such calculated values were replotted in (b).

Discussion

This study established that anipamil i.v. dose-dependently reduced arrhythmias due to LAD occlusion in conscious and anaesthetized rats. This agrees with a previous study in which anipamil was antiarrhythmic if given orally 4 h before occlusion (Curtis & Walker, 1986). The present study further defined this antiarrhythmic effect and showed it to be dose-dependent and occurring in more than one rat model. It also illustrated the long duration of action of anipamil since anipamil given either 4 h or

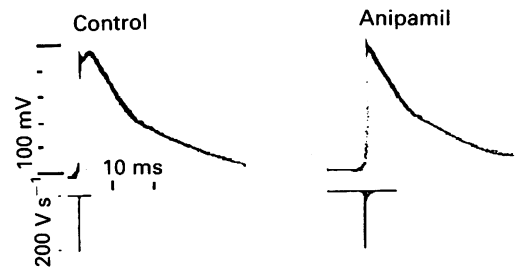

Figure 4 Epicardial action potential recorded *in vivo* before and after administration of anipamil. An example of intracellular potentials recorded from the epicardial surface of a rat heart before and after administration of anipamil (2.5 mg kg^{-1}) is shown.

Table 4 Action potential characteristics recorded *in vivo* before and after 2.5 mg kg^{-1} anipamil i.v. (A)

Time period	*RMP (mV)*	*AP (mV)*	*MRR (V s^{-1})*	*AP duration (ms) at 50% repolarization*
Control	75 ± 5	85 ± 5	164 ± 10	12 ± 3
5 min after A	73 ± 5	90 ± 5	166 ± 12	13 ± 3
30 min after A	72 ± 4	90 ± 4	171 ± 10	12 ± 4

Epicardial potentials were recorded by a floating microelectrode technique in 5 open-chest pentobarbitone anaesthetized rats. Where possible, a single cell potential was held before and after administration of anipamil. When multiple impalements were made only average values for individual rats were used in the calculation of group $\bar{x}$ ± s.e.mean.

15 min before occlusion was equally efficacious. Antiarrhythmic effects were not associated with a reduction in occluded zone size, although they were associated with possible anti-ischaemic effects as inferred from ECG evidence. Such evidence has previously been reported for anipamil following its oral administration (Curtis *et al.*, 1986).

The antiarrhythmic effectiveness of anipamil can usefully be compared with those of verapamil enantiomers. In conscious rat (−)-, (±)- and (+)-enantiomers had ED_{50} values of 2, 6 and 20 mg kg^{-1}, respectively (Curtis *et al.*, 1984; Curtis & Walker, 1986), which are close to that for anipamil (between 1 and 5 mg kg^{-1}). Antiarrhythmic efficacy of both verapamil and anipamil was associated with hypotension and bradycardia and this depression of the cardiovascular system increased the possibility of death due to cardiac output failure following coronary occlusion.

The mechanisms underlying anipamil's antiarrhythmic activity may involve more than one action. We previously suggested that the antiarrhythmic efficacy of calcium antagonists in conscious rats relates to inhibition of calcium currents in ventricular tissue (Curtis *et al.*, 1984; Curtis & Walker, 1986). Such a mechanism may also be involved in the antiarrhythmic action of anipamil, but unfortunately the full pharmacological profile of this drug is unknown, although it is a calcium antagonist (Dillon & Nayler, 1988). Effects on intracellular potentials were suggestive of Class IV rather than Class I or III actions since a reduced calcium current during Phase 2 of the action potential would accentuate repolarization processes only.

The present experiments suggested that the putative anti-ischaemic actions of anipamil participated in antiarrhythmic actions. If delays in the development of ischaemia-induced changes in the ECG (Figure 3) indicated an anti-ischaemic action, one would expect anipamil to be more efficacious against arrhythmias appearing very early after the onset of ischaemia. Figure 2 suggests that this was partly the case since arrhythmias occurring in the period when ECG changes were noticeably delayed appeared more sensitive to the antiarrhythmic actions of anipamil. However, particularly with the 5 mg kg^{-1} dose and incidence of VF, anipamil reduced arrhythmias in all time periods thus concordance between ECG effects and the time dependency of arrhythmias was not complete. If all of the antiarrhythmic actions of anipamil were due to the same process as that which delayed the development of R-wave or S-T segment elevation, then one would expect Figure 3a and Figure 2b and d to show overall concordance. This was not the case since, in both cases, the slopes of the time-dependency were depressed by anipamil whereas the maxima they achieved were only depressed in Figure 2b and d. Thus, both an anti-ischaemic and direct antiarrhythmic action appeared to be involved.

Anti-ischaemic actions may have accounted for the reduction in reperfusion arrhythmias seen in rats subjected to 3 or 5 min ischaemic periods, but not for the reductions after more prolonged periods of ischaemia (Table 2). A complete analysis would require a more extensive time series study to differentiate clearly between secondary antiarrhythmic actions due to anti-ischaemic effects, and those due to direct actions. Antiarrhythmic actions against reperfusion arrhythmias are complicated by the fact that such arrhythmias depend on a number of factors (see review by Manning & Hearse, 1984). These include not only the extent, intensity and duration of the preceding period of ischaemia, but also the speed and efficacy of reperfusion. A drug influencing any of the foregoing could be anti-arrhythmic by virtue of reducing the initial arrhythmic stimulus and not by direct anti-arrhythmic actions.

In conclusion, anipamil given intravenously, has antiarrhythmic actions against both occlusion and reperfusion arrhythmias in the conscious rat. These antiarrhythmic actions may involve both primary antiarrhythmic, and secondary antiarrhythmic actions. The latter is presumed to be due to 'anti-ischaemic' effects. The duration of the antiarrhythmic actions of anipamil was very prolonged.

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Modulation of sodium current kinetics by chlorpromazine in freshly-isolated striatal neurones of the adult guinea-pig

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1 The neurones of the striatum were freshly dissociated from the adult guinea-pig brain by enzymatic and mechanical treatments. Sodium channel current kinetics in these neurones were measured using a whole cell variation of the patch-clamp technique.

2 Chlorpromazine, a neuroleptic, in micromolar concentrations reversibly reduced the amplitude of the sodium currents. Activation and inactivation time constants were not affected. The inhibition followed one-to-one binding stoichiometry.

3 The concentration-response curve shifted to the left when the holding potential was less negative. The EC_{50} shifted from 4.8 μM to 0.9 μM when the holding potential was changed from -120 mV to -70 mV.

4 The steady-state activation curve of the sodium current was not affected by chlorpromazine, whereas the steady-state inactivation curve was shifted in the negative direction. Consequently, the window current which is normally present at a potential range around -50 mV was decreased in the presence of chlorpromazine.

5 Successive sodium currents evoked by a train of depolarizing pulses (30 ms duration) to -10 mV showed a cumulative decrease in size during the application of chlorpromazine. However, such 'use-dependent' block was not observed when the pulse duration was reduced to 1 ms.

6 The recovery from inactivation in the presence of chlorpromazine, was expressed as a second order process. The faster component was similar to the recovery time course of the normal sodium channels. The slower component accounted for the use-dependent effect of chlorpromazine.

7 The results indicate that chlorpromazine binds to the resting sodium channels producing steady-state block at a very negative holding potential. When the membrane is depolarized, chlorpromazine binds to the inactivated form of the sodium channels with much higher affinity and stabilizes them in the inactivated state, slowing their kinetics.

Introduction

The opening of the Na^+ channels is often blocked by various categories of chemical agents, and these agents have proved to be useful in understanding the physiology of channel gating (Narahashi, 1974). We have recently shown that micromolar concentrations of chlorpromazine (Cpz), a neuroleptic, blocks Na^+ channels in mouse cultured neuroblastoma cells (Ogata *et al.*, 1989b). A potent voltage-dependent blocking action on the cardiac ventricular Na^+ channels explains the well-known 'quinidine-like' side effect of neuroleptics (Ogata & Narahashi, 1989; Ogata *et al.*, 1989a). Furthermore, Cpz also exerted a potential blocking action on the different types of neuronal Ca^{2+} channels in a manner distinct from the action on the Na^+ channels (Narahashi *et al.*, 1989).

Recently, several investigators have succeeded in acutely dissociating the brain neurones from newborn or adult rodents and in recording voltage-gated as well as receptor-operated ionic currents from these neurones by use of the patch-clamp technique (Huguenard & Alger, 1986; Kay & Wong, 1986; Kaneda *et al.*, 1988). We have recently developed a technique to dissociate single striatal neurones from adult guinea-pig brain. This preparation allowed precise voltage- and space-clamp controls which are indispensable for accurate measurements of voltage-gated Na^+ currents. The aim of this study

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was, therefore, to examine the effect of Cpz on the gating kinetics of the Na^+ channels in acutely dispersed striatal neurones of the guinea-pig.

Methods

Adult guinea-pigs of either sex (200 g) were decapitated, and the forebrain region was sliced in the sagittal direction (300 μm thick) according to the technique described elsewhere (Abe & Ogata, 1982). The technique for dispersion of the brain neurones was basically the same as the techniques described by others (Kay & Wong, 1986; Kaneda *et al.*, 1988). The caudate-putamen was dissected out under a binocular microscope (see Figure 1a) and incubated in a medium (36°C) of the following composition (in mM): NaCl 120, KCl 5, $CaCl_2$ 1.8, $MgCl_2$ 1, piperazine-N,N'-bis[2-ethanesulphonic acid]-Na (PIPES-Na) 5 mM and glucose 25 mM with 0.5% trypsin (Sigma) added. During the incubation, the slices were continuously bubbled with 100% O_2. After 1 h of incubation, the slices were gently agitated with a fire-polished Pasteur pipette (inner tip diameter, 100 μM).

Electrical recording

The methods for electrical recording used in the present study were similar to those described in a

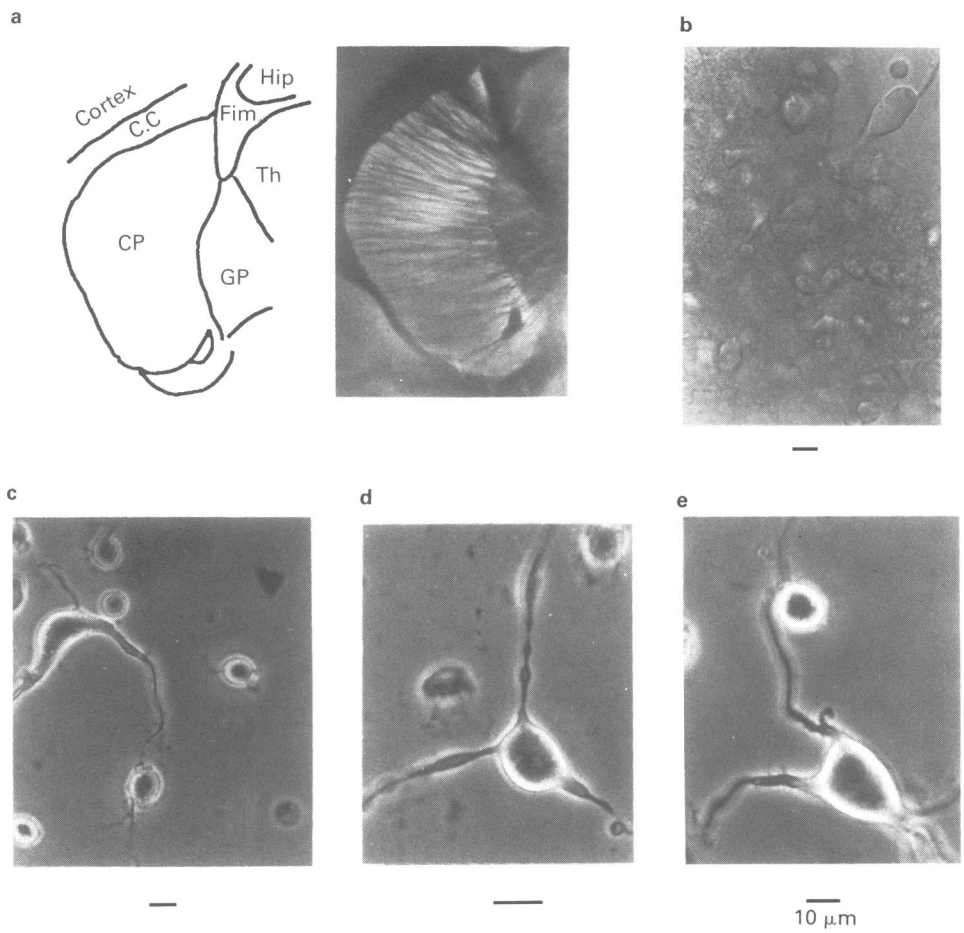


Figure 1 Striatal neurones of the adult guinea-pig. (a) Anatomical schema of the striatal region and a photograph of a striatal slice. C.C., corpus callosum; Fim, fimbria; Hip, hippocampus; CP, caudate putamen (striatum); GP, globus pallidum; Th, thalamus. (b) Nomarski interference microscopic observation of the tissue fragments produced by mild agitation. (c–e) Phase-contrast photomicrographs of typical examples of the freshly dissociated adult striatal neurones (see text, for explanation).

previous paper (Ogata & Narahashi, 1989). Briefly, a whole-cell variation of the patch-clamp technique (Hamill *et al.*, 1981) was used. Suction pipettes with a resistance of 1.2–2 MΩ were made of borosilicate glass capillary tubes using a 2-stage vertical puller and were fire polished. Inverted command pulses were applied to the bath through an Ag-AgCl pellet/3 M KCl agar bridge. The pipette potential was maintained at the ground level. Membrane currents passing through the pipette were recorded by a current-to-voltage convertor designed by M. Yoshii (Narahashi *et al.*, 1987) and stored digitally with a personal computer (PC-9801, NEC, Tokyo, Japan). Capacitative and leak currents were subtracted digitally by the P-P/4 procedure (Benzanilla & Armstrong, 1977). Fits to an exponential time course and a simple Boltzmann distribution were determined with the computer by a non-linear sum of the least squares fitting routine. The liquid junction potential between internal and external solutions recorded as zero current-potential was about −13 mV. The data shown here were compensated for this effect.

Solutions

The external and the internal (pipette-filling) solutions were designed to separate the Na^+ channel current (I_{Na}) from other voltage-dependent currents. The external solution contained (in mM): NaCl 100, tetraethylammonium (TEA)-Cl 25, $CaCl_2$ 1.8, CsCl 5, $MgCl_2$ 1, glucose 25, HEPES 5 and $CoCl_2$ 2. The solution was titrated to pH 7.4 with NaOH. The internal solution contained (in mM): Cs-glutamate 125, NaCl 10, $MgCl_2$ 2.5, HEPES 5, EGTA 5 and glucose 5. The pH of the internal solution was adjusted to 7.0 with CsOH. External TEA and replacement of external and internal K^+ with Cs^+ effectively suppressed the K^+ current, and external Co^{2+} totally suppressed the Ca^{2+} current. Cpz at a concentration of 10 mM was freshly prepared in each experiment and diluted to the desired concentration just before use. Unless otherwise specified, experiments were carried out at room temperature (22°C). Results are expressed as means ± s.e.mean. Significant differences from control were evaluated by Student's *t* test.

Results

The cells retained a good viability after enzymatic dissociation and stable Na^+ currents could be recorded. A nomarski interference microscopic observation of the tissue fragments produced by mild agitation provided a good estimate of the cellular composition within the tissue. As shown in Figure 1b, within the mildly agitated tissue fragment, individual cells were visible yet the cellular architecture of the tissue was preserved. Due to the thickness of the preparation, only a small percentage of the cells was in focus. However, in actual observations, a sharp outline of most of the cells could be observed by bringing them into focus.

The striatum of the adult guinea-pig contained two major morphological cell types depending on the shape and size of the soma as shown in Figure 1c. The overwhelming majority (greater than 95%) of the striatal neurones were of medium size (12–16 μm in diameter when measured across their greatest dimension) and had a round cell body with fine processes having poor arborization. A typical example of a medium-sized cell is shown in Figure 1d. Another 3–5% had polymorphic and large cell bodies (22–26 μm shortest dimension and 30–45 μm largest dimension). A typical example of a large cell is shown in Figure 1e together with a medium-sized round cell. These observations are consistent with histological data based on microscopic studies on the striatum (e.g., Lu & Brown, 1977; Chang *et al.*, 1982). Processes appeared to be thicker than their actual size, since most of the processes were out of focus due to their three-dimensional orientation. The medium-sized neurones were particularly advantageous for space-clamp control in the voltage-clamp experiment, and therefore, in the experiments described in this paper, we used only the medium-sized neurones.

Steady-state block of Na^+ current

Striatal neurones developed fast and transient inward currents. Since these currents were blocked by a low concentration of tetrodotoxin (0.1 μM), disappeared in Na^+-free solution and persisted during perfusion of inorganic Ca^{2+} blockers such as 2 mM Co^{2+}, it was concluded that these currents were carried by Na^+. Figure 2 shows I_{Na} recorded from a medium-sized striatal neurone and the effect of Cpz on this current. Figure 2a illustrates the experimental protocol used for subtraction of the capacitative and leak currents. The test pulse (V_p) was followed by 4 repetitive pulses whose amplitude was reduced to 1/4 of V_p and the polarity was reversed ($V_{-P/4}$). Each interval was 0.5 s. Since the leak conductance measured at a potential range of −150 to +50 mV was linear in our experimental situation in which internal and external K^+ were substituted by equimolar amounts of Cs^+, the summation of current traces generated by the above 5 consecutive pulses effectively subtracted both the capacitative and leak components from the test pulse current.

I_{Na} produced by a step depolarization to −10 mV was inhibited by Cpz 4 μM by about 50% (Figure 2b). The current trace obtained in the presence of Cpz

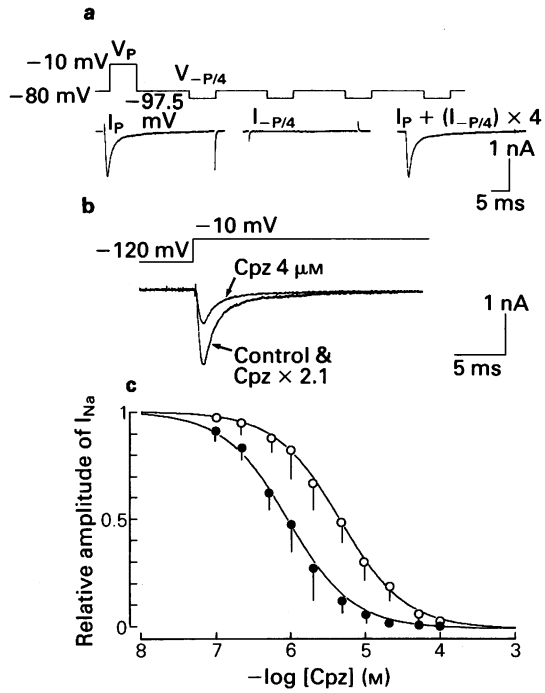


Figure 2 Effects of chlorpromazine (Cpz) on I_{Na} in the freshly isolated guinea-pig striatal neurone. (a) Illustrates a P-P/4 protocol used to eliminate capacitative and leak components from the current obtained in response to test pulses. (b) Illustrates the effect of Cpz on I_{Na} induced by a step depolarization to −10 mV. The current trace obtained in the presence of Cpz is scaled to the control current (Cpz × 2.1). (c) Concentration-response relationships after Cpz block of I_{Na}. The amplitudes of peak I_{Na} in the presence of Cpz elicited by 30 ms test pulses to −10 mV from a holding potential (V_h) of −120 mV (○) or −70 mV (●) were normalized to the control amplitude. Each point represents the mean from 5–7 experiments and vertical lines show s.e.mean. Curves were drawn according to the following equation:

$$I_{Na} = 1/(1 + [Cpz]/K_d)$$

where K_d is the dissociation constant of the binding reaction Cpz + X = CpzX. Here, X represents the channel in the unblocked configuration. In this and subsequent figures (except Figure 6), a P-P/4 protocol has been employed. Upward and downward deflections represent outward and inward currents, respectively.

was scaled to the control by multiplying the amplitude by 2.1. Both the activation and decaying phases completely overlapped with those of the control current in all 10 cells examined. The action of Cpz was usually apparent within 1 min after the drug was added, stabilized within 5 min or less, and full recovery occurred 5–10 min after washing. The blocking action of Cpz was not affected by lowering the medium temperature to 11°C.

Figure 2c shows the concentration-dependence of the blocking action of Cpz on I_{Na}. The peak amplitudes of I_{Na} in response to depolarizing test pulses to −10 mV were plotted as a function of Cpz concentration. When a holding potential (V_h) was −120 mV, the half-maximal inhibition of I_{Na} by Cpz occurred at 4.9 μM (open circles in Figure 2c). The lines in Figure 2c are theoretical curves corresponding to binding reactions where one Cpz molecule is necessary to block a single Na^+ channel. The concentration-dependence of the current amplitude was best fitted with 1 : 1 stoichiometry (theoretical curves for 2 : 1 and 3 : 1 which deviated from the measured curve were not illustrated for clarity). Thus, the cooperation of one Cpz molecule appears to be required to block a single Na^+ channel.

The concentration-response curve shifted to the left when the membrane was held at a more depolarized level (filled circles in Figure 2c). At a V_h of −70 mV, the half-blocking concentration was reduced from 4.9 μM to 0.9 μM. A one-to-one binding curve provided a good fit to the data as in the case of −120 mV V_h.

Figure 3 shows the effect of Cpz on the relationship between the depolarizing command potential and the peak amplitudes of I_{Na}. I_{Na} was activated at −50 mV, peaked at −20 mV and reversed its polarity at +60 mV. Cpz (5 μM) reduced the peak current amplitudes to 50.8 ± 4.6% ($n = 5$) of the control. There was no detectable voltage-dependence for the block, as shown in Figure 2c. The curve obtained by multiplying the values of the current-voltage curves in the presence of Cpz (open squares) by 1.85 times overlapped with the control current-voltage curve (open circles).

Effects of Cpz on activation kinetics

The instantaneous current upon repolarization (tail current) was measured to study the steady-state activation curve of I_{Na}. Figure 4a shows the tail currents upon clamp-back to a fixed potential (−80 mV) from −20 mV measured at various times following the appearance of I_{Na}. The steady-state activation (m_∞) curve is usually compiled from the tail current measured at a fixed time after the onset of step depolarization. However, as shown in Figure 3b, the time to the peak of I_{Na} changed depending on the magnitude of the step depolarization. Therefore, we measured the time-dependence of the tail current amplitude by changing the duration of step depolarization (see Figure 4a), and determined the maximum amplitude of the tail current at various levels of step depolarization.

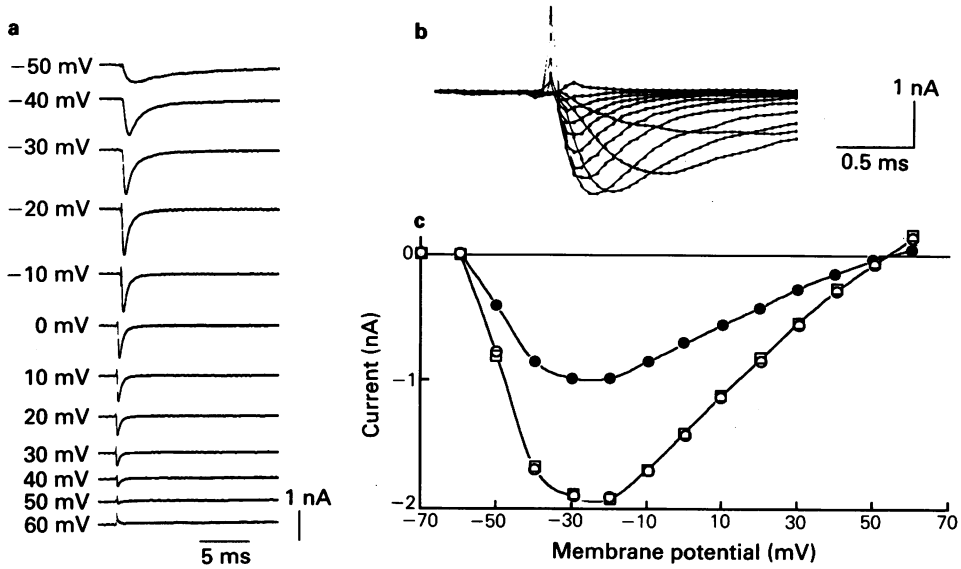


Figure 3 Effects of chlorpromazine (Cpz) on the current-voltage relationship for I_{Na}. (a) Shows a family of I_{Na} elicited by 20 ms step depolarizations to −50–+60 mV from V_h of −80 mV. Step depolarizations were applied at a constant rate of 0.03 Hz. The same recordings were shown superimposed in a faster time scale in (b). (c) The peak amplitudes of I_{Na} plotted as a function of test potentials, in controls and in the presence of Cpz; (○) control, (●) Cpz 5 μM and (□) Cpz × 1.85.

The time course I_{Na} can be described by:

$$\exp(-t/\tau_m) = 1 - [I(t)/I'(t)]^{1/n} \quad (1)$$

where τ_m is the time constant of activation kinetics, n is an integer, and I(t) and I'(t) represent the recorded current amplitude and the current amplitude estimated from the extrapolation of the decay phase of I_{Na} at time t (Ogata & Narahashi, 1989). The plot of values, $1 - [I(t)/I'(t)]^{1/n}$, on a semi-logarithmic scale against t should fall on a straight line if an appropriate value of n is given. As shown in Figure 4b, the plot fell on a straight line when n was 3, indicating that the activation process is best described by m^3 kinetics. τ_m measured at −30 mV was 0.16 ± 0.02 ms ($n = 4$). Cpz (2–5 μM) had no effect on the τ_m ($P > 0.05$, $n = 4$).

The m_∞ curves were made by calculating the cube root of the peak amplitude of the tail current (Figure 4c). The slope factor for e-fold change in the control m_∞ measured at the mid-point potential (−25.5 ± 2.0 mV, $n = 4$) was 11.7 ± 2.3 mV. Cpz (2.5 μM) had no effect on the m_∞ curves in any of 3 cells examined.

Steady-state inactivation

Since a membrane depolarization, which increases the proportion of the inactivated state of the channel, augmented the channel block by Cpz (Figure 2c), Cpz might preferentially bind to inactivated channels with higher affinity than to the resting channels and stabilize them in the inactivated state. We, therefore, tested this possibility by measuring the steady-state availability of Na^+ channels. Figure 5a shows an experimental protocol. Inactivation characteristics were studied by varying the conditioning potential ($V_{conditioning}$) and applying test pulses (V_{test}) to −10 mV. A plot of peak current amplitude against $V_{conditioning}$ gives a measure of the voltage-dependence of inactivation. To avoid distortion of the measurement by fluctuation of control I_{Na} during a course of recording, control I_{Na} ($V_{reference}$) was measured 10 s before each $V_{conditioning}$. The 10 s interval was sufficient for recovery from inactivation. Experiments in which the amplitude of control I_{Na} changed more than 10% were discarded. The steady-state inactivation (h_∞) was calculated by the ratio, $I_{test}/I_{reference}$.

The inactivation curve had a slope parameter (k) of 6.3 mV and a mid-point voltage ($V_{1/2}$) of −65 mV (Figure 5b). The h_∞ curve in the presence of Cpz (2.5 μM) revealed a parallel shift of about 12 mV (12.6 ± 3.8 mV, $n = 4$) toward a more negative potential, indicating that the blocking action of Cpz is voltage-dependent. The slope factor which reflects the voltage sensitivity of the inactivation gating was not affected by Cpz. The actual size of I_{Na} available

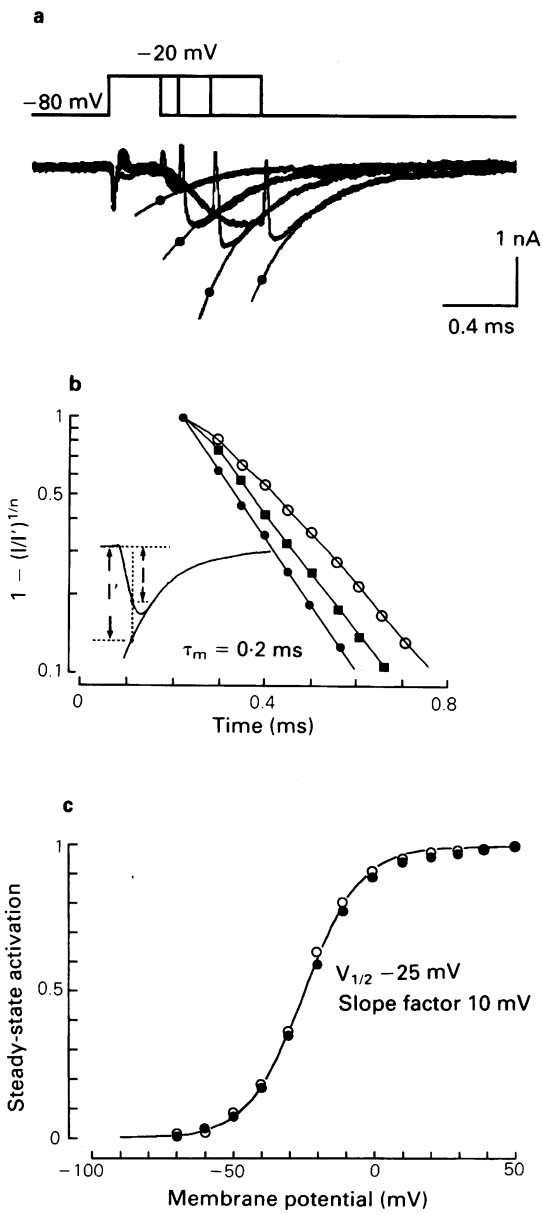


Figure 4 The steady-state activation (m∞) curves for I_{Na} in the presence and absence of chlorpromazine (Cpz). (a) Tail currents measured after the depolarizing command potentials of various duration. The membrane was depolarized to −20 mV from V_h of −80 mV for a period of ΔT. The initial portion of the tail current was obscured due to the P-P/4 procedure. However, this did not affect the exponential fitting of the decaying phase of the tail current. The deactivation time course of the tail current was expressed by a single exponential function (solid lines). The dots on the solid lines represent the initial amplitude of the tail current determined by extrapolating the falling phase to the time of repolarization. (b) The time course of I_{Na} activation was assessed by equation (1) described in the text. I and I′ represent the recorded current amplitude and the current amplitude estimated by the extrapolation of the decay phase, fitted by a single exponential function for test potential of −30 mV from a V_h of −80 mV (see inset). The value, $1 - [I(t)/I'(t)]^{1/n}$, was plotted against time on a semi-logarithmic scale. (○) $n = 1$, (■) $n = 2$, (●) $n = 3$. (c) The cube root of the maximal amplitude of the tail current was plotted as a function of the levels of step depolarization. The smooth curves were drawn according to the equation:

$$m\infty = 1/(1 + \exp[-(V_{test} - V_{1/2})/\kappa])$$

where V_{test} is the potential level of the step depolarization and $V_{1/2}$ is the V_{test} where I_{Na} is one-half maximal, and κ is the slope factor (10 mV). (○) Control, (●) in the presence of Cpz 2.5 μM.

at very negative conditioning potentials (−100 to −130 mV) decreased by about 32% (see the curve drawn by dotted line). This decrease was equivalent to that expected from the concentration-response curve (Figure 2c).

Dissociation constant for binding of Cpz to the inactivated Na^+ channels

The binding of Cpz to the inactivated Na^+ channel is electrically silent, since the channels are already non-conducting and not available for opening. It is, therefore, impossible to measure directly K_i, the dissociation constant for the binding of Cpz to the inactivated Na^+ channels. We estimated it using several equations. There is a relationship between K_i, K_{rest} and $K_{apparent}$ (the dissociation constant that is determined by a mix of the actual binding reactions to resting and inactivated channels) described by (Bean *et al.*, 1983; Bean, 1984):

$$K_{apparent} = 1/[h\infty/K_{rest} + (1 - h\infty)/K_i]$$

The h∞ at V_h of −70 mV was 0.77 (Figure 5) and K_{rest} and $K_{apparent}$ were 4.9 and 0.9 μM, respectively (Figure 2c). Therefore, calculated K_i was 0.24 μM.

The K_i can also be deduced from the voltage-dependence of I_{Na} availability (Bean *et al.*, 1983). The shift in midpoint of the h∞ curve in the presence of Cpz, ΔV (−15 mV, Figure 5), is expressed, assuming one-to-one binding (Figure 3c), as:

$$\Delta V = k \ln\{(1 + [Cpz]/K_{rest})/(1 + [Cpz]/k_i)\}$$

in which k is the slope factor of the h∞ curve (6.3 mV, Figure 5) and [Cpz] is the concentration of Cpz. The calculated value of K_i was 0.14 μM, approximately the value estimated from the concentration-response curve (0.24 μM).

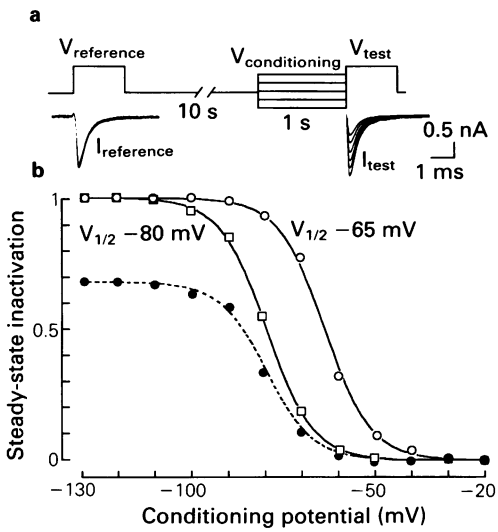


Figure 5 Steady-state inactivation (h∞) curves for I_{Na} in the presence and absence of chlorpromazine (Cpz). (a) Experimental protocol. Two identical step depolarizations to −10 mV for 30 ms were applied 10 s before ($V_{reference}$) and immediately subsequent (V_{test}) to the conditioning pulse ($V_{conditioning}$). The potential level of $V_{conditioning}$ was changed from −130 mV to −20 mV in 10 mV steps. (b) The peak current amplitude measured during V_{test} was normalized to the control amplitude measured during $V_{reference}$ and plotted as a function of $V_{conditioning}$. The smooth curves were drawn according to the equation:

$$h\infty = 1/1 + \exp[(V_{conditioning} - V_{1/2})/\kappa])$$

where $V_{1/2}$ is the conditioning potential when I_{Na} is one-half maximal, and κ is the slope factor (6.3 mV). The curve indicated by (●) is drawn by multiplying the h∞ in the presence of Cpz by 0.68, the reduction of I_{Na} at −120 mV by Cpz. (○) Control, (□) Cpz 2.5 μM.

Use-dependent block

In addition to the steady-state channel block, Cpz produced block of Na^+ channels during repetitive stimulation. Figure 6a shows the time courses of I_{Na} elicited by a train of depolarizing pulses. Eight consecutive pulses to −10 mV (30 ms duration) were applied from a V_h of −120 mV with various pulse intervals. The peak current amplitude for each pulse in the train was normalized to that of the first pulse and plotted against the pulse number. In control experiments (circles), the amplitude of I_{Na} remained constant at a pulse interval of 100 ms, indicating that normal Na^+ channels fully recover from inactivation within 100 ms after repolarization to −120 mV. In the presence of Cpz, a marked cumulative decrease of I_{Na} was seen in a wide range of pulse intervals

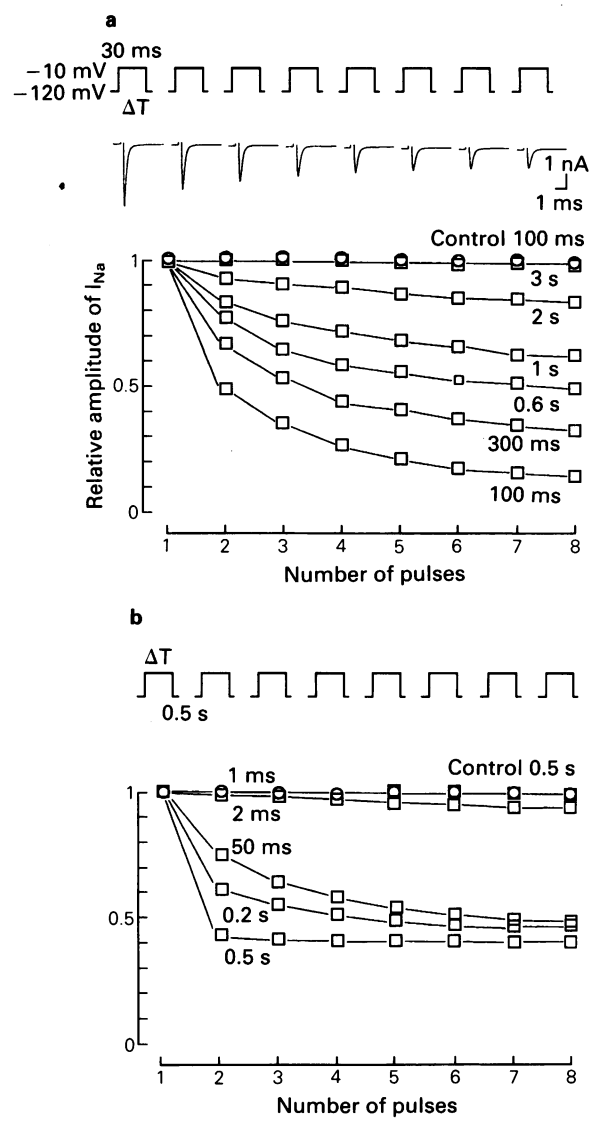


Figure 6 Time course of I_{Na} elicited by a train of step depolarizations applied at various pulse intervals. (a) Eight consecutive pulses to −10 mV (30 ms duration) were applied from a V_h of −120 mV at various pulse intervals ranging from 100 ms to 3 s. Inset: current traces in response to repetitive step depolarizations applied with 300 ms intervals in the presence of chlorpromazine (Cpz, 2.5 μM). The peak current amplitude for each pulse in the train was normalized to that of the first pulse and plotted against the pulse number. (○) Control; (□) in the presence of Cpz (2.5 μM). Numerals attached to the traces indicate interpulse intervals of repetitive stimulation. (b) Eight consecutive pulses to −10 mV (duration, 1–500 ms) were applied from a V_h of −120 mV with a fixed interval (500 ms). (○) Control; (□) in the presence of Cpz (2.5 μM).

from 100 ms to 2 s. Hereafter, this type of block seen during repetitive pulsing will be referred to as 'use-dependent block'. An interval of 3 s was required to avoid use-dependent block of I_{Na}. Similar results were obtained in all 8 cells examined.

Figure 6b shows an experiment similar to that in Figure 6a, but here, the protocol was designed to assess the time-dependence of the blocking process. The pulse duration was variable and the pulse interval was fixed at 500 ms. An interval of 500 ms was long enough to allow the normal channels to recover from inactivation but short enough for the drug-bound channels to reprime (as shown later). In the control experiments, I_{Na} remained constant in all the cases where the pulse duration was 50, 200 or 500 ms (only the plots for 500 ms are illustrated for clarity). In the presence of Cpz, the use-dependent block was not produced when pulse duration was 1 ms in any of the 3 cells examined. However, lengthening of the pulse duration to longer than 50 ms produced a marked use-dependent block. When the pulse interval was 50 or 200 ms, I_{Na} was progressively reduced during repetitive depolarization. When the pulse interval was increased to 500 ms, the second I_{Na} reached a steady-state value.

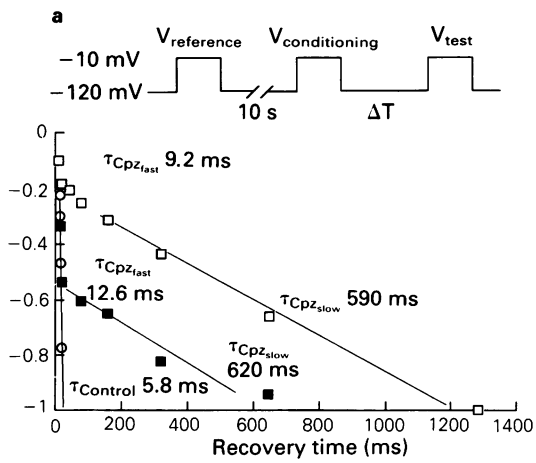
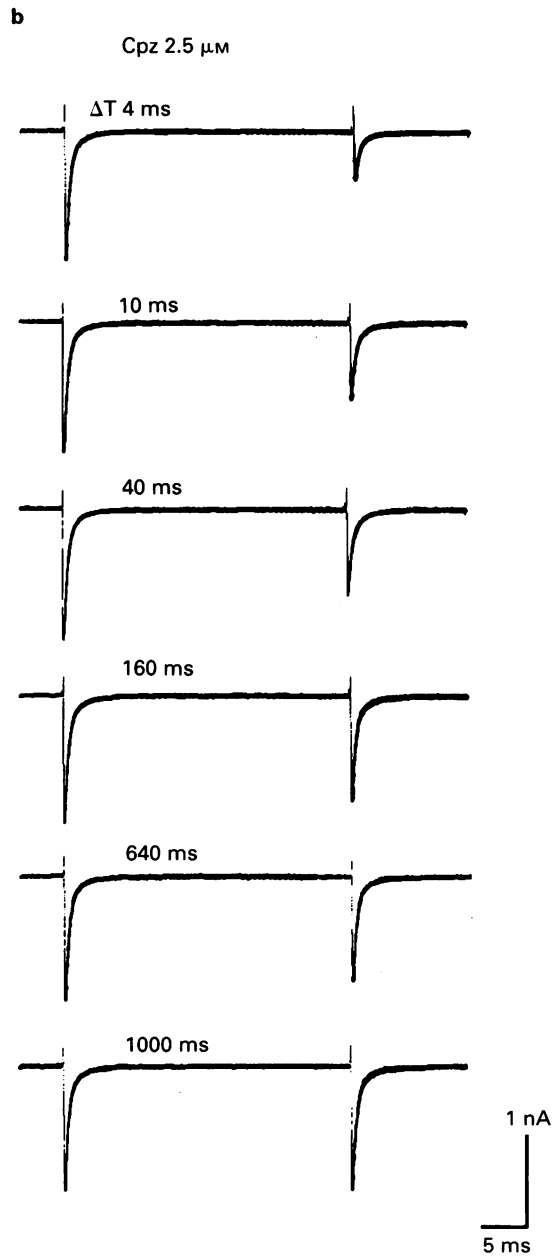


Figure 7 Dissociation of chlorpromazine (Cpz) from the inactivated Na^+ channel. (a) Recovery from inactivation was assessed using a double pulse protocol shown in the inset. A conditioning pulse to −10 mV ($V_{conditioning}$) was followed by a variable recovery period, and then by a test pulse to −10 mV (V_{test}) for 30 ms. Each two-pulse sequence was given at 30 s intervals. The peak amplitude of the test current was normalized to that of the preceding control current evoked by $V_{reference}$ and $\log(1 - I_{test}/I_{reference})$ was plotted as a function of the recovery period (ΔT). (○) Control; in the presence of Cpz 1 μM (■) or 2.5 μM (□). (b) Illustrates the currents evoked by $V_{reference}$ and V_{test} in the presence of 2.5 μM Cpz (the periods of $V_{conditioning}$ and ΔT were not sampled).

Kinetics of the use-dependent block

The results so far obtained suggest that Cpz preferentially binds to the Na^+ channel in its inactivated state and stabilizes it. Therefore, recovery from inactivation is expected to be delayed in the presence of Cpz. This was examined by use of a double-pulse protocol (Figure 7). A 500 ms conditioning pulse to



−10 mV was followed by a variable recovery period, and then by a test pulse to the same potential. The duration of the conditioning pulse of 500 ms was sufficient to produce maximal steady-state block (see Figure 6b). Each set of two pulses was given at an interval of 30 s. The peak current for each test pulse (V_{test}) was normalized to that for the preceding control pulse ($V_{reference}$) and plotted as a function of the recovery period (ΔT).

The test currents in the drug-free solution completely recovered within 20 ms with a time constant of 5.6 ± 0.6 ms ($n = 5$) (open circles). In the presence of Cpz (2.5 μM), the time course of recovery was markedly slowed, and a recovery period longer than 1 s was necessary to obtain full recovery (open squares). The recovery time course was characterized by two exponentials. The time constants of recovery were 8.9 ± 0.7 ms and 602 ± 64 ms ($n = 5$) for the fast and slow components, respectively. The fast time constant approximated the time constant of the control. Lowering the Cpz concentration to 1 μM resulted in a reduction of the amplitude of the slow component (filled squares). Figure 7b illustrates the $I_{reference}$ and I_{test} recorded at various recovery periods (ΔT).

Figure 8 illustrates how Na^+ channel inactivation was accelerated by Cpz during maintained membrane depolarization. As shown in the inset of Figure 8, a test pulse to −10 mV was preceded by a conditioning pulse to −10 mV with various durations. An interpulse-interval of 500 ms was put between the conditioning and test pulses to allow drug-unbound normal channels to reprime fully from the inactivated state to the resting state (see Figure 6b in which a test pulse of 500 ms duration had no effect on the Na^+ current evoked by subsequent test pulses in the control medium). The peak amplitude of the test current was normalized to that of the reference current measured 10 s before the conditioning pulse, and plotted against the conditioning pulse duration (ΔT) on a normal (upper half) and semi-logarithmic (lower half) scale. The Na^+ current in the presence of Cpz decreased to a steady-state level with a two exponential time course (τ_{fast}, 16.0 ± 1.8 ms; τ_{slow}, 162.5 ± 13.8 ms).

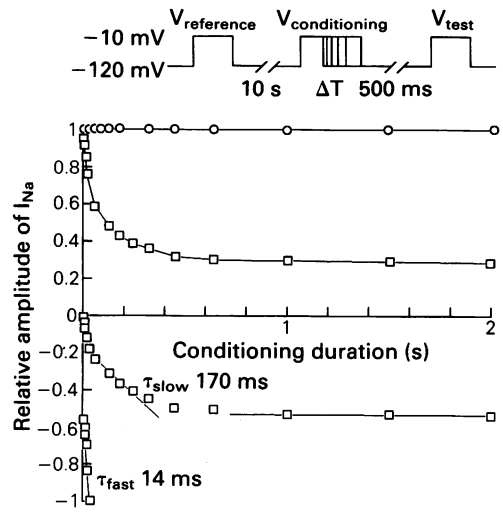


Figure 8 Association of chlorpromazine (Cpz) to the inactivated Na^+ channel. Inset shows an experimental protocol. A conditioning pulse ($V_{conditioning}$) to −10 mV of varying duration was followed by a 500 ms recovery period and then by a test pulse (V_{test}) of 30 ms duration. An identical pulse to V_{test} was applied 10 s before the conditioning pulse ($V_{reference}$). The peak amplitudes of test currents were normalized to those of control I_{Na} evoked by $V_{reference}$, and plotted against the conditioning duration on a normal (upper half) or semi-logarithmic scale (lower half). The lines were drawn by eye. (○) Control; (□) in the presence of Cpz (2.5 μM).

Discussion

The present study shows that the Na^+ channel gating kinetics are retained after enzymatic dissociation of the cells in the mammalian brain. The use of small (12–16 μm) neurones that have relatively fine processes (Figure 1) contributed to the quality of voltage-clamp control, as evidenced by a gradual increase of peak I_{Na} in the negative slope region of the I–V curve without 'threshold phenomenon' (Figure 3b), a single exponential decay of the tail currents (Figure 4a), and a consistent peak-latency of I_{Na} during multiple changes of the current amplitude (Figure 5a).

Interaction of Cpz with resting Na^+ channels

Our data indicate that Cpz has a potent blocking action on I_{Na} of the guinea-pig striatal neurones. Since the block was seen at a low temperature (11°C), this action appears to be due to a direct action on the ion channel rather than to some indirect effect. We have shown that the block of I_{Na} in the guinea-pig ventricular myocytes was the result of a direct action on the ion channel (Ogata *et al.*, 1989a). In this respect, Benishin *et al.* (1988) demonstrated that the inhibition of the calcium-activated K^+ channel in rat forebrain synaptosomes by neuroleptics is not mediated by calmodulin.

It appears that the blocking action of Cpz can be interpreted on the basis of the modulated receptor hypothesis introduced for local anaesthetic block of Na^+ channels (Hille, 1977; Hondeghem & Katzung, 1977). At very negative (−120 mV) V_h, where most

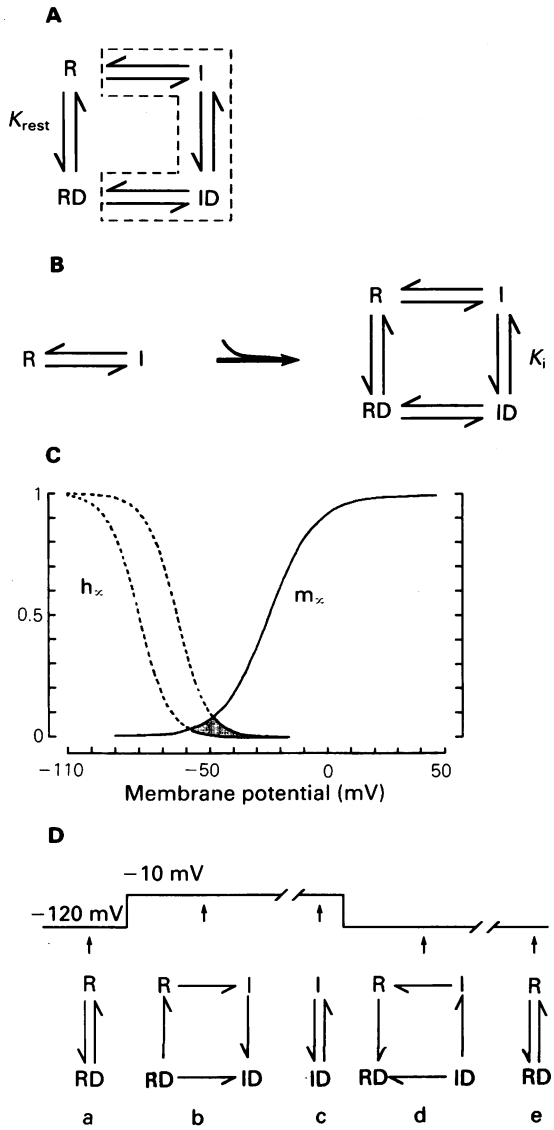


Figure 9 Modulated-receptor model for chlorpromazine (Cpz) binding. The size of the symbols indicating the channel state represent the relative population of the channel at that state. The scheme ignores interactions between Cpz and the open state of the channel, since our data suggested that these are negligible for interpretation of our experimental results. It should be noted that the illustrations are given for the sake of intuitive explanation and do not provide a correct picture of the channel states. (A) Illustrates the channel state at a very negative V_h in the presence of Cpz to explain resting block. The channel states enclosed by the broken line indicate that the channel population is negligibly small or absent. (B) Illustrates the shift of h_∞ curve by Cpz. The m_∞ and h_∞ curves shown in Figures 7 and 8 are drawn together in (C) to indicate a decreased 'window current' (shaded area) by Cpz. (D) The channel states at a very negative V_h (a), during a prolonged membrane depolarization (b and c), and after clamp-back to the initial V_h (d and e) to explain the association and dissociation of Cpz with the inactivated channel. R, resting state; RD, resting state with Cpz bound; I and ID, corresponding forms of the inactivated state; K_{rest} and K_i, the dissociation constants for resting and inactivated channels, respectively.

of the channels are in a resting state, i.e., ready to open upon depolarization ($h_\infty = 1$), (see Figure 5b), Cpz reduced the amplitude of I_{Na} (Figure 2b). This block is, therefore, thought to be due to the binding of Cpz to the resting channels (Figure 9A). The dissociation constant for this binding (K_{rest}) can be obtained from the concentration-response curve measured at a V_h of -120 mV (4.9 μM, see Figure 2c).

Interaction of Cpz with open Na^+ channels

I_{Na} in the presence of Cpz overlapped with control I_{Na} when the peak amplitude was normalized to the control (Figure 2b), indicating that Cpz does not affect τ_m or τ_h, the rates at which Na^+ conductance approaches its maximum or declines to its minimum. The above observation also suggests that Cpz does not interact with the open configuration of the channel (Colquhoun & Hawkes, 1983; Lee & Tsien, 1983). This idea is strengthened by a lack of voltage-dependence of the I–V curve (Figure 3c). Further supporting evidence was provided by the observation that, in the presence of Cpz, the use-dependent block was not produced when repetitive pulses to -10 mV were delivered with a pulse duration of 1 ms (Figure 6b). Since τ_m at a membrane potential of -10 mV was smaller than τ_m at -30 mV (0.2 ms, see Figure 4b) and τ_h was 1.6 ms (calculated from the trace in Figure 2b), a step depolarization to -10 mV for 1 ms is long enough to initiate the activation but too short to produce an appreciable amount of inactivation. Therefore, if Cpz interacted with the open Na^+ channel, I_{Na} should have become smaller during repetitive pulses due to the use-dependent (current-dependent) block.

Interaction of Cpz with inactivated Na^+ channels

Due to the shift in the h_∞ curve toward a more negative potential (Figure 5b), the action of Cpz is markedly enhanced under conditions where the membrane is depolarized. The dissociation constant for the binding to the inactivated state of Na^+ channels, K_i, (0.24 or 0.14 μM) appears to be 20–30 times smaller than K_{rest} (4.9 μM) and below the clinical concentration of this drug (Cooper *et al.*, 1976). Therefore, the action of Cpz may become significant in

pathological conditions in which the membrane is depolarized.

One possible explanation for the shift of the $h\infty$ curve is the change in the gating kinetics of the channel. Since $h\infty$ is described by $\alpha_h/(\alpha_h + \beta_h)$, where α_h and β_h are the rate constants for channel opening and closing, the reduction of $h\infty$ could be attributed to a change in the ratio, β_h/α_h. However, the observation that the decay time constant of I_{Na} which can be expressed by $1/(\alpha_h + \beta_h)$ was not affected by Cpz does not favour this idea.

The shift in $h\infty$ might be best explained by an increased population of the quiescent channels due to a production of drug-bound inactivated channels. As illustrated in Figure 9B, channels are distributed to R and I at depolarized membrane potentials in the control solution. Application of Cpz causes binding of Cpz to R and I with K_{rest} and K_i. Since $K_{rest} \gg K_i$ (see below), $I \rightarrow ID$ is much greater than $R \rightarrow RD$. The overwhelming binding of Cpz to I causes a proportional decrease in R due to the equilibrium between resting and inactivated channels, thus reducing $h\infty$.

As shown in Figure 9C, there was a significant degree of overlap between the inactivation and activation curves in the control solution, suggesting that Na^+ channels of the guinea-pig striatal neurones may be capable of supporting a steady influx of Na^+ (window current) over a narrow range of potentials. Application of Cpz which shifted the $h\infty$ curve to the left (Figure 5b) without affecting the $m\infty$ curve (Figure 4) cancels most of the window current (shaded area in Figure 9C). This effect of Cpz might be significant in the regulation of steady-state excitability of the neurone.

Kinetics of the use-dependent block

As was observed in the guinea-pig ventricular myocyte (Ogata & Narahashi, 1989; Ogata *et al.*, 1989a), the action of Cpz was promoted by repetitive activation of the channel (Figure 6). This use-dependent effect appears to be due to the slow re-priming of the Cpz-bound channels, since the block of open-channels did not appear to play a dominant role, at least in the concentrations we used (see above). As shown in Figure 7, recovery from inactivation was expressed as two exponentials. Since the fast component had a time course comparable to that of the recovery of the normal Na^+ channels, this may reflect the recovery of the unbound channel fraction (I in Figure 9D, c). Alternately the slow component might represent the recovery of the drug-bound channel fraction which was temporally produced during a conditioning depolarization (ID in Figure 9D, c). The dissociation of Cpz from the inactivated channels is shown schematically in Figure 9D ($c \rightarrow d \rightarrow e$) in terms of the change of the channel equilibrium states.

When the duration of the test pulse was 30 ms, the use-dependent block did not attain a steady-state value during eight consecutive test pulses (Figure 6a). Since the use-dependent block reflects the successive decrease of the channel populations, R + RD in Figure 9D, a, the above observation might indicate that the 30 ms duration was too short to reach an equilibrium between I and ID (Figure 9D, c). The experiment in Figure 6b measures the time-dependence of this equilibration. If the test pulse duration is long enough to allow the channels to be redistributed to I and ID in equilibrium (from a to c in Figure 9D), the amplitudes of I_{Na}s evoked by test pulses subsequent to the first test pulse should become constant, with their absolute values determined by the interpulse interval. Thus, it appears that the shift of the channel population from 'a' to 'c' in Figure 9D takes more than 200 ms.

The question is what is the pathway of the movement of RD to ID and which portion of the pathway is rate-limiting. An experimental approach to this question is shown in Figure 8. The time course of I_{Na} in this figure reflects the time constant of the association of Cpz with the inactivated Na^+ channels, because during a 500 ms interpulse interval, normal Na^+ channels are fully reprimed (see Figure 7a). The time course of I_{Na} was expressed as:

$$I_{Na}(t) = 0.33 + 0.28 \exp(-t/\tau_{fast}) + 0.39 \exp(-t/\tau_{slow}),$$

where τ_{fast} and τ_{slow} are 14 ms and 170 ms, respectively. The first term is the steady-state value of the use-dependent block. In other words, it reflects the amount of R in Figure 9D, d. The two time constants represent the rates by which the state 'a' shifts to 'c' in Figure 9D. The channel fraction R moves to ID via $R \rightarrow I \rightarrow ID$. The time required for the shift of the channel from R to I is estimated from the values of τ_m and τ_h and is thus negligible (in the order of 1 or 2 ms). The pathway from I to ID does not appear to be the rate-limiting step of the slow use-dependent effect, since repetitive pulse of 30 ms duration produced an appreciable amount of use-dependent block (Figure 6a). If we assume that RD directly moves to ID, its time course will be described by a single exponential function. If RD moves to ID via R and I, its time course will be expressed by a multi-exponential function. Taken together, the most likely explanation for the above two exponential time courses might be that the fast component mainly reflects the process, $I \rightarrow ID$ and the second com-

ponent represent the process, RD → R or RD → ID. The prolonged time course of the use-dependent block thus appears to be due to the slow kinetics of the drug-bound Na^+ channels.

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Effects of anipamil on cardiovascular status and regional blood flow in anaesthetized rats

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1 Anipamil, a long-acting analogue of verapamil, was tested at 1, 2.5 and 5 mg kg^{-1} i.v. for its effects on cardiovascular status and blood flow in different organs and vascular beds in rats anaesthetized with pentobarbitone.

2 Blood flow was determined by microsphere (^{57}Co or ^{113}Sn) injection before, and 1 h after, administration of anipamil at the above doses.

3 Anipamil produced a dose-dependent fall in blood pressure and heart rate. This was associated with a fall in peripheral resistance. At the highest dose there was, in addition, evidence of myocardial depression.

4 Both blood flow and conductance (flow corrected for blood pressure) were notably increased by anipamil in heart, liver and skeletal muscle beds, but were decreased, especially at the high doses, in kidneys, intestine and spleen.

5 The profile of the actions of anipamil on haemodynamic performance and flow was consistent with its proposed calcium antagonist actions. This profile was similar to that of verapamil.

Introduction

Anipamil is a verapamil analogue with a long duration of action (Raschack, 1984). It has been shown to be active against arrhythmias induced in conscious rats by occlusion of a coronary artery (Curtis *et al.*, 1986). Furthermore, it has anti-ischaemic actions in a variety of preparations (Gries & Raschack, 1984).

In *in vivo* experiments, anipamil lowered blood pressure and caused bradycardia (Curtis *et al.*, 1986). Dillon & Nayler (1988) recently found that anipamil binds to the same membrane sites as known calcium antagonists. In view of the above, it was considered of interest to ascertain the actions of anipamil in terms of its effects on blood pressure, heart rate, cardiac output and regional blood flow in order to determine whether or not its profile of cardiovascular actions was similar to those of other calcium antagonists. A preliminary report of parts of this study has been given previously (Walker *et al.*, 1988).

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Methods

The effects of 1, 2.5 or 5 mg kg^{-1} of anipamil i.v. on blood pressure, heart rate and regional blood flow were investigated in anaesthetized male Sprague-Dawley (University of British Columbia) rats weighing between 340–380 g. The procedures used have been described in detail elsewhere (Pang, 1983). Briefly, rats were anaesthetized with pentobarbitone (60 mg kg^{-1}, i.p.) and the iliac vein and arteries as well as the right carotid artery cannulated with PE-50 tubing. The carotid cannula was advanced into the left ventricle. Pressures were recorded with Statham pressure transducers connected to a Grass Polygraph (MP-7). Heart rate was electronically obtained by means of a Grass Tachograph (model 7P4F) while a Grass differentiator (model 7P20C) was used to process the ventricular pressure so as to obtain maximum dp/dt.

Suspensions of microspheres (15 μm diameter, New England Nuclear, Ma, U.S.A.) labelled either with ^{57}Co or ^{113}Sn were injected either before, or 1 h after, administration of anipamil. Half of the animals

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received ^{57}Co microspheres before anipamil while ^{113}Sn was given 1 h after. In the rest of the animals this order of giving microspheres was reversed. No differences could be ascribed to the order in which labelled microspheres were given. Anipamil (Knoll AG, Ludwigshafen, W. Germany) was dissolved in warm (50°C) distilled water (acidified to pH 3.0 with HCl) and suitably diluted so as to give a maximum injection volume of 10 ml kg^{-1}. Similar volumes of acidified warmed water were used for control injections and all injections were given over 1 min.

The injection of the appropriate microsphere suspension (30,000–40,000 microspheres in 200 μl) was made according to our usual procedure (Pang, 1983) with reference blood samples being withdrawn (Harvard infusion/withdrawal pump, Ma, U.S.A.) from the iliac arterial cannula at a rate of 0.35 ml min^{-1} for determination of cardiac output. After injection of the second set of microspheres the rat was killed and dissected into its component organs which were then weighed and their radioactive content determined as described previously (Pang, 1983). In the case of skin and muscle only representative samples were taken. The sample of skin was obtained from the dorsal and ventral areas and the muscle sample was taken from the chest, abdomen, limbs, diaphragm and back.

Data and statistical analysis

Blood flow in each of the organs was expressed either as total blood flow in the organ or tissue sample, or as conductance, i.e. flow divided by mean blood pressure. The latter normalisation procedure compensated for changes in blood pressure and allowed changes of resistance in different vessel beds to be assessed. Data were expressed either in absolute values in the pre- or post-drug periods, or as changes (pre-drug minus post-drug). Drug effects could, therefore, be assessed relative either to the pre-drug period, or to vehicle-treated controls. Statistical tests were performed on group means by analysis of variance, for determining significant sources of variance, and by Duncan's multiple range test for difference between means using an SPSSS package on the UBC Amdahl mainframe computer. A P value of 0.05, or less, was taken as being statistically significant and all values are given as mean ± s.e.mean.

Results

General cardiovascular changes induced by anipamil

Figure 1 summarises the changes from the pre-drug values, shown in Table 1, that anipamil induced in blood pressure, cardiac output, dp/dt_{max}, heart rate, stroke volume and total peripheral resistance. Anipamil caused a dose-dependent fall in blood pressure (Figure 1a) which was associated with bradycardia (Figure 1b), especially at the highest dose. The fall in blood pressure was associated with an increase in cardiac output at the lower doses (Figure 1a) but with a decrease at the highest dose. The calculated peripheral resistance showed statistically significant vasodilatation occurred with the two lower doses (Figure 1c).

The maximum rate of rise of ventricular pressure (dp/dt_{max} in Figure 1b) fell with the highest doses of anipamil, whereas stroke volume always rose with treatment (Figure 1c). The overall haemodynamic response pattern was consistent with anipamil causing a decreased peripheral resistance at the two lower doses, whereas at the highest dose direct depressant effects on the heart also became pronounced.

Table 1 Control values (pre-drug) for the various treatment groups

Group	*BP* (mmHg)	*CO* (ml min^{-1})	*Heart rate* (beats min^{-1})	dP/dt_{max} (mmHg s^{-1})	*SV* (ml)	*TPR* (mmHg min ml^{-1})
Pre-vehicle	93 ± 3	90 ± 9	300 ± 30	7670 ± 360	0.31 ± 0.02	1.1 ± 0.1
Pre-Anipamil: (mg kg^{-1})						
Pre-1	91 ± 2	87 ± 8	330 ± 15	7480 ± 360	0.27 ± 0.03	1.1 ± 0.1
Pre-2.5	111 ± 5	90 ± 6	335 ± 10	9390 ± 870	0.27 ± 0.02	1.3 ± 0.1
Pre-5	112 ± 5	98 ± 5	360 ± 20	9530 ± 550	0.28 ± 0.02	1.2 ± 0.1

BP = blood pressure, CO = cardiac output, SV = stroke volume, TPR = total peripheral resistance. Each value is mean ± s.e.mean from 9 rats. Measurements were made 1 min before beginning drug treatment.

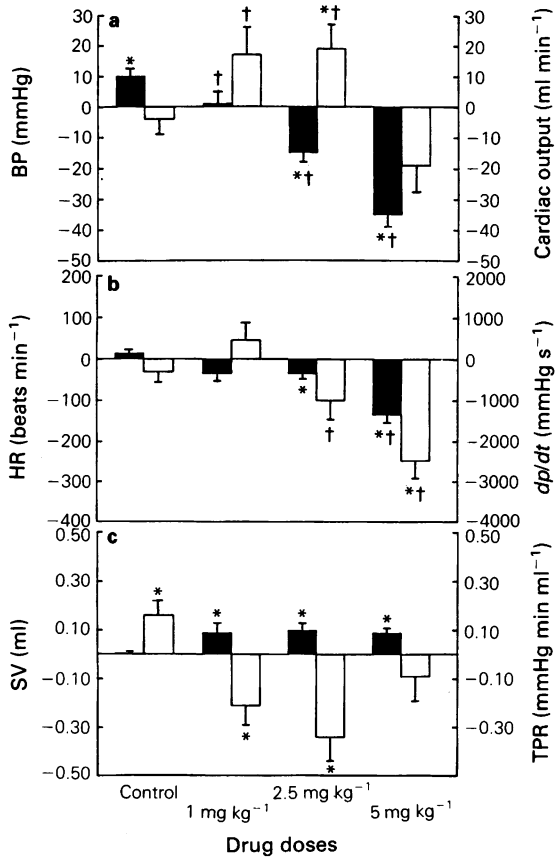

Figure 1 Effects of anipamil and vehicle on haemodynamic variables expressed as post-drug minus pre-drug control values for individual animals exposed to vehicle, 1.0, 2.5 or 5.0 mg kg^{-1} i.v. anipamil. The effect on blood pressure (filled columns) and cardiac output (open columns) is shown in (a), heart rate (filled columns) and dp/dt_{max} (open columns) in (b) and stroke volume (filled columns) and total peripheral resistance (open columns) in (c). Each value is mean ± s.e.mean. Statistical significance is shown by (*) for comparison with pre-drug values and (†) for comparison with vehicle control.

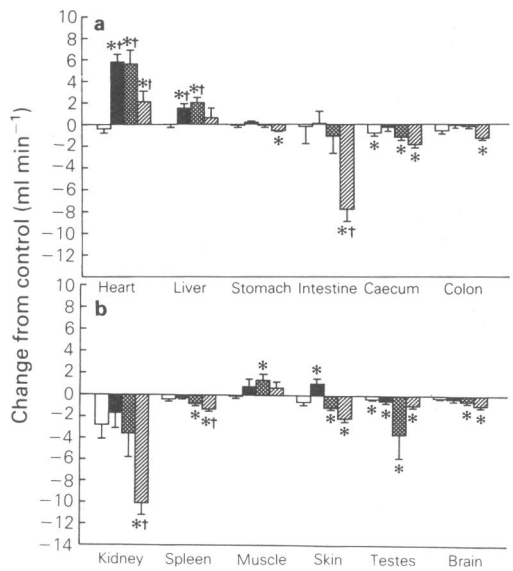

Figure 2 Effects of anipamil on blood flow in organs or tissue samples expressed as post-drug minus pre-drug values. Vehicle-treated controls are shown as open columns and 1, 2.5 and 5 mg kg^{-1} anipamil i.v. as closed, cross-hatched and hatched columns, respectively. All values are mean with s.e.mean shown by vertical bars; $n = 9$. Statistical comparisons are with pre-drug values (*) or vehicle-treated group (†).

The changes induced by anipamil have to be viewed against the changes which occurred with time in this preparation. Thus, for the vehicle-treated control animals, the time between the pre-drug and the post-drug sampling period (1 h) was associated with statistically significant increases in total peripheral resistance and blood pressure.

The effects of anipamil on blood flow were expressed as changes in blood flow (Figure 2) or as flow normalised for changes in blood pressure, i.e. conductance, in Figure 3. Tables 2 and 3 give the pre-drug values from which changes in flow and conductance were calculated. In all figures statistical significance is indicated for two types of comparison. The first comparison was between the pre- and post-drug values, while the second was between the post-drug periods for treated versus control groups. In Figure 2 it can be seen, for the vehicle-treated control group, that flows in all organs in the post-drug administration periods tended to be lower than those in the corresponding pre-drug periods. While individual differences only reached statistical significance for caecum and testes, the statistical chance for flow in all post-drug periods being less than the corresponding pre-drug periods (as was the case in Figure 2), was 0.00024. This indicates that a general reduction in flow with time occurred in pentobarbitone-anaesthetized rats. A general decrease in flow was in keeping with the higher blood pressure and total peripheral resistance found in the post-drug period for control animals (Figure 1a). With anipamil treatment a different pattern of flow changes was observed in that increases in flow occurred in some organs and vessel beds. These increases reached statistical significance for heart, liver, muscle and skin. When compared with the

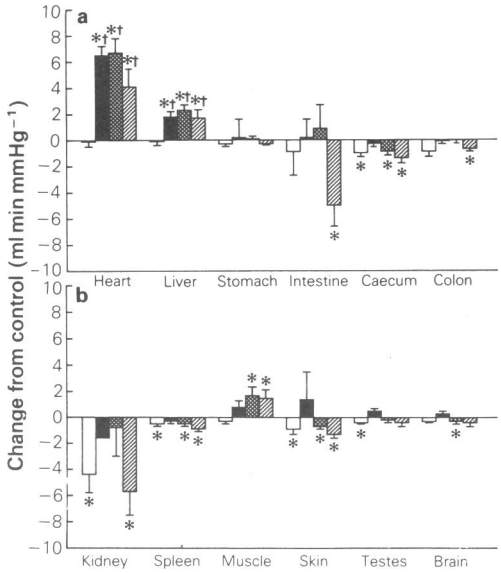

Figure 3 Effects of anipamil on conductance in organs or tissue samples expressed as post-drug minus pre-drug values. Vehicle-treated controls are shown as open columns and 1, 2.5 and 5 mg kg^{-1} anipamil i.v. as closed, cross-hatched and hatched columns, respectively. All values are mean with s.e.mean shown by vertical bars; $n = 9$. Statistical comparisons are with pre-drug values (*) or the vehicle-treated group (†).

vehicle-treated control group, statistical significance was only achieved for heart and liver. In other organs the pattern was more reminiscent of the vehicle-treated animals since flow was generally less in the post-drug period. These decreases, as opposed to the increases noted above, were greater with higher doses and were statistically greater than vehicle-treated controls for intestine, kidneys and spleen at the highest dose level of anipamil.

When flow was normalised for changes in blood pressure so as to give conductance values (Figure 3), a similar pattern to that described above was found. In vehicle-treated rats conductance was uniformly reduced in the post-drug period, indicating a general increase in resistance in all vessel beds. Most noticeable increases in conductance in the post-drug period were seen in the heart and liver since, for the three doses of anipamil, the increases in conductance in the two organs were statistically greater than control. In muscle, conductance in post-drug periods was always statistically greater than in the pre-drug periods. A similar tendency for a decrease in conductance in the post-drug period was particularly marked after the high dose of anipamil.

In summary, anipamil caused vasodilatation in muscle, heart and liver vascular beds but the high dose, particularly, accentuated the tendency for a decrease in flow with time which occurred in this anaesthetized rat preparation.

Discussion

Anipamil caused a dose-dependent reduction in blood pressure as a result of a reduced total peripheral resistance. In addition, at the highest dose, there was an added component of myocardial depression, as witnessed by attenuation of the maximum rate of rise in ventricular pressure and reduced heart rate. When administered at 1 and 5 mg kg^{-1}, i.v., in conscious rats anipamil also prolonged P-R intervals, as well as lowering blood pressure and heart rate (Walker, unpublished observation); further evidence

Table 2 Absolute blood flow to the various organs and tissue samples

Organ	*Pre-vehicle*	*Pre-*1 mg kg^{-1}	*Pre-*2.5 mg kg^{-1}	*Pre-*5 mg kg^{-1}
Heart	5.0 ± 0.7	4.4 ± 0.2	5.4 ± 0.4	5.2 ± 0.2
Liver	1.9 ± 0.3	2.1 ± 0.4	1.8 ± 0.3	1.9 ± 0.5
Stomach	1.1 ± 0.2	1.0 ± 0.2	1.2 ± 0.1	1.3 ± 0.2
Intestine	13.1 ± 0.2	9.9 ± 1.3	12.1 ± 0.8	14.0 ± 1.2
Caecum	2.7 ± 0.5	1.8 ± 0.2	2.7 ± 0.3	2.8 ± 0.3
Colon	2.7 ± 0.5	2.2 ± 0.3	1.7 ± 0.3	2.3 ± 0.3
Kidney	18.0 ± 1.8	15.5 ± 1.3	20.0 ± 1.7	18.8 ± 1.6
Spleen	1.2 ± 0.2	1.2 ± 0.1	1.4 ± 0.1	1.9 ± 0.2
Muscle (40 g)	1.7 ± 0.3	1.8 ± 0.2	1.8 ± 0.2	1.9 ± 0.2
Skin (40 g)	3.7 ± 0.7	3.4 ± 0.4	3.5 ± 0.3	3.5 ± 0.5
Testes	1.5 ± 0.2	1.7 ± 0.3	1.5 ± 0.1	1.6 ± 0.2
Brain	1.6 ± 0.2	1.5 ± 0.2	1.6 ± 0.1	1.9 ± 0.2

Values (ml min^{-1}) are mean ± s.e.mean of determinations in 9 rats anaesthetized with pentobarbitone. All values are the resting values before injection of drug or control solution.

Table 3 Absolute conductance in the various organs and tissue samples

Organ	*Pre-vehicle*	*Pre*-1 mg kg^{-1}	*Pre*-2.5 mg kg^{-1}	*Pre*-5 mg kg^{-1}
Heart	5.3 ± 0.7	4.9 ± 0.2	4.9 ± 0.2	4.7 ± 0.2
Liver	2.0 ± 0.3	2.2 ± 0.4	1.6 ± 0.2	1.6 ± 0.4
Stomach	1.3 ± 0.2	1.1 ± 0.2	1.1 ± 0.1	1.2 ± 0.1
Intestine	14.2 ± 1.1	11.1 ± 1.5	11.0 ± 0.7	13.2 ± 2.0
Caecum	2.9 ± 0.4	2.0 ± 0.2	2.5 ± 0.3	2.6 ± 0.4
Colon	3.0 ± 0.3	2.5 ± 0.3	1.6 ± 0.2	2.1 ± 0.3
Kidney	19.4 ± 1.8	17.1 ± 1.3	18.3 ± 1.7	17.1 ± 1.5
Spleen	1.3 ± 0.1	1.3 ± 0.1	1.3 ± 0.1	1.7 ± 0.2
Muscle (40 g)	1.8 ± 0.3	2.0 ± 0.2	1.6 ± 0.1	1.7 ± 0.2
Skin (40 g)	3.9 ± 0.7	3.7 ± 0.4	3.2 ± 0.4	3.2 ± 0.3
Testes	1.6 ± 0.2	1.9 ± 0.3	1.3 ± 0.1	1.5 ± 0.3
Brain	1.7 ± 0.1	1.7 ± 0.3	1.5 ± 0.1	1.8 ± 0.3

Values (ml min^{-1} mmHg^{-1} × 100) are mean ± s.e.mean of determinations in 9 rats anaesthetized with pentobarbitone. All values are the resting values before injection of drug or control solution.

of direct cardiac effects. Overall the haemodynamic effects of anipamil were, therefore, reminiscent of those due to high dose verapamil (Curtis *et al.*, 1986). A degree of vascular selectivity was exhibited by anipamil but overall the drug appears to have a small ratio of vascular to cardiac actions since the highest dose caused both severe negative inotropic and chronotropic effects, which led to a reduction of flow in most organs. The highest and most cardiac depressant dose of anipamil also reduced the overall decrease in total peripheral resistance, presumably because of marked activation of vasopressor systems in an attempt to maintain blood pressure in the presence of reduced cardiac output. In a recent study Dillon & Nayler (1988) reported that anipamil has a dose-dependent negative inotropic effect in spontaneously beating, or electrically-paced, rat isolated hearts. The same authors also examined the binding characteristics of anipamil to membranes from rat myocardium and found that anipamil binds to phenylalkylamine binding sites in a manner similar to verapamil and related calcium antagonists.

Although heart rate and maximum rate of rise in ventricular pressure were reduced by the administration of 2.5 mg kg^{-1} anipamil, an increase in cardiac output was still observed. This increase was possibly the result of an increase in venous tone. It has previously been shown in our laboratory that the administration of verapamil, nifedipine or flunarizine cause dose-dependent increases in mean circulatory filling pressure (Waite *et al.*, 1988). Mean circulatory filling pressure is a measure of total body venous tone (Guyton *et al.*, 1973). An increase in venous return after verapamil (Taira *et al.*, 1980) or nifedipine (Ogilvie, 1985) has also been reported to occur in anaethetised dogs. The suggestion that anipamil caused an increase in cardiac output by increasing venous tone is supported by other reports to the effect that a number of calcium antagonists can increase cardiac output while reducing heart rate (Hof *et al.*, 1982; Flaim & Zelis, 1982).

Comparison of the flow and conductance changes revealed an interesting pattern of vasodilatation and vasoconstriction with different doses of anipamil. In vehicle-treated controls there was increasing vasoconstriction with time which was overcome to an extent by the lowest doses of anipamil. However, at the highest dose of anipamil, further constriction occurred. The low dose of anipamil (1 mg kg^{-1}) did not greatly change the flow in most vascular beds, except for the heart and liver where flow was markedly increased. Although the 2.5 mg kg^{-1} dose of anipamil increased cardiac output, flow in a number of organs was reduced (particularly in caecum, spleen, skin, testes and brain) while it was increased in others (liver, heart and muscle). Such patterns suggest that anipamil selectively dilated blood vessels in coronary and skeletal muscle beds. The increase in hepatic arterial flow was possibly related to reduced portal venous flow. It has been shown that a decrease in portal venous flow leads to an increase in hepatic arterial flow such that total hepatic blood flow remains constant (Lautt, 1980).

Conductance values were calculated in order to normalise, or correct, blood flow for concomitant changes in blood pressure; such values more accurately reflect the true pattern of vasodilatation and vasoconstriction. In a comparison of conductance and flow, the pattern of vasodilatation in heart, liver and skeletal muscle was better revealed. In particular, calculation of conductance showed that the lower doses of anipamil, in fact, caused vasodilatation in the intestine, a finding which was not apparent from analysis of flow data. Generally, comparisons

between conductances in vehicle-treated and anipamil groups indicated that the lower doses of anipamil increased conductance in a number of organs to which flow had been reduced.

The overall haemodynamic and blood flow effects of anipamil had similarities and differences with other calcium antagonists. For example, verapamil caused a fall in blood pressure in anaesthetized cats which was dependent to some extent on cardiac depression (Hof, 1983). Verapamil also increased coronary blood flow but was noticeably more effective in causing vasodilatation in other vascular beds (Hof, 1983). In anaesthetized cats, PY 108-068, a dihydropyridine derivative, increased flow to heart, muscle and the small intestine, as did nifedipine (Hof *et al.*, 1982).

In conclusion, it would appear that anipamil produces cardiovascular effects which are consistent with its proposed calcium antagonist actions. The haemodynamic and flow effects of anipamil are qualitatively similar to those produced by verapamil (unpublished observations and Curtis & Walker, 1986).

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Endothelin stimulates short circuit current in a cultured epithelium

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1 Endothelin, a novel potent vasoconstrictor peptide produced by vascular endothelial cells stimulated anion secretion by a cultured secretory epithelium derived from the rat epididymis as measured by changes in short-circuit current (SCC).

2 Stimulation of the SCC was observed when endothelin was added to the basolateral or the apical side of the epithelium. The response to basolateral application was greater than that to apical application. The EC_{50} values were found to be 1.3 nM and 3.0 nM for basolateral and apical application, respectively. These values were about half an order to one order of magnitude higher than that required for its vasoconstrictor action.

3 The stimulation of SCC by endothelin was accompanied by a rise in the intracellular adenosine 3′:5′-cyclic monophosphate (cyclic AMP) content in epididymal monolayers.

4 The effect of endothelin on SCC was mediated through an increase in prostaglandin synthesis by the tissues and was not blocked by an antagonist of angiotensin II (Sar^1 Ile^8 angiotensin II) or of adrenaline (propranolol).

5 It is speculated that endogenous endothelin plays an important role in the control of water and electrolyte transport in the epididymis.

Introduction

Yanagisawa *et al.* (1988) have recently isolated endothelin, a potent vasoconstrictor peptide from cultured endothelial cells. These authors proposed that this peptide may mediate the endothelium-dependent vasoconstrictor action of various chemical and physical stimuli such as noradrenaline, thrombin, hypoxia, etc. (Rubanyi & Vanhoutte, 1985, Vanhoutte, 1987). The amino acid sequence has been determined and from this it appears that endothelin does not belong to any previously known peptide family. However, endothelin shows local homologies to a certain group of peptide neurotoxins that act on voltage-dependent Na channels (Yanagisawa *et al.*, 1988).

It is known that various vasoactive peptides such as vasoactive intestinal peptide (Schwartz *et al.*, 1974), angiotensin (Levens *et al.*, 1981), bradykinin (Cuthbert & Margolius, 1982) and atrial natriuretic peptide (Mohrmann *et al.*, 1987) do have effects on electrolyte and fluid transport in epithelia, and because there is evidence that endothelin acts on ion channels in cell membranes (see Yanagisawa *et al.*, 1988), we have studied the effect of endothelin on electrogenic anion secretion by a cultured epithelial monolayer derived from the rat epididymis. This epithelium has been shown to secrete anions electrogenically when stimulated with secretory agonists that increase the level of intracellular cyclic AMP (Cuthbert & Wong, 1986; Wong, 1988a, b, c).

Methods

Measurement of short circuit current (SCC)

Primary monolayer cultures of rat cauda epididymis were grown on millipore filters by methods described previously (Cuthbert & Wong, 1986; Wong, 1988a, b, c). After 4 days of culture, the monolayer became confluent and was ready for the measurement of electrogenic anion secretion by the short circuit current technique. Monolayers (area 0.4 to 0.7 cm^2) were clamped between the two halves of the Ussing chambers with a 0.7 cm^2 window. The tissue was short circuited (voltage clamped at zero potential) using W-P Instruments Dual Voltage Clamp Amplifier (DVC 1000) and the short circuit current displayed on a pen recorder (Kipp and Zonen). Very often, the two channels of the amplifier were used simultaneously on parallel monolayers so that studies could be made under control and experimental conditions. At the end of the experiments, the

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area of the monolayers was accurately measured with an IBM computer equipped with a digitizer (Hipad™). In most experiments, monolayers were incubated in Krebs-Henseleit (K-H) solution (Wong, 1988a). In some experiments, a Cl-free solution was used in which NaCl, KCl and $CaCl_2$ were replaced with the corresponding gluconate salts. Endothelin was from Auspep, Australia and was reconstituted in dilute acetic acid and kept below −20°C before use. Diphenylamine-2-carboxylate (DPC) was purchased from Riedel-deHaën, W. Germany, piroxicam and prostaglandin E_2 were from Sigma, Co. (St Louis, MO, U.S.A.), lysylbradykinin from Cambridge Research Biochemicals, Cambridge, U.K., adrenaline was obtained from David Bull Laboratories, angiotensin II from Ciba Geigy, Switzerland and Sar^1, Ile^8, angiotensin II from Beckman Instruments Inc., California, U.S.A.

Measurement of intracellular adenosine 3':5'-cyclic monophosphate (cyclic AMP)

Epididymal monolayers were grown on 24-well plates (Linbro, Flow Lab. Inc., U.S.A.). After 4 days of culture, monolayers were washed twice with Krebs Henseleit (K-H) solution and then incubated in 0.5 ml of the same buffer containing isobutylmethylxanthine (IBMX 1 mM) for 10 min at 32°C. Secretory agonists were added to the wells and incubated for a further 10 min. At the end of the incubation, 10 μl perchloric acid (60% w/v) was added to the well to terminate the reactions. The content of each well was mixed well and transferred to a 1.5 ml microfuge tube which was then centrifuged at 10,000 *g* for 5 s; 300 μl of the supernatant was transferred to a test tube and the excess acid was neutralized by 1 *M* KOH. Aliquots (50 μl) of the mixture were assayed for cyclic AMP with a cyclic AMP assay kit (Amersham, Buckinghamshire, U.K.). This method is based on the competition between unlabelled cyclic AMP and a fixed quantity of the tritium-labelled compound for binding to a protein which has a high specificity and affinity for cyclic AMP.

Statistical analysis

Comparisons between groups of data were made by Student's *t* test.

Results

Effect of endothelin on short circuit current

When incubated in normal Krebs-Henseleit solution, the epididymal monolayers exhibited a potential difference (PD) of 2–4 mV, a short circuit current of 1–2 $\mu A\,cm^{-2}$ and a transepithelial resistance of about 500 $\Omega\,cm^2$ (Wong, 1988c). Addition of endothelin to the basolateral side caused a dose-dependent increase in the SCC (Figures 1 and 2). A maximal response (ΔSCC, $3.20 \pm 0.12\,\mu A\,cm^{-2}$, mean ± s.e.mean, $n = 30$ cultures) was obtained with 8 nM endothelin. The EC_{50} value (concentration producing 50% maximal response) was 1.3 nM (geometric mean, $n = 5$, 95% confidence limits = 1.19, 1.41). Addition to the apical side produced a smaller response (maximal response, $1.18 \pm 0.05\,\mu A\,cm^{-2}$, $n = 30$ cultures). The EC_{50} value was 3.0 nM (geometric mean, $n = 5$, 95% confidence limits = 2.40, 3.60).

Effects of chloride removal and diphenylamine-2-carboxylate

The short circuit current response to endothelin was studied in the absence of extracellular Cl (Cl replaced by gluconate). When monolayers were incubated in K-H solution containing Cl, the SCC response (ΔSCC) to endothelin (8 nM, basolateral application) was $3.17 \pm 0.14\,\mu A\,cm^{-2}$ ($n = 4$ cultures). In monolayers incubated in Cl-free solution, ΔSCC was $0.24 \pm 0.02\,\mu A\,cm^{-2}$ ($n = 4$). The difference is significant statistically ($P < 0.001$, Student's unpaired *t* test). In some experiments, monolayers were stimulated with endothelin (8 or 16 nM) added to the basolateral bathing medium. When the SCC had reached the peak level, diphenylamine-2-carboxylate (DPC, 0.1 mM) added to the apical bathing solution lowered the SCC to or below the prestimulated level (4 experiments) (Figure 1).

Effect of repeated stimulation with endothelin

The response of the tissue to apical application of endothelin can be due to the presence of receptors in the apical membrane or to the diffusion of endothelin to the basolateral side where they interact with basolateral membrane receptors. To investigate these possibilities, endothelin (1 nM) was first added to the basolateral side to produce approximately the same response as apical application of 8 nM endothelin (Figure 2). When the response subsided, endothelin (8 nM) was again added to the basolateral side to determine whether the first response had caused tachyphylaxis to the second one (Figure 3a). These results were compared with those obtained in parallel monolayers in which the tissues were first challenged with apical application (8 nM) followed by

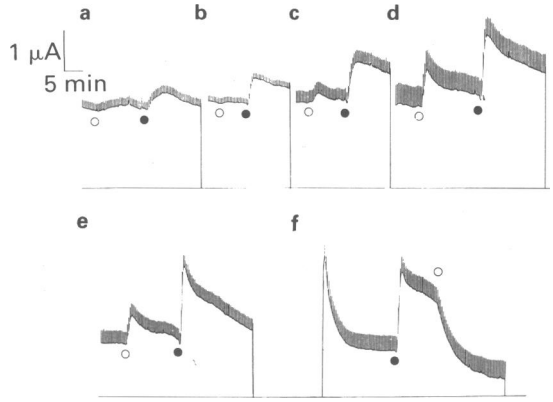

Figure 1 Short circuit currents (SCC) of 6 separate monolayers, area 0.4–0.7 cm^2. Endothelin was added to the apical (○) and then the basolateral sides (●) to produce the following concentrations: (a) 0.8, (b) 1.6, (c) 4, (d) 8, and (e) 16 nM. In (f), the tissue was first challenged with endothelin (16 nM) added to the basolateral side (●) followed by apical application of diphenylamine-2-carboxylate 0.1 mM, (○). Transient current pulses were the result of intermittently clamping the potential at 0.2–0.3 mV. The horizontal lines indicate zero SCC. Each record is representative of 5 different experiments.

basolateral application (8 nM) (Figure 3b). After the tissue had first responded to basolateral application of 1 nM endothelin, the second response (ΔSCC measured at the peak of the response) to basolateral application of 8 nM endothelin was $0.90 \pm 0.05\ \mu A\ cm^{-2}$ ($n = 4$) (Figure 3a). This

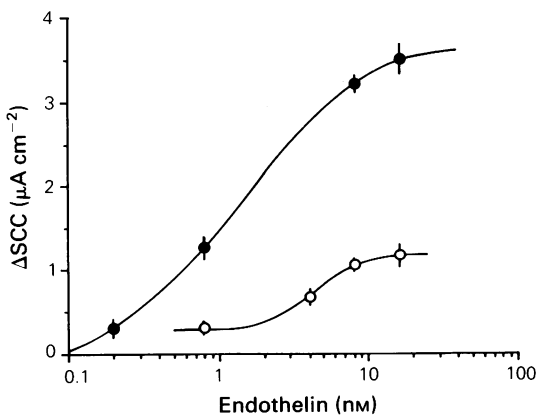

Figure 2 The effect of endothelin on the short circuit current (SCC) of rat epididymal monolayers, area 0.4–0.7 cm^2. ΔSCC is the increase in current measured at the peak of the response. Endothelin was added to the basolateral (●) or apical bathing medium (○). Each point shows the mean of 5 experiments; vertical lines indicate s.e.mean.

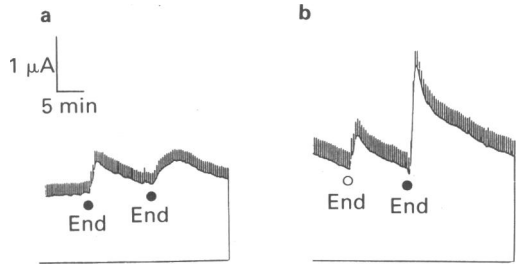

Figure 3 Short circuit currents (SCC) of 2 separate monolayers, area 0.4 cm^2 measured simultaneously. (a) Monolayer first stimulated with endothelin (1 nM, ●) added to the basolateral side followed by basolateral addition (8 nM, ●); (b) monolayer first stimulated with endothelin (8 nM, ○) added to the apical side followed by basolateral application (8 nM, ●). Transient current pulses were the result of intermittently clamping the potential at 0.3 mV. The horizontal lines indicate zero SCC. These records are representative of 4 different sets of experiments.

response was significantly ($P < 0.001$, Student's unpaired t test) lower than that observed in control monolayers (derived from the same batch of cells) which were treated with basolateral application of endothelin (8 nM) for the first time (ΔSCC, $3.18 \pm 0.16\ \mu A\ cm^{-2}$, $n = 4$). This indicated that repeated basolateral application caused tachyphylaxis of the basolateral endothelin receptor. In contrast, however, in tissues that had been stimulated first with 8 nM endothelin added apically, the second response (ΔSCC) to basolateral addition of 8 nM endothelin was $3.17 \pm 0.22\ \mu A\ cm^{-2}$ ($n = 4$). This value is not significantly different from the responses obtained from control monolayers without pretreatment with apical endothelin ($P > 0.95$, Student's unpaired t test) suggesting that apical application did not result in tachyphylaxis of the basolateral endothelin receptors.

In another group of experiments, monolayers were first treated with 8 nM endothelin to induce tachyphylaxis of the basolateral endothelin receptors, the effects of other secretory agonists on the short circuit current were then tested. Figure 4 shows that although the response to the second basolateral application of endothelin (8 nM) was prevented by tachyphylaxis of the basolateral endothelin receptors, the responses to bradykinin, prostaglandin and adrenaline were unaffected under these circumstances. This would support the contention that the endothelin receptors are distinct from those of bradykinin, prostaglandin and adrenaline (Figure 4).

Effects of prostaglandin synthesis inhibitor

The rise in SCC stimulated by endothelin was completely blocked by pretreating the tissues with an

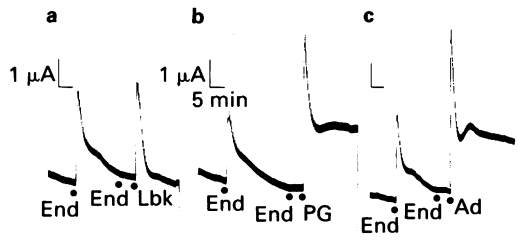

Figure 4 Short circuit currents (SCC) of 3 separate monolayers, area 0.7 cm^2. In each case, endothelin (8 nM) was first added to the basolateral side. When the current returned to the basal level, the tissue was challenged with endothelin for the second time followed by lysylbradykinin (Lbk 0.1 μM) (a), prostaglandin E_2 (PG 0.5 μM) (b), or adrenaline (Ad 0.23 μM) (c) added to the basolateral side. Transient current pulses were the result of intermittently clamping the potential at 0.3 mV. The horizontal line indicates zero SCC. Each record is representative of 3 different experiments.

inhibitor of prostaglandin synthesis, piroxicam (5 μM) (Figure 5 and Table 1), indicating that the stimulation as mediated by prostaglandins synthesized locally in the tissue. In contrast, stimulation of the SCC by adrenaline was not affected by piroxicam pre-treatment (Figure 5 and Table 1).

Effects of antagonists on secretion induced by endothelin and other agonists

Previous work in our laboratory has shown that Cl secretion by epididymal epithelium is stimulated by basolateral application of angiotensin II and adrenaline (Wong, 1988a), and therefore the effects of antagonists of angiotensin II (Sar[1], Ile[8] Angiotensin II) and adrenaline (propranolol) were studied. Angiotensin II (500 ng ml^{-1}) added basolaterally caused an increase in the SCC that was completely abolished by Sar[1], Ile[8] angiotensin II (5 μg ml^{-1}), an angiotensin II antagonist. However, pretreating the monolayers with the antagonist (5 μg ml^{-1}) did not affect the SCC response to endothelin (8 nM) (Table 1). Similarly, pretreating the tissues with propranolol (2 μM) did not affect the endothelin response although the same dose of propranolol almost completely blocked the SCC response to adrenaline (0.23 μM) (Table 1) (Wong & Chan, 1988).

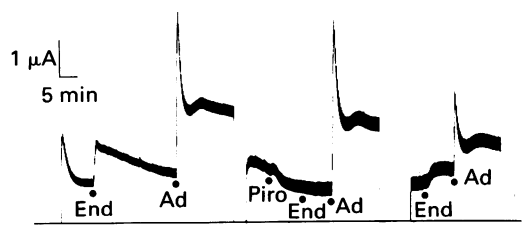

Figure 5 Effects of piroxicam (Piro) (prostaglandin synthesis inhibitor) on the short circuit current (SCC) response to endothelin (End) and adrenaline (Ad) in an epididymal monolayer, area 0.6 cm^2. Monolayer first stimulated with endothelin (End) (8 nM) and then with adrenaline (Ad) (0.23 μM) both added to the basolateral side. After washing, both agents were added again in the presence of piroxicam (5 μM) added to the basolateral side. Finally, control responses were repeated after washing. Transient current pulses were the results of intermittently clamping the potential at 0.3 mV. The horizontal line indicates zero SCC. This record is representative of 3 different sets of experiments.

Secretory response to endothelin mediated by cyclic AMP

Evidence has been obtained that stimulation of anion secretion in the epididymis is mediated by

Table 1 Effect of angiotensin II antagonist (Sar[1], Ile[8] Ang II, 5 μg ml^{-1}), propranolol (2 μM) and piroxicam (5 μM) on the angiotensin II-(Ang II, 500 ng ml^{-1}), endothelin-(8 nM) or adrenaline-(0.23 μM) induced rise in the short circuit current (SCC)

Agent	*ΔSCC*	*Number of experiments*
Ang II	3.70 ± 0.15	6
Ang II + Sar[1], Ile[8] Ang II	0*	6
Endothelin	2.52 ± 0.27	4
Endothelin + Sar[1], Ile[8] Ang II	2.54 ± 0.28	4
Adrenaline	10.32 ± 1.70	4
Adrenaline + propranolol	0.12 ± 0.02*	4
Endothelin	2.81 ± 0.16	4
Endothelin + propranolol	2.85 ± 0.18	4
Endothelin	3.10 ± 0.20	3
Endothelin + piroxicam	0*	3
Adrenaline	10.83 ± 0.25	3
Adrenaline + piroxicam	10.63 ± 0.28	3

Results are expressed as the change in current, ΔSCC (μA cm^{-2}) measured at the peak of the response. Inhibitors were added to the bath 2 min (Sar[1], Ile[8] Ang II and propranolol) or 15 min (piroxicam) before addition of the agonists. All agents were added to the basolateral bathing medium. Each value shows the mean ± s.e.mean of the number of experiments shown.

* $P < 0.001$ compared to the control of the respective group (Student's unpaired t test).

Table 2 Effect of various secretory agonists on the intracellular adenosine 3':5'-cyclic monophosphate (cyclic AMP) content (*p*mol/10^5 cells) of epididymal monolayers derived from the rat cauda epididymis

Agents	*Cyclic AMP*	*Number of experiments*
Control	22.6 ± 2.0	16
Adrenaline (0.23 μM)	61.5 ± 5.4*	5
PGE_2 (0.5 μM)	40.5 ± 4.8*	6
Ang II (500 ng ml^{-1})	40.4 ± 5.3*	6
Endothelin (16 nM)	52.7 ± 5.8*	5

Each value is the mean ± s.e.mean of the number of experiments shown.
* $P < 0.001$ compared to control (Student's unpaired *t* test).

intracellular cyclic AMP as the second messenger (Cuthbert & Wong, 1986; Wong, 1988a). The cyclic AMP content in epididymal monolayers incubated in various agonists was measured. Adrenaline (0.23 μM), PGE_2 (0.5 μM), angiotensin II (500 ng ml^{-1}) or endothelin (16 nM) stimulated a rise in the concentration of intracellular cyclic AMP (Table 2).

Discussion

These results show that endothelin, a novel potent vasoconstrictor substance released from the endothelium of blood vessels, increased short circuit current in cultured rat epididymal epithelium. This increase is likely to be caused by an increase in net anion secretion since the response was greatly reduced when epididymal monolayers were incubated in a Cl-free solution. Secondly, the response was inhibited by the chloride channel blocker, DPC (0.1 mM) added to the apical solution (Figure 1). This blocker has been shown to inhibit anion secretion by cultured rat epididymal epithelium and the intact epididymis of rats *in vivo* (Wong, 1988b).

The EC_{50} value obtained in rat epididymis is about half an order to one order of magnitude higher than that required for vascular smooth muscle contraction (Yanagisawa *et al.*, 1988) but is about one order of magnitude lower than that required for stimulation of contraction in the human isolated urinary bladder (Maggi *et al.*, 1989). The receptors mediating the effect in the epididymis are distinct from those of angiotensin, bradykinin and adrenaline (Figure 4) although the effects of endothelin, bradykinin (Cuthbert & Wong, 1986) and angiotensin II (Wong, 1988a) are mediated by an increase in prostaglandin synthesis.

The cellular mechanisms that are responsible for the stimulation of anion secretion are unknown. The intracellular messenger mediating the secretory response appears to be cyclic AMP (Table 1). In the epididymal cells, anion secretion is mediated by the active uptake of Cl and HCO_3 across the basolateral membrane and exit of the anions through the apical anion channels. The uptake of anions at the basolateral membrane is accomplished by a Na-K-2Cl symport, and a pair of antiports involving Na-H exchange and Cl-HCO_3 exchange (Wong 1988a). Stimulation of secretion can be achieved by stimulation of any of these ion carriers and/or ion channels. Recent work has shown that endothelin stimulates the Na-H exchanger in the rat mesangial cells hence causing an intracellular alkalinization. This phenomenon is associated with the stimulation of mitogenesis in the cells (Simonson *et al.*, 1989).

The experiments with repeated stimulation with endothelin suggest that the responses of the tissues to apical application could not have been due to diffusion of endothelin from the apical to the basolateral side. The basolateral receptors underwent tachyphylaxis upon repeated stimulation (Figure 3a). If the responses to apical application (Figure 1) were due to diffusion of endothelin to the basolateral side, then tachyphylaxis would have reduced the responses to subsequent basolateral addition. However, this was not the case (Figure 3b) suggesting that receptors for endothelin are likely to be present in both the apical and basolateral membranes but with predominance in the latter. In this respect, the disposition of endothelin receptors is unlike that of ATP and adrenaline in that the latter two receptors display apical/basolateral 'sidedness' (Wong, 1988c).

Our present results suggest that in addition to its effects on smooth muscle (Yanagisawa *et al.*, 1988; Maggi *et al.*, 1989), endothelin may also play a role in regulating electrolyte and fluid transport in the epididymis and possibly other transporting epithelia. Koseki *et al.* (1989) studied the distribution of endothelin receptors by autoradiography and receptor binding assays and found that not only are the endothelin receptors found in the arteries and heart, they are also present in many organs such as the brain, kidney, lung, adrenal gland and intestine. These findings are consistent with the notion that endothelin may play the role of a paracrine hormone which is widely distributed and subserves diverse physiological functions.

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Receptors mediating the effects of substance P and neurokinin A on mucus secretion and smooth muscle tone of the ferret trachea: potentiation by an enkephalinase inhibitor

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1 The effects of substance P (SP) and neurokinin A (NKA) were examined on tracheal smooth muscle tone, mucus volume output, lysozyme output and albumin transport across the ferret *in vitro* whole trachea in the presence and absence of the enkephalinase inhibitor, thiorphan.

2 SP (0.001–3 μM) and NKA (0.01–10 μM) contracted the tracheal smooth muscle and increased mucus volume, lysozyme and albumin outputs into the tracheal lumen. The EC_{50} values for SP and NKA for all of the variables measured were significantly reduced, and all of the maximum responses were significantly enhanced by thiorphan (10 μM).

3 In the presence of thiorphan, SP (1 μM) and NKA (10 μM) produced albumin concentrations in the secreted mucus (8.9 and 7.2 $\mu g \mu l^{-1}$) which were greater than those in the submucosal buffer (4.2 $\mu g \mu l^{-1}$).

4 In the presence of thiorphan, NKA was approximately 5 times more potent than SP at contracting the tracheal smooth muscle. Conversely SP was 23, 15 and 22 times more potent than NKA at stimulating mucus volume, lysozyme and albumin outputs respectively.

5 Thus, there is neutral endopeptidase in the ferret trachea *in vitro* which cleaves exogenously applied SP and NKA, thereby reducing the magnitude and potency of their actions. SP and NKA contract the ferret tracheal muscle probably by an action at NK_2 (or NK_3)-receptors but stimulate mucus volume output, lysozyme output and albumin transport across the tracheal wall probably by an action on NK_1 receptors.

Introduction

The tachykinins are small peptides which are likely to play important roles in the regulation of airway function. Included in these tachykinins are substance P (SP) and neurokinin A (NKA) which are localised to C-fibre nerve endings in the airways of many species including man (Lundberg *et al.*, 1984). Immunohistological studies suggest that SP- and NKA-immunoreactivity is localised to sensory nerves in airway smooth muscle, around blood vessels and ganglia and within and under the epithelium (Lundberg *et al.*, 1985; Ghatei *et al.*, 1987). There is a sparse innervation of SP and NKA immunoreactive nerves around submucosal glands (Lundberg *et al.*, 1984); however receptors for SP are present on guinea-pig (Hoover & Hancock, 1987) and human (Carstairs & Barnes, 1986) bronchial submucosal glands.

SP and NKA have potent effects on the airways of several species. These peptides contract airway smooth muscle (Lundberg & Saria, 1987), are vasodilators of the tracheobronchial circulation (Laitinen *et al.*, 1987; Salonen *et al.*, 1988) and increase microvascular permeability (Rogers *et al.*, 1988). In addition SP is a potent stimulant of macromolecule output from canine and human submucosal glands (Coles *et al.*, 1984; Barnes *et al.*, 1986). Little is known about the effects of NKA on submucosal gland secretion.

SP and NKA are degraded by several peptidases including neutral endopeptidase (NEP), which is also called enkephalinase (Hudgin *et al.*, 1981). This peptidase is a cell membrane-bound enzyme that is present in airway epithelium, glands and smooth muscle from many species including man (Llorens &

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Schwartz, 1981; Johnson *et al.*, 1985). Recently inhibitors of NEP, thiorphan and phosphoramidon, have been shown to increase both the maximum responses and potency of SP and NKA on human (Black *et al.*, 1988) and ferret (Sekizawa *et al.*, 1987) airway smooth muscle *in vitro*, and on guinea-pig airways *in vivo* (Dusser *et al.*, 1988). Thiorphan also potentiates the SP-induced secretion of macromolecules from the ferret trachea (Borson *et al.*, 1987).

In the present study we have used the ferret *in vitro* trachea (Webber & Widdicombe, 1987) to examine the effect of SP and NKA on the volume of mucus produced, the lysozyme content of the secreted mucus (as an indicator of serous cell output) and tracheal smooth muscle tone in the presence and absence of thiorphan. Recently we have demonstrated that albumin is actively transported across the ferret tracheal wall (Webber & Widdicombe, 1989) and the effect of SP and NKA on this transport process has also been investigated.

Methods

The ferret in vitro *trachea*

Ferrets of either sex, weighing 0.5–1.5 kg, were anaesthetized by an intraperitoneal injection of sodium pentobarbitone (Sagatal, May & Baker, 50 mg kg^{-1}). The trachea was exposed and cannulated about 5 mm below the larynx with a perspex cannula containing a conical collecting well (Webber & Widdicombe, 1987). The ferret was then killed with an overdose of anaesthetic injected into the heart. The chest was opened along the midline and the trachea exposed to the carina, cleared of adjacent tissue, removed and cannulated just above the carina. The trachea was mounted, laryngeal end down, in a jacketed organ bath with Krebs-Henseleit buffer restricted to the submucosal side. The composition of the Krebs-Henseleit solution was (mM): NaCl 120.8, KCl 4.7, KH_2PO_4 1.2, $MgSO_4 7H_2O$ 1.2, $NaHCO_3$ 24.9, $CaCl_2$ 2.4, glucose 5.6. The buffer was maintained at 37°C and gassed with 95% O_2/5% CO_2. The lumen of the trachea remained air-filled. Secretions were carried by gravity and mucociliary transport to the lower cannula, where they pooled and could be withdrawn periodically into a polyethylene catheter which was inserted into the lower cannula to form an airtight seal. The catheters containing the secretions were sealed at both ends with bone wax, numbered and stored frozen until required.

After defrosting, the secretions were washed out of the catheters into labelled plastic vials with 0.5 ml distilled H_2O. The vials were frozen and stored for use in the albumin and lysozyme assays. Preliminary experiments had shown that frozen storage for up to 6 months does not affect the enzymatic activity of lysozyme or the albumin content. Secretion volumes were estimated by the differences in weights of the catheters with secretions and dried without secretions, and the secretion rates were expressed as $\mu l\,min^{-1}$ (assuming 1 g of secretion is equivalent to 1 ml).

During experiments the carinal cannula was attached to a pressure transducer which was connected to a pen recorder. Changes in smooth muscle tone produced changes in tracheal pressure which were registered by the pressure transducer and recorded on the pen recorder, thus enabling assessment of changes in smooth muscle tone of the trachea during mucus collection.

Before the start of an experiment each trachea was allowed to equilibrate for 20 min, and during this time changes of bathing medium were made every 5 min.

Addition of peptides

Five different concentrations of SP or NKA, covering at least a tenfold concentration range, were added to the submucosal buffer bathing the trachea singly and in a random sequence. Each concentration was left in contact with the trachea for 30 min and during this time any increase in smooth muscle tone was recorded. After 30 min the secretion produced was withdrawn and processed as described above. The trachea was then washed twice and fresh buffer containing no peptide was placed in the organ bath. One or two control periods of 30 min were allowed between each drug addition, depending on how quickly the secretion rate returned to its basal level.

After five concentrations of peptide had been added to the trachea, the buffer surrounding the trachea was replaced with buffer containing thiorphan (10 μM). This buffer was left in contact with the trachea for 1 h and was replenished every 30 min. Any change in smooth muscle tone during this period was measured and any mucus produced was withdrawn at the end of 1 h. The same five concentrations of peptide were then added to the trachea in the same sequence as described above. However, this time the peptide was in a buffer containing thiorphan (10 μM). All mucus samples obtained were assayed for lysozyme and albumin.

It is possible that any change in response of the trachea to the peptides after thiorphan may be due to changes in tissue sensitivity which are independent of any effect on enkephalinase. This was tested in separate experiments by using the same procedure as described above except that thiorphan was not

added to the buffer for the second determination of peptide concentration-response curves.

Assay for lysozyme

The lysozyme concentrations of the mucus samples obtained were measured by a turbidimetric assay which relies on the ability of lysozyme to break down the cell wall of the bacterium *Micrococcus lysodeikticus*. Addition of lysozyme to a solution of the bacteria reduces the turbidity of the solution, thereby leading to a fall in optical density (OD) measured at 450 nm.

A stock suspension of *M. lysodeikticus* of 3 mg ml^{-1} was prepared. When diluted 10 fold (the dilution in the assay) this suspension gives an OD of approximately 0.6 at 450 mm. To produce a standard curve, various concentrations of hen egg white lysozyme (0.5 to 100 ng ml^{-1}) were incubated in duplicate in 1.5 ml potassium phosphate buffer (50 mM, pH 7.4) containing *M. lysodeikticus* (0.3 mg ml^{-1}), sodium azide (1 mg ml^{-1}) and bovine serum albumin (BSA, 1 mg ml^{-1}). The BSA was included in the assay for its protein stabilizing effects and the sodium azide was added to prevent the growth of bacteria in the incubating solutions. The reaction mixtures were incubated for 18 h at 37°C. After incubation the OD of each solution was measured at a wavelength of 450 nm with potassium phosphate buffer pH 7.4 containing BSA (1 mg ml^{-1}) as a blank. The standard curve was constructed by plotting the fall in OD (reduction in turbidity) against the concentration of lysozyme in the solution.

To estimate the concentration of lysozyme in a mucus sample, 20 μl of sample was incubated in 1.5 ml potassium phosphate buffer (50 mM), pH 7.4), exactly as described above for the known concentrations of lysozyme used in the preparation of the standard curve. The lysozyme concentrations (equivalent to hen egg white lysozyme) of the 20 μl samples and hence of the original mucus samples were estimated from the standard curve. The rate of output of lysozyme was then calculated by dividing the total amount of lysozyme in a mucus sample by the time over which the sample was accumulated.

Albumin transport

To examine the effect of SP and NKA on the transport of bovine serum albumin (BSA) across the ferret trachea, BSA was added to the buffer bathing the submucosal surface of the trachea in a concentration of 4 mg ml^{-1}. Fluorescent BSA (0.02–0.03 mg ml^{-1}) was also added to the buffer as a marker and enabled an estimate to be made of the total amount of albumin which appeared in the mucus samples.

The fluorescence of the mucus samples was measured with a fluorimeter, using an excitation wavelength of 550 nm and an emission wavelength of 490 nm. The fluorescent albumin concentration of the mucus samples was estimated from a standard curve relating fluorescence (arbitrary units) to the concentration of fluorescent albumin (range 25 ng ml^{-1} to 3 μg ml^{-1}). The total concentration of albumin in the mucus samples was obtained by multiplying the fluorescent albumin concentration (estimated from the standard curve) by the ratio of non-fluorescent to fluorescent albumin used in the experiment. The rate of output of albumin was determined by dividing the total amount of albumin in a mucus sample by the time over which that sample accumulated.

Intraluminal fluorescent albumin concentration might not reflect that of fluorescent albumin in the organ bath since the fluorescent label might be split off or transferred. Therefore, as described elsewhere (Webber & Widdicombe, 1989), nine mucus samples selected randomly were analysed for albumin using affinity chromatography. Linear regression analysis showed a highly significant correlation between the concentration of albumin using this method and the concentration calculated for the same samples by fluorescence measurements (slope = 1.1, $r = 0.97$, $P < 0.01$). This suggested that the fluorescent label was still attached to the albumin and that the fluorescence of the mucus samples provided an accurate indication of the total albumin concentration.

Analysis of results

The concentration-response curves presented in Results were obtained by pooling the results from experiments with a single peptide. The EC_{50} values and maximum responses (±95% confidence limits) were estimated from fitting concentration-response curves to the data points by a computerised, non-linear, least squares estimate. EC_{50} is the concentration of peptide which produced a response equal to 50% of the maximum obtained response to that peptide.

Differences between EC_{50} values or maximum responses obtained in the absence and presence of thiorphan were analysed for statistical significance using Student's unpaired *t* test.

Results

Effects of thiorphan

Thiorphan (10 μM) had no significant effect on mucus volume output (0.12 ± 0.06 μl min^{-1} compared to control immediately before thiorphan of

$0.09 \pm 0.05\,\mu l\,min^{-1}$, $n = 12$), lysozyme output ($28 \pm 6\,ng\,min^{-1}$ compared to $19 \pm 9\,ng\,min^{-1}$, $n = 12$), albumin output ($2.9 \pm 0.9\,\mu g\,min^{-1}$ compared to $2.7 \pm 0.6\,\mu g\,min^{-1}$, $n = 12$), albumin concentration ($3.6 \pm 0.8\,\mu g\,\mu l^{-1}$ compared to $3.7 \pm 0.4\,\mu g\,\mu l^{-1}$, $n = 12$) or intraluminal tracheal pressure ($+2.7 \pm 2.2\,mmH_2O$, $n = 12$).

Effects of substance P and neurokinin A

Intraluminal pressure SP (0.01 to 3 μM) and NKA (0.01 to 3 μM) produced concentration-dependent increases in intraluminal tracheal pressure. These concentration-response curves were shifted to the left by thiorphan (10 μM) (Figure 1a and b) with significantly increased maximum responses (47 and 94% for SP and NKA respectively) and decreased EC_{50} values (10 fold for SP and 15 fold for NKA, Table 1).

Mucus volume output The mean mucus volume output in control periods before addition of SP was $0.08 \pm 0.05\,\mu l\,min^{-1}$ ($n = 28$) and in control periods before NKA was $0.07 \pm 0.04\,\mu l\,min^{-1}$ ($n = 28$). In the absence of the enkephalinase inhibitor thiorphan, SP (0.01 to 3 μM) and NKA (0.03 to 10 μM) produced small concentration-dependent increases in mucus volume output (Figure 2a and b). The concentration-response curves for SP and NKA-induced mucus volume output were shifted to the left by thiorphan (Figure 2a and b) with significantly increased maximum responses (133 and 240% for SP and NKA respectively) and decreased EC_{50} values (13 fold for SP and 2 fold for NKA, Table 1).

Lysozyme output The mean rate of output of lysozyme in control periods before SP was $32 \pm 5\,ng\,min^{-1}$ ($n = 28$) and in controls before NKA was $27 \pm 6\,ng\,min^{-1}$ ($n = 28$). SP (0.01 to 3 μM) and NKA (0.1 to 10 μM) produced small

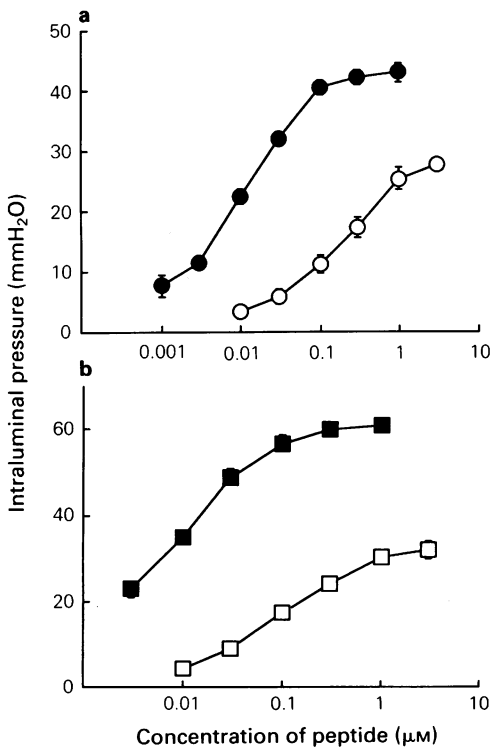

Figure 1 The effect of thiorphan (10 μM) on substance P (SP) and neurokinin A (NKA)-induced contractions of the ferret *in vitro* trachea (intraluminal pressure). Concentration-response curves in (a) are for SP alone (○) and SP in the presence of thiorphan (●), and in (b) are for NKA alone (□) and NKA in the presence of thiorphan (■). All points are means of six determinations with s.e.means shown as vertical bars.

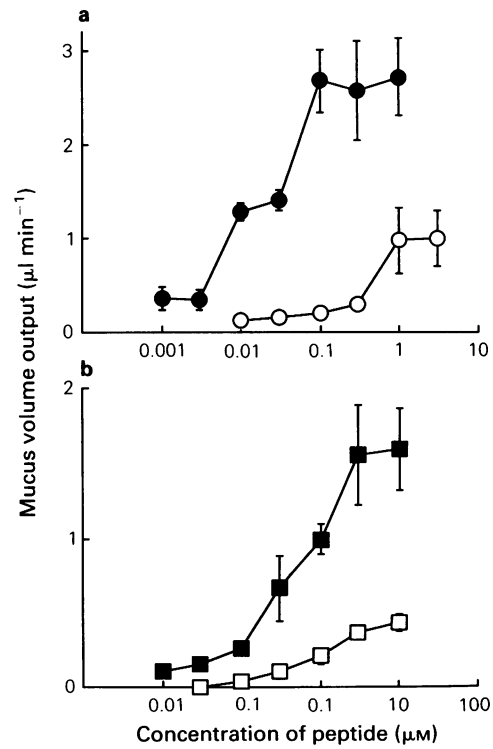

Figure 2 The effect of thiorphan (10 μM) on the mucus volume output from the ferret trachea produced by substance P (SP) and neurokinin A (NKA). Concentration-response curves in (a) are for SP alone (○) and SP in the presence of thiorphan (●), and in (b) are for NKA alone (□) and NKA in the presence of thiorphan (■). All points are means of six determinations with s.e.means shown as vertical bars.

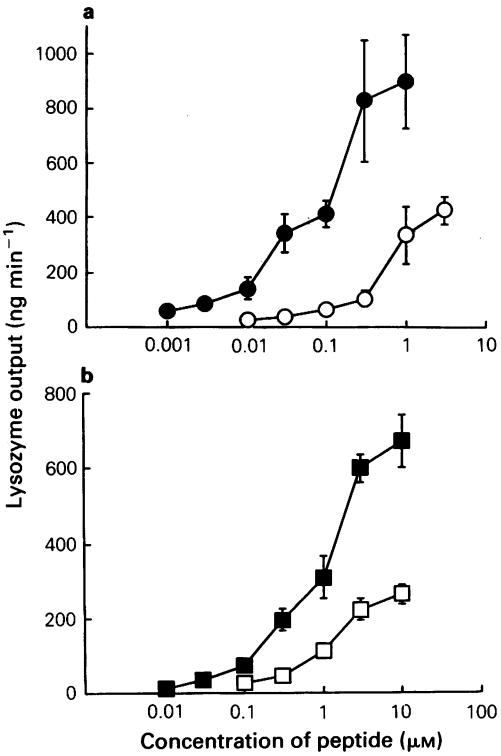

Figure 3 The effect of thiorphan (10 μM) on the rate of output of lysozyme from the ferret trachea produced by substance P (SP) and neurokinin A (NKA). Concentration-response curves in (a) are for SP alone (○) and SP in the presence of thiorphan (●), and in (b) are for NKA alone (□) and NKA in the presence of thiorphan (■). All points are the means of six determinations with s.e.means shown as vertical bars.

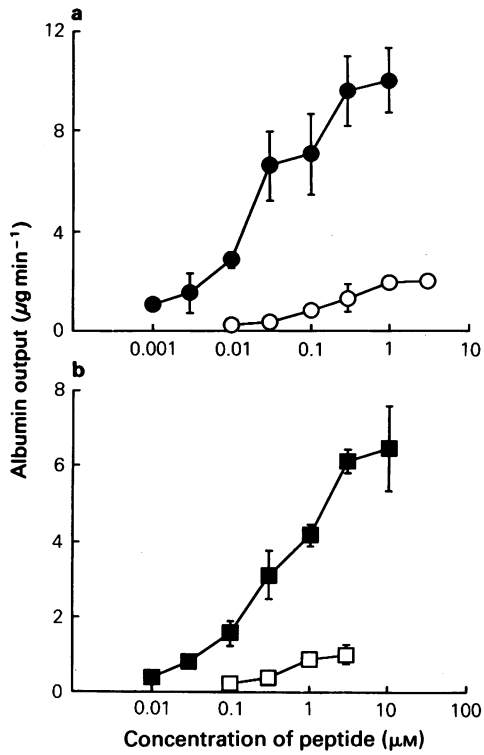

Figure 4 The effect of thiorphan (10 μM) on the rate of output of albumin from the ferret trachea produced by substance P (SP) and neurokinin A (NKA). Concentration-response curves in (a) are for SP alone (○) and SP in the presence of thiorphan (●), and in (b) are for NKA alone (□) and NKA in the presence of thiorphan (■). All points are the means of six determinations with s.e.means shown as vertical bars.

concentration-dependent increases in lysozyme output. The concentration-response curves for SP and NKA-induced lysozyme outputs were shifted to the left by thiorphan (Figure 3a and b) with significantly increased maximum responses (89 and 120% for SP and NKA respectively) and decreased EC_{50} values (eight fold for SP and three fold for NKA, Table 1).

Albumin output and concentration The mean rate of output of albumin in controls before SP was $0.23 \pm 0.06\,\mu g\,min^{-1}$ ($n = 28$) and in controls before NKA was $0.27 \pm 0.09\,\mu g\,min^{-1}$ ($n = 28$). In the absence of thiorphan, SP (0.01 to 3 μM) and NKA (0.1 to 3 μM) produced small, concentration-dependent increases in albumin output (Figure 4a and b). The concentration-response curves for SP and NKA-induced albumin output were shifted to the left by thiorphan (10 μM) (Figure 4a and b) with significantly increased maximum responses (368 and 318% for SP and NKA respectively) and decreased EC_{50} values (ten fold for SP and three fold for NKA, Table 1).

In the absence of thiorphan, the mean concentration of albumin in the mucus collected during control periods before addition of SP (3 μM) was $2.8 \pm 0.6\,\mu g\,\mu l^{-1}$ ($n = 4$). This albumin concentration was not significantly changed ($3.1 \pm 0.5\,\mu g\,\mu l^{-1}$) by addition of SP (3 μM). Similarly in controls before NKA (3 μM) the concentration of albumin was $3.4 \pm 0.7\,\mu g\,\mu l^{-1}$ ($n = 4$), which was not significantly changed ($3.7 \pm 0.4\,\mu g\,\mu l$) by NKA (3 μM).

In contrast, in the presence of thiorphan (10 μM) the concentration of albumin in control periods before SP (1 μM) was $3.2 \pm 0.6\,\mu g\,\mu l^{-1}$; this albumin concentration was significantly increased to

Table 1 EC_{50} values and maximum responses for the effects of substance P (SP) and neurokinin A (NKA) in the presence and absence of thiorphan (10 μM), on intraluminal pressure and mucus volume, lysozyme and albumin outputs from the ferret trachea

	Substance P				*Neurokinin A*			
	EC_{50} (μM)		*Maximum*		*EC_{50} (μM)*		*Maximum*	
	Control	*+ Thiorphan*	*Control*	*+ Thiorphan*	*Control*	*+ Thiorphan*	*Control*	*+ Thiorphan*
Mucus volume (μl min^{-1})	0.4 (0.3–0.6)	0.03 (0.01–0.05)*	1.2 (1.0–1.4)	2.8 (2.7–2.9)*	1.0 (0.7–1.3)	0.5 (0.3–0.8)*	0.5 (0.4–0.6)	1.7 (1.5–1.9)*
Lysozyme (ng min^{-1})	0.6 (0.4–1.0)	0.08 (0.06–0.1)*	530 (500–560)	1000 (850–1150)*	2.0 (1.6–2.4)	0.8 (0.6–1.0)*	350 (310–390)	770 (745–795)*
Albumin (μg min^{-1}	0.2 (0.1–0.4)	0.02 (0.008–0.05)*	2.2 (2.0–2.4)	10.3 (10.1–10.5)*	1.3 (1.0–1.6)	0.5 (0.3–0.7)*	1.7 (1.6–1.8)	7.1 (6.8–7.4)*
Luminal pressure (mmH_2O)	0.2 (0.08–0.5)	0.02 (0.008–0.05)*	30 (26–34)	44 (40–48)*	0.06 (0.04–0.1)	0.004 (0.002–0.008)*	32 (28–36)	62 (57–67)*

The 95% confidence limits for the EC_{50} values and maximum responses are shown in parentheses. Control EC_{50} values and maximum responses are those obtained in the absence of thiorphan.

* Significantly different ($P < 0.05$) from the control EC_{50} or maximum response.

Table 2 EC_{50} values and maximum responses for consecutive concentration-response curves for substance P (SP) and neurokinin A (NKA)-induced increases in intraluminal pressure, and mucus volume, lysozyme and albumin outputs from the ferret trachea

	Substance P				*Neurokinin A*			
	EC_{50}^1 (μM)	*EC_{50}^2 (μM)*	*Maximum^1*	*Maximum^2*	*EC_{50}^1 (μM)*	*EC_{50}^2 (μM)*	*Maximum^1*	*Maximum^2*
Mucus volume (μl min^{-1})	0.4 (0.2–0.6)	0.4 (0.3–0.4)	1.5 (1.2–1.8)	1.6 (1.4–1.8)	1.2 (0.9–1.5)	1.3 (1.0–1.6)	0.4 (0.3–0.5)	0.4 (0.3–0.5)
Lysozyme (ng min^{-1})	0.7 (0.4–1.0)	0.6 (0.4–0.8)	510 (500–520)	550 (520–580)	1.7 (1.4–2.0)	1.6 (1.4–1.8)	300 (280–320)	330 (300–360)
Albumin (μg min^{-1})	0.4 (0.2–0.6)	0.5 (0.3–0.7)	1.7 (1.2–2.2)	1.9 (1.6–2.2)	1.8 (1.6–2.0)	1.9 (1.7–2.1)	1.9 (1.7–2.1)	1.6 (1.3–1.9)
Luminal pressure (mmH_2O)	0.3 (0.1–0.5)	0.4 (0.2–0.6)	35 (28–42)	33 (30–36)	0.08 (0.06–1.0)	0.08 (0.06–1.0)	27 (24–30)	29 (24–39)

EC_{50}^1 and maximum^1 refer to the first curve and EC_{50}^2 and maximum^2 to the second curve. The 95% confidence limits for EC_{50} values and maximum responses are shown in parentheses.

$8.9 \pm 2.1\,\mu g\,\mu l^{-1}$ by SP (1 μM). Similarly, the albumin concentration in controls before NKA (10 μM) was $2.9 \pm 0.7\,\mu g\,\mu l^{-1}$, and this was increased to $7.2 \pm 2.1\,\mu g\,\mu l^{-1}$ by NKA (10 μM).

The concentration of albumin in the submucosal buffer was $4.2 \pm 0.6\,\mu g\,\mu l^{-1}$ ($n = 8$).

Comparison of potencies of substance P and neurokinin A

The EC_{50} values and maximum responses for SP and NKA-induced mucus volume, lysozyme and albumin output and for smooth muscle contraction in the presence and absence of thiorphan (10 μM) are shown in Table 1. The EC_{50} for NKA-induced smooth muscle contraction (0.004 μM) in the presence of thiorphan is significantly lower than that for SP (0.02 μM). In contrast the EC_{50} values for the NKA-induced mucus volume (0.5 μM), lysozyme (0.8 μM) and albumin outputs (0.5 μM) in the presence of thiorphan are significantly higher than those for SP (0.03, 0.08 and 0.02 μM respectively).

Consecutive concentration-response curves for substance P and neurokinin A Table 2 shows the EC_{50} values and maximum responses obtained for SP and NKA-induced mucus volume, lysozyme and albumin outputs, and for smooth muscle contraction when two concentration-response curves were constructed for a single peptide consecutively. There are no significant differences between the EC_{50} values or maximum responses for SP or NKA, obtained from the first or second concentration-response curves (Table 2).

Discussion

SP and NKA dose-dependently increased intraluminal tracheal pressure suggesting contraction of the tracheal smooth muscle. These tachykinins contract tracheal and bronchial smooth muscle *in vitro* and *in vivo* in a variety of other species including man (Lundberg & Saria, 1987). The contractile effects of SP and NKA on the ferret trachea were greatly potentiated by the enkephalinase inhibitor thiorphan with increases in potency and maximum response. Previously, thiorphan was shown to increase the potency and maximum responses of SP and NKA on human (Black *et al.*, 1988) and ferret (Sekizawa *et al.*, 1987) airway smooth muscle *in vitro*, and on guinea-pig airways *in vivo* (Dusser *et al.*, 1988). Since the tachykinins were administered to the submucosal side of the trachea in the present study, it seems likely that there are enkephalinases present within the smooth muscle which, in the absence of thiorphan, break down the tachykinins thereby reducing their effect. Indeed, enkephalinase activity has been identified in ferret tracheal smooth muscle (Sekizawa *et al.*, 1987).

In the absence of thiorphan, SP and NKA produced small increases in mucus volume from the ferret trachea. In contrast, SP is a potent stimulant of glycoprotein secretion from canine tracheal explants (Coles *et al.*, 1984) and of fucose (a marker for mucus glycoprotein) output from isolated human bronchi (Barnes *et al.*, 1986), both effects being obtained in the absence of enkephalinase inhibitors. It is not clear why, in the absence of thiorphan, SP is a weak secretagogue in the ferret but a potent secretagogue in dog and human airways. The most likely explanation is a species variation in the amount of enkephalinase present in *in vitro* airway preparations. Both the potency and maximum responses for SP and NKA-induced secretion of mucus from the ferret trachea were enhanced by addition of thiorphan. Similarly, thiorphan potentiated the SP-induced release of macromolecules from explants of ferret trachea (Borson *et al.*, 1987). Enkephalinases have been identified in the submucosal glands of the ferret trachea by immunocytochemical (Sekizawa *et al.*, 1987) and biochemical (Borson *et al.*, 1986) methods.

SP and NKA may increase mucus output by acting on specific receptors on the myoepithelial cells of the submucosal glands. Contraction of these cells would squeeze existing mucus out of the secretory tubules and gland ducts onto the airway surface (Shimura *et al.*, 1987). Alternatively SP and NKA may interact with receptors on the submucosal glands to promote cellular secretion by an exocytotic mechanism (Gashi *et al.*, 1986). We cannot exclude either of these mechanisms in the present study and both may be occurring. If the tachykinins were working only by contracting myoepithelial cells there should be a rapid tachyphylaxis of SP and NKA-induced mucus volume output, however this was not observed in the present study.

At least part of the mucus volume increase produced by SP and NKA in the present study must come from serous cells in the submucosal glands, since the tachykinins also stimulated the output of lysozyme, which is a specific marker for serous cell secretion (Tom-Moy *et al.*, 1983). Like mucus volume, SP-, and NKA-induced lysozyme output was weak in the absence of thiorphan but was greatly potentiated by pretreatment with the enkephalinase inhibitor. In contrast, SP potently degranulates serous cells in submucosal glands of ferret tracheal explants (Gashi *et al.*, 1986). As with mucus volume this difference probably occurs because of varying amounts of enkephalinase in the two preparations.

Albumin is actively transported across the ferret

trachea *in vitro* from the submucosal side into the lumen (Webber & Widdicombe, 1989). Further evidence suggests this active transport occurs across the tracheal epithelium (Price *et al.*, 1989). In the present study SP and NKA produced small increases in albumin output from the trachea in the absence of thiorphan. However, there was no change in the albumin concentration of the secreted mucus compared to control values, even at the highest concentrations of SP and NKA. After thiorphan pretreatment SP and NKA were potent stimulators of albumin output into the tracheal lumen. Furthermore, the peptides also increased the albumin concentration of the secreted mucus to a level which exceeded that in the submucosal buffer. Thus SP and NKA are not only potent stimulants of albumin transport across the ferret tracheal epithelium but can stimulate this transport to occur against a concentration gradient. Previously β-agonists (Webber & Widdicombe, 1989) and prostaglandin E_1 (Deffebach *et al.*, 1989) have been shown to have a similar action. The increase in responsiveness of albumin transport to SP and NKA after thiorphan suggests that tachykinin breakdown by enkephalinase is being prevented whilst the peptides cross the tracheal mucosa from the submucosal buffer to the epithelium. This breakdown may be prevented at any site within the trachea since enkephalinases are not only present in ferret tracheal smooth muscle and submucosal glands but are also in the epithelium (Sekizawa *et al.*, 1987). SP and NKA also stimulate chloride transport across the canine tracheal epithelium (Al-Bazzaz *et al.*, 1985; Tamaoki *et al.*, 1988) and this transport may be linked with albumin transport, although this has not been investigated in the present study.

It is possible that the changes in the response of the trachea to SP and NKA with thiorphan may be due to changes in tissue sensitivity with time and not due to any effect of thiorphan. However, this seems unlikely since concentration-response curves to SP or NKA constructed consecutively with no thiorphan present produced near-identical EC_{50} values and maximum responses (Table 2).

There are at least three different receptors mediating the actions of the tachykinins. These receptors were characterised using biological (Regoli *et al.*, 1987) and binding assays (Lee *et al.*, 1986) and the classification is based on agonist potency ratios. Thus, NK_1-receptors are found in tissues where SP is more potent than NKA or NKB. In contrast NK_2-receptors occur where the potency order is NKA > NKB > SP and NK_3-receptors are found when the potency order is NKB > NKA > SP.

In the present study NKA was approximately 5 times more potent than SP at contracting the ferret tracheal smooth muscle in the presence of thiorphan (Table 1). Similarly, NKA is more potent than SP at contracting guinea-pig trachea (Uchida *et al.*, 1987; Advenier *et al.*, 1987) and human bronchi (Martling *et al.*, 1987; Advenier *et al.*, 1987) *in vitro* and at contracting guinea-pig airways *in vivo* (Manzini *et al.*, 1988). Therefore, it is probable that NKA and SP contract both tracheal and bronchial smooth muscle from several species by an action on the NK_2-receptor, although since NKB was not used in several of these studies, including the present one, NK_3-receptors cannot be ruled out.

In contrast to the action of the tachykinins on airway smooth muscle, SP was approximately 20 and 10 times more potent than NKA at stimulating mucus volume and lysozyme output from the ferret trachea (Table 1). These results are consistent with the tachykinins stimulating NK_1-receptors on the submucosal glands to produce an increase in the volume of secreted mucus part of which is coming from serous cells. There is little other information concerning the receptors mediating the effects of tachykinins on mucus secretion in the airways. However, SP is more potent than NKA at increasing microvascular leakage within guinea-pig airways (Rogers *et al.*, 1988) and is more potent than NKA at increasing conductance across canine airway epithelium (Rangachari & McWade, 1985). Therefore, effects of tachykinins on the airways apart from smooth muscle contraction but including mucus secretion are probably mediated through NK_1-receptors. Furthermore, SP is more potent than NKA at stimulating salivation in the anaesthetized guinea-pig (Manzini *et al.*, 1988), so secretory actions of the tachykinins other than mucus secretion are also probably NK_1-receptor mediated. In addition, in the present study, SP was 25 times more potent than NKA at stimulating albumin transport across the epithelium. This is consistent with the NK_1-receptor mediated action of the tachykinins on ion-transport across canine tracheal epithelium (Rangachari & McWade, 1985).

In summary, SP and NKA contract ferret tracheal smooth muscle and increase mucus volume, lysozyme output and albumin transport across the epithelium. All of the actions of SP and NKA are greatly potentiated by thiorphan suggesting inhibition of tachykinin breakdown by enkephalinase enzymes within the trachea. The SP- and NKA-induced muscle contractions are probably mediated by NK_2-receptors whereas the other actions of the tachykinins on the trachea are probably mediated by NK_1-receptors.

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EDRF-mediated dilatation in the rat isolated perfused kidney: a microangiographic study

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1 X-ray microangiographic techniques were used to study the influence of endothelium-derived relaxing factor (EDRF) on vasomotion in the isolated, intact, buffer-perfused kidney of the rat. The main renal (R_0), segmental (R_1) and interlobar (R_2) arteries (control diameters ca. 600, 400 and 300 μm respectively) were studied quantitatively.

2 Inhibition of basal EDRF activity by haemoglobin (1 μM) did not elevate perfusion pressure or constrict R_0, R_1 and R_2 in control preparations, implying a low level of spontaneous myogenic tone. In preparations preconstricted by 0.3 μM methoxamine, haemoglobin caused a further rise in perfusion pressure and amplified constrictor responses in R_1 and R_2 while also inducing 'paradoxical' dilatation of R_0.

3 A spatially heterogeneous pattern of diameter responses (constriction of R_2 and R_1 with minimal dilatation of R_0) was observed with two concentrations of methoxamine (0.3 μM and 3 μM). The magnitude of these responses was, however, smaller with 3 μM than 0.3 μM methoxamine, even though it increased perfusion pressure to a greater extent (88 mmHg cf. 24 mmHg). This 'paradoxical' behaviour indicates more pronounced constriction of distal arteries (which could not be resolved quantitatively) with 3 μM methoxamine.

4 In contrast to the heterogeneity of constrictor responses induced by methoxamine, the dilator action of acetylcholine was spatially homogeneous: log IC_{50} values calculated from the diameter changes induced in R_0, R_1 and R_2 were similar and, moreover, equivalent to that calculated from the corresponding alterations in perfusion pressure. The fall in perfusion pressure induced by an approximately median effective concentration of acetylcholine (0.3 μM) was completely reversed by haemoglobin, consistent with the involvement of EDRF, although, reversal of the acetylcholine-induced dilatation of R_0, R_1 and R_2 was not observed.

5 The results are consistent with the idea that constriction of distal vessels can attenuate and even directionally reverse intrinsic constrictor responses in the proximal R_0, R_1 and R_2 'feed' arteries by producing an overriding increase in 'upstream' pressure. This effect explains the paradoxical dilatation of R_0 induced by haemoglobin in the presence of 0.3 μM methoxamine, the smaller magnitude of the diameter changes induced in R_0, R_1 and R_2 by 3 μM as compared to 0.3 μM methoxamine, and the failure of haemoglobin to reverse the acetylcholine-induced dilatation of R_0, R_1 and R_2.

Introduction

The endothelium-dependent relaxation of arterial smooth muscle which can be induced by a wide variety of compounds such as acetylcholine (ACh), is mediated by stimulated release of endothelium-derived relaxing factor (EDRF). EDRF is an unstable endogenous vasodilator (Furchgott & Zawadski, 1980; Griffith *et al.*, 1984), identical with or closely related to nitric oxide (Ignarro *et al.*, 1987; Palmer *et al.*, 1987; Furchgott, 1988), and is avidly bound by haemoglobin (Kelm *et al.*, 1988). Endothelium-dependent relaxation has been demonstrated widely in large arteries but the role of EDRF in intact organs remains largely uninvestigated.

We have recently shown that haemoglobin inhibits ACh-induced relaxation in resistance vessels of the rabbit buffer-perfused isolated ear *in situ* (Griffith

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et al., 1988). Similar conclusions have been reached by other workers on rabbit heart (Stewart et al., 1987) and rat mesenteric (Furchgott et al., 1987) preparations. This suggests that the dilator action of ACh in resistance vessels, as in large vessels, is mediated by stimulated release of EDRF. Indeed, when endothelium is removed mechanically from isolated segments of resistance arteries (De Mey & Gray, 1985; Owen & Bevan, 1985) the dilator response to ACh is abolished. Basal release of EDRF (i.e. spontaneous release in the absence of any exogeneous pharmacological agent) also exerts a major effect on pressure-flow and diameter-flow relationships in the vascular network of the rabbit ear (Griffith et al., 1987; 1989) and the rat mesentery (Randall & Hiley, 1988).

In the present study we have investigated the influence of haemoglobin on baseline perfusion pressure, constrictor responses induced by the α_1-adrenoceptor agonist methoxamine, and dilator responses induced by ACh in the rat isolated perfused kidney. Microangiographic techniques (Griffith et al., 1987; 1988; 1989) were used to measure diameter changes in the three primary sets of arteries of the rat kidney (down to approximately 200 μm in size). In combination with simultaneous measurement of pressure changes, this information allows inferences to be made about the responses of more distal vessels which cannot be visualized directly in this preparation by microangiography.

Methods

Preparation of the isolated perfused kidney of the rat

Male Wistar rats (U.W.C.M., Cardiff) weighing between 250–300 g were anaesthetized with chloroform, the thorax was opened and heparin was injected into the left ventricle (0.3 ml, 20,000 units ml^{-1}), following which the animals were killed by exsanguination. The left kidney was then removed after cannulation of the renal artery, and perfused with Krebs-Henseleit solution (composition in mM: Na^+ 143, K^+ 5.9, Ca^{2+} 2.6, Mg^{2+} 1.2, Cl^- 128, $H_2PO_4^-$ 2.2, HCO_3^- 24.9, SO_4^{2-} 1.2, glucose 10) oxygenated with 95% O_2/5% CO_2 at a constant flow rate of 2.25 ml min^{-1}.

The kidneys were mounted in front of a 4 μm microfocal X-ray source in such a manner that the effluent from the renal vein would not flow over the surface of the kidney and thus obscure the radiographic images. Microangiographs were obtained with an exposure time of 8 to 14 s at a kilovoltage of between 30 and 55 kV. The radiographic contrast material used was Omnipaque (iohexol, 300 mg iodine ml^{-1}), which caused a small rise in perfusion pressure attributable to its greater viscosity than Krebs-Henseleit solution. However, these rises were less than 5% of mean perfusion pressure during the X-ray exposure. Iohexol was introduced into the kidneys for 8 s before the radiographic exposure to ensure optimal opacification of arteries with minimal filling of veins and thus reduce problems due to vessel overlap on the radiographic images. At the conclusion of each experiment, Micropaque ($BaSO_4$ suspension) was injected into each kidney to provide a high-contrast topographical 'map' of its arterial system (Figure 1).

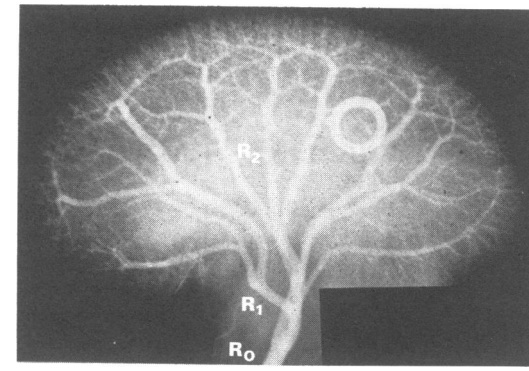

Figure 1 High contrast topographical 'map' of the arterial system of rat isolated kidney obtained following injection of a $BaSO_4$ suspension (Micropaque). Diameter measurements were made of the main renal artery R_0, the segmental, R_1 and the interlobar arteries, R_2. The calibrated grid had an external diameter of 3 mm.

Quantitation

The microangiographs were analysed by a semi-interactive image analysis system as previously described (Griffith et al., 1987; 1988). A calibrated gold grid was placed on the surface of each kidney to determine magnification factors (approximately $\times 3$) accurately. The radiographs were further magnified electronically by the image analysis system. The diameter of each vessel studied was measured 6 times and the results averaged. The accuracy of this protocol has been shown to be better than 5% (Griffith et al., 1988). Quantitation of diameters was possible only for the main renal (R_0), segmental (R_1) and interlobar (R_2) arteries. In the case of the R_1 and R_2 arteries, three or four vessels were measured in each kidney and the results averaged to give a single representative value. Although the arcuate arteries could be visualised in some experiments, accurate measurement was not generally possible. As previously shown in the rabbit ear, intermittent expo-

sure to radiographic contrast medium did not impair endothelium-dependent relaxation or constrictor responsiveness (Griffith *et al.*, 1988).

Experimental protocol

Following equilibration, changes in the diameters of R_0, R_1 and R_2 were investigated after administration of 1 μM haemoglobin to (1) resting preparations, (2) preparations constricted by the α_1-adrenoceptor agonist methoxamine (0.3 μM) and (3) preparations dilated by 0.3 μM ACh after preconstriction by methoxamine (3 μM). In addition, diameter changes induced by cumulative addition of ACh (0.03–30 μM) to methoxamine (3 μM)-constricted preparations were studied. In all cases microangiographs were obtained following stabilization of perfusion pressure after each pharmacological intervention.

Drugs

Acetylcholine chloride was obtained from Sigma Chemical Co., Poole, UK. Methoxamine hydrochloride was a gift from the Wellcome Foundation Ltd, UK, iohexol was obtained from Nyegaard, UK, and Micropaque was obtained from Nicholas, UK. Haemoglobin, which is a specific inhibitor of EDRF activity in peripheral arteries (Martin *et al.*, 1985; Edwards *et al.*, 1986; Nishiye *et al.*, 1989), was prepared following lysis of red blood cells and purified by gel filtration on a Sephadex G200 column as previously described (Edwards *et al.*, 1986).

Statistical analysis

Data are given as mean ± s.e.mean. Logarithmic concentration-response curves to ACh were plotted in each preparation where response was (a) the fall in pressure across the whole kidney and (b) the increase in diameter of each of the three generations of arteries R_0, R_1 and R_2. IC_{50} values were determined graphically in each case for the individual preparations and geometric mean IC_{50} values of the various data groups calculated and compared by one-way analysis of variance. The effect of haemoglobin on basal perfusion pressure and constrictor or dilator responses in each generation of artery was assessed by paired Student's t test. $P < 0.05$ was taken as significant.

Results

Effects of haemoglobin in control preparations

Administration of 1 μM haemoglobin produced no significant change in baseline perfusion pressure (95 ± 8 cf. 95 ± 10 mmHg, $n = 9$). Consistently, there was no significant change in the diameters of the R_0, R_1 and R_2 arteries (Figure 2, left).

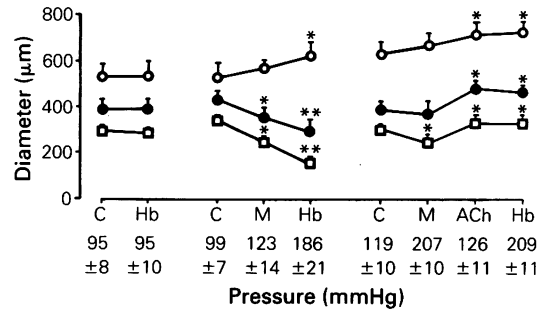

Figure 2 Steady-state diameters (in μm) of R_0 (○), R_1 (●) and R_2 (□) and corresponding perfusion pressures after administration of 1 μM haemoglobin (Hb) ($n = 9$, left), 0.3 μM methoxamine (M) followed by 1 μM haemoglobin ($n = 10$, centre) and 3 μM methoxamine followed by 0.3 μM acetylcholine (ACh) and then 1 μM haemoglobin ($n = 11$, right). Significant differences from control diameter are indicated (* $P < 0.05$; ** $P < 0.01$). Statistically, haemoglobin alone had no effect on the diameters of R_0, R_1 and R_2; 0.3 μM methoxamine constricted R_1 and R_2 while 3 μM methoxamine constricted only R_2. Methoxamine nevertheless increased steady-state perfusion pressure in a concentration-dependent fashion. When administered after 0.3 μM methoxamine, haemoglobin increased the constriction of R_1 and R_2 but, 'paradoxically', resulted in dilatation of R_0 (centre). Haemoglobin reversed the fall in perfusion pressure induced by 0.3 μM ACh but did not significantly alter the dilatation of R_0, R_1 and R_2 induced by ACh (right).

Effects of methoxamine

Generations R_0, R_1 and R_2 responded in a qualitatively similar way to the two concentrations of methoxamine studied. The diameter of the R_0 arteries increased slightly (although not significantly) in the presence of 0.3 μM methoxamine but significant constriction occurred in the R_1 and R_2 arteries ($P < 0.05$, $n = 10$) (Figure 2, centre). In the presence of higher concentrations of methoxamine (3 μM), the diameter of R_0 increased slightly (although again not significantly) while constriction was observed in R_1 and R_2. However, this was significant only in the case of R_2 ($P < 0.05$, $n = 10$) (Figure 2, right). Quantitatively, therefore, the changes in diameter were less marked with the higher concentration of methoxamine.

By contrast, steady-state perfusion pressure varied in a concentration-dependent manner, increasing by 24% from 99 ± 7 resting to 123 ± 14 mmHg following administration of 0.3 μM methoxamine, and by

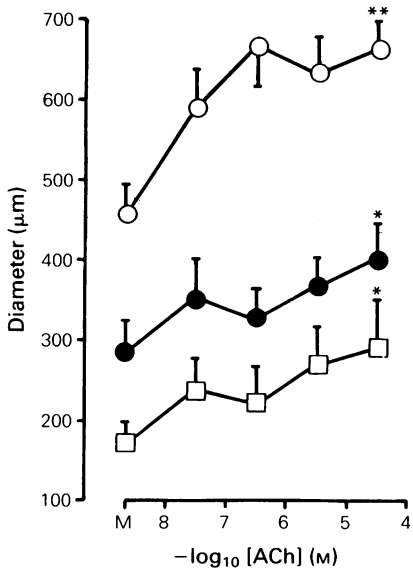

Figure 3 Averaged diameters of generations R_0 (○), R_1 (●) and R_2 (□) (in μm) as a function of acetylcholine (ACh) concentration (0.3–30 μM) in preparations constricted by 3 μM methoxamine (M) ($n = 14$). Microangiographs were taken following stabilization of perfusion pressure after each increase in the concentration of ACh. Error bars reflect both experimental factors and also biological variation in the size of the vessels selected for study. Diameters after addition of the highest concentration of ACh were in each case significantly larger than those obtained in the presence of methoxamine alone (* $P < 0.05$, ** $P < 0.01$).

73% from 119 ± 10 resting to 207 ± 10 mmHg following 3 μM methoxamine (Figure 2).

Effects of haemoglobin in the presence of 0.3 μM methoxamine

Following administration of 1 μM haemoglobin in the presence of 0.3 μM methoxamine, the diameters of R_1 and R_2 further decreased compared to control ($P < 0.01$, $n = 10$), while that of R_0 increased significantly above control ($P < 0.05$, $n = 10$) (Figure 2). Haemoglobin increased steady-state perfusion pressure in the presence of 0.3 μM methoxamine by 51% from 123 ± 14 mmHg to 186 ± 21 mmHg.

Effect of haemoglobin on dilatation induced by an approximately median effective concentration of acetylcholine

Mean perfusion pressure in the presence of 3 μM methoxamine was 207 ± 10 mmHg which fell to 126 ± 11 mmHg in the presence of 0.3 μM ACh and rose to 209 ± 11 mmHg in the presence of 1 μM haemoglobin ($n = 10$) (Figure 2, right). Haemoglobin therefore completely reversed the dilatation induced by the addition of ACh in terms of perfusion pressure. The mean steady-state diameters of the constricted R_0, R_1 and R_2 arteries were significantly increased by 0.3 μM ACh ($P < 0.05$) (Figure 2, right), but in contrast to its effect on perfusion pressure, haemoglobin did not reverse the diameter increases induced by ACh in any of generations R_0, R_1 and R_2.

Concentration-response curves to acetylcholine

Concentration-dependent dilatation was induced by ACh over the range 0.03–30 μM in preparations preconstricted by 3 μM methoxamine ($n = 14$). Log_{10} IC_{50} values calculated from the ACh-induced increases in diameter in R_0, R_1 and R_3 (Figure 3), were statistically similar to each other and equivalent to that calculated from the ACh-induced falls in perfusion pressure (Figure 4) when compared by one-way analysis of variance (Table 1). The diameters of R_0, R_1 and R_2 were increased by ca. 20% of their constricted value by ACh, so that the associated decreases in hydraulic resistance (proportional to 1/(diameter)^4 in Poiseuille flow) would be ca. 80%.

Discussion

We have used X-ray microangiography to study arterial responses in the rat isolated, perfused kidney. To obtain adequate spatial resolution with this technique an X-ray source size of the order of microns must be employed. This constraint necessarily limits the magnitude of the photon flux which can be generated by the microscope and consequently, in a 3-dimensional vascular bed such as the rat kidney, the size of vessel which can be studied quantitatively. The smallest generation of arteries studied here (R_2, diameter ca. 200 μm) is, therefore, significantly larger than in the 2-dimensional bed of the rabbit ear in which vessels of ca. 70 μM diameter can be adequately resolved (Griffith *et al.*, 1988).

Haemoglobin appears to be a specific inhibitor of EDRF activity at the concentration employed (1 μM) (Martin *et al.*, 1985; Edwards *et al.*, 1986; Kelm *et al.*, 1988; Nishiye *et al.*, 1989). The finding that it failed to elevate baseline perfusion pressure or to constrict the main renal (R_0), segmental (R_1) and interlobar (R_2) arteries in unconstricted preparations could indicate, either that basal EDRF activity was negligible, or that spontaneous myogenic tone was low in these rat isolated preparations. The latter, in fact, seems to be the case as haemoglobin elevated perfusion pressure in preparations constricted by

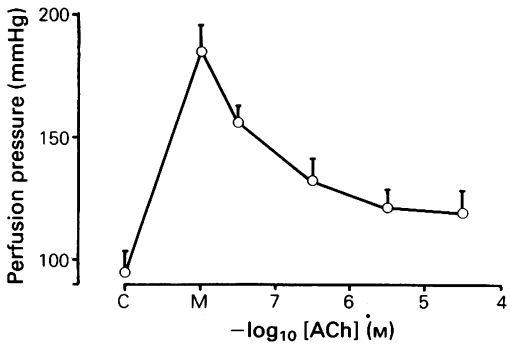

Figure 4 Change in perfusion pressure (mmHg) in rat isolated kidneys constricted by 3 μM methoxamine as a function of acetylcholine concentration (0.3–30 μM) ($n = 14$). The $\log_{10}$ IC_{50} value derived from these data is given in Table 1.

0.3 μM methoxamine, an α_1-agonist which would not be expected to stimulate EDRF release. Although there are considerable species and regional differences in the ability of α_2-adrenoceptors to mediate EDRF release (Cocks & Angus, 1983; Miller & Vanhoutte, 1985; Egleme *et al.*, 1984; Matsuda *et al.*, 1985), there is no evidence that α_1-adrenoceptors can do so in any species or artery type. The lack of effect of haemoglobin in the absence of a constrictor agonist in this preparation differs from previous experiments with the rabbit isolated ear, in which inhibition of basal EDRF activity induced a small but significant rise in perfusion pressure in the absence of exogeneous constrictor agents and in which haemoglobin therefore unmasked myogenic tone (Griffith *et al.*, 1987).

Table 1 Concentrations of acetylcholine resulting in half-maximal dilatation

Generation	$-log_{10}(IC_{50})$
R_0	6.5 ± 0.2
R_1	6.2 ± 0.4
R_2	6.3 ± 0.4
Mean value	6.3 ± 0.1
Pressure response	6.4 ± 0.3

Molar $\log_{10}$ IC_{50} values for inhibition of methoxamine (3 μM)-induced vasoconstriction by acetylcholine. These were calculated separately for the diameter changes induced by acetylcholine in each of the three generations of arteries (i.e. R_0, R_1 and R_2) and for the corresponding falls in perfusion pressure of the intact preparations ($n = 14$). All $\log_{10}$ IC_{50} values were statistically similar.

The tendency of the main renal artery (R_0) to dilate after administration of either 0.3 or 3 μM methoxamine (particularly in the presence of haemoglobin), contrasts with the observation that such vessels constrict in response to this agonist when studied as isolated ring preparations (Winquist, 1988). The lobar (R_1) and segmental (R_2) arteries, by contrast, exhibited the expected constrictor response. Diameter changes induced by 3 μM methoxamine, while being directionally similar, were smaller in magnitude than those induced by 0.3 μM methoxamine, even though the converse was the case in terms of their effects on perfusion pressure. The findings suggest that a rise in 'upstream' intraluminal pressure, secondary to 'downstream' constriction in vessels not visualised by the microangiographic technique, can attenuate intrinsic constrictor responses in the proximal R_1 and R_2 arteries and even reverse those in R_0. The failure of haemoglobin to influence the dilatation of R_0, R_1 and R_2 induced by acetylcholine in preparations constricted by 3 μM methoxamine can be explained by a similar mechanism. Analysis of responses in proximal R_0, R_1 and R_2 'feed' vessels thus allows inferences to be made about the behaviour of the distal vasculature of these rat isolated kidney preparations.

When increasing concentrations of acetylcholine were administered after 3 μM methoxamine, the R_0, R_1 and R_2 arteries dilated in a concentration-dependent fashion, IC_{50} values being similar in the three generations even though the effects of methoxamine were spatially heterogeneous. The fall in perfusion pressure induced by 0.3 μM acetylcholine was completely reversed by haemoglobin, consistent with the involvement of EDRF. Spatial homogeneity in the sensitivity of these different sized vessels to acetylcholine is analogous to findings in the rabbit isolated ear, in which IC_{50} values for acetylcholine and substance P are identical in the central artery and its first three branch generations, in spite of spatial differences in agonist-induced constrictor responses (Griffith *et al.*, 1988). This contrasts with the behaviour of isolated resistance arteries in which the potency of acetylcholine and other endothelium-dependent agents has been shown to increase with diminishing vessel size (De Mey & Gray, 1985; Owen & Bevan, 1985).

In summary, the findings demonstrate that basal EDRF activity exerts a significant influence in this rat isolated kidney preparation only when tone is elevated exogeneously. The results with methoxamine, acetylcholine and haemoglobin also indicate that, because of 'paradoxical' effects, net responses may differ between conventional arterial preparations and intact perfused organ preparations. This is a consequence of the hydraulic connection between vessels in an intact vascular network: the

response of any given artery to a pharmacological intervention will be determined both locally and by the behaviour of distant vessels.

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Potent NMDA-like actions and potentiation of glutamate responses by conformational variants of a glutamate analogue in the rat spinal cord

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1 Neuropharmacological actions of all possible-state isomers of α-(carboxycyclopropyl)glycine (CCG), conformationally restricted analogues of glutamate, were examined for electrophysiological effects in the isolated spinal cord of the newborn rat.

2 Eight CCG stereoisomers demonstrated a large variety of depolarizing activities. Among them, the (2**R**, 3**S**, 4**S**) isomers of CCG (D-CCG-II) showed the most potent depolarizing activity, followed by the (2**S**, 3**R**, 4**S**) isomer (L-CCG-IV).

3 The depolarization evoked by L-CCG-IV, D-CCG-II and other D-CCG isomers was effectively depressed by N-methyl-D-aspartate (NMDA) antagonists. D-CCG-II was about 5 times more potent than NMDA in causing a depolarization.

4 The (2**S**, 3**S**, 4**S**) isomer of CCG (L-CCG-I) was more potent than L-glutamate in causing a depolarization of spinal motoneurones. The depolarization was slightly depressed by NMDA antagonists, but residual amplitudes of responses to L-CCG-I in the presence of NMDA antagonists were almost insensitive to 6,7-dinitro-quinoxaline-2,3-dione (DNQX) or 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), suggesting that L-CCG-I might be a novel potent agonist.

5 After application of the (2**S**, 3**S**, 4**R**) isomer of CCG (L-CCG-III), responses to L-glutamate, D- and L-aspartate were markedly enhanced. The enhancement lasted for a period of several hours without a further application of L-CCG-III.

6 L-CCG-III also caused a depolarization, but it seemed unlikely that the potentiation of the glutamate response was directly related to the depolarization evoked by L-CCG-III.

7 The potentiation might be due to inhibition of uptake processes, but L-CCG-III was superior to L-(−)-*threo*-3-hydroxyaspartate, a potent uptake inhibitor of L-glutamate and L-aspartate, in enhancing the response to L-glutamate in terms of amplitude and duration of responses.

8 CCG isomers should provide useful pharmacological tools for analysis of glutamate neurotransmitter systems.

Introduction

cis- and *trans*-L-α-(carboxycyclopropyl)glycine (CCG) have been isolated from young fruits of the plants, *Aesculus parviflora* and *Blighia sapida* (Fowden *et al.*, 1969). Both natural CCGs cause a depolarization of neurones in the dorsal horn of the rat spinal cord when administered iontophoretically (McLennan *et al.*, 1982). CCG is a conformationally restricted glutamate analogue in which the cyclopropyl group fixes the glutamate chain in an extended or folded form. The glutamate molecule is relatively flexible and so the carbon chain of glutamate is able to conform completely with that of CCG. Thus, it is reasonable to assume that an acyclic transmitter molecule has different conformations and is capable of fitting to the different types of receptors. Therefore, glutamate is supposed to be able to bind to different types of glutamate receptors: kainate-, quisqualate- and N-methyl-D-

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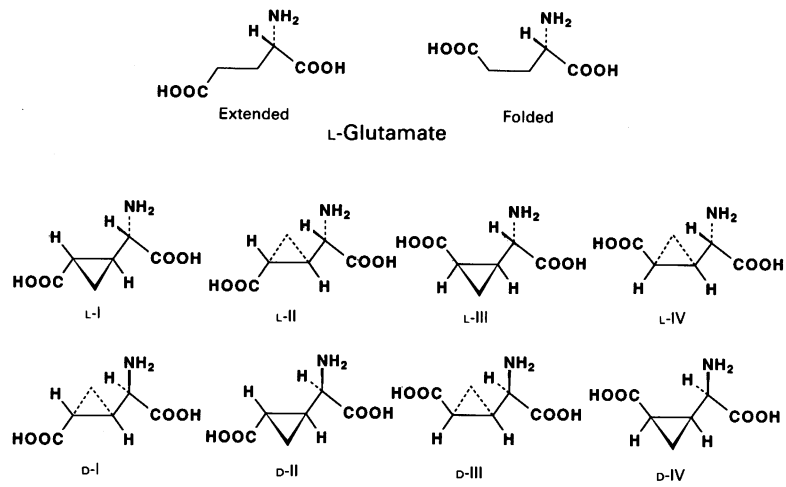

Figure 1 Chemical structures of eight diastereomers of α-(carboxycyclopropyl)glycine (CCG). L-CCG-I and D-CCG-I are abbreviated to L-I and D-I, respectively, and so forth.

aspartate (NMDA)-type receptors. However, the interaction of glutamate with its receptors is not well understood with respect to their preference for particular conformers of the glutamate molecule.

At present, there is no way to determine directly the conformation of transmitter molecules activating their receptors. The only possible technique to analyze the active conformations of transmitter molecules is by chemical modification of agonists and the characterization of the different actions of the various analogues. CCG would be expected to be a good compound for this study. Ohfune and his colleagues (Kurokawa & Ohfune, 1985; Yamanoi *et al.*, 1988) synthesized four isomers of L-CCG expecting that these compounds would provide useful information about the interaction between the conformation of glutamate molecules and activation of glutamate receptor subtypes. In our initial experiments (Shinozaki & Ishida, 1988; Shinozaki *et al.*, 1989), all isomers of L-CCG caused a depolarization of the rat isolated spinal motoneurone with various depolarizing activities. The (2S, 3R, 4S) isomer of L-CCG (L-CCG-IV), a folded form of L-CCG, was the most potent among these isomers, and had a preferred action at the NMDA-type receptor. In addition, the (2S, 3S, 4S) isomer (L-CCG-I), an extended form of L-CCG, was more potent than L-glutamate (Shinozaki & Ishida, 1988; Shinozaki *et al.*, 1989), and L-CCG-I had a preferred action at the non-NMDA-type receptors, although it was much less potent than either kainate or quisqualate. Thus, L-CCG isomers gave us an insight into the conformation of glutamate molecules for activating their receptors.

CCG has eight stereoisomers theoretically, including L- and D-forms (Figure 1). Some of these isomers of CCG would be expected to provide additional information about the interaction of glutamate molecules with excitatory amino acid receptor subtypes, and thus to be potentially valuable compounds for analyzing mechanisms of the physiological effect of glutamate. In experiments in the rat isolated spinal cord, we have now examined the different responses to these CCG isomers arising from their conformational variation. The potent NMDA-like actions, marked potentiation of glutamate responses and a new-type depolarization were observed with some of the compounds and of particular interest.

Methods

The newborn rat spinal cord has been widely used for experiments on the mammalian CNS because of the ease of changing drug concentrations of the bathing solution (Otsuka & Konishi, 1974; Ault *et al.*, 1980; Agrawal & Evans, 1986). The methods used for the electrophysiological experiments in the newborn rat isolated spinal cord (1–5 day-old Wistar rats, $n = 94$) were essentially similar to those described previously (Ishida & Shinozaki, 1988a,

1988b; Shinozaki & Ishida, 1988). Under ether anaesthesia, the lumbar-sacral spinal cord was isolated, hemisected sagittally and placed in a 0.15 ml bath perfused at a fixed flow rate of 5–6 ml min^{-1} with artificial cerebrospinal fluid (composition in mM: NaCl 138.6, KCl 3.4, $CaCl_2$ 1.26, $NaHCO_3$ 21.0, NaH_2PO_4 0.58, glucose 10.0, and tetrodotoxin (TTX) 0.5 μM, pH 7.4) which was oxygenated with a gas mixture of 95% O_2 and 5% CO_2. TTX was added to the bathing solution in order to block spontaneous depolarization and indirect drug effects. In some cases, $MgCl_2$ was added to the perfusing fluid in a concentration of 1.0 mM to examine the depolarizing activity of NMDA-type agonists (Mayer *et al.*, 1984; Nowak *et al.*, 1984). The potential changes generated in the motoneurones were recorded extracellularly from the L_3–L_5 ventral roots with a suction electrode. The tip of the suction electrode was a glass capillary, with an inner diameter that fitted tightly onto the recorded ventral roots in order to enhance the isolation impedance, which severely affected recording of the responses. After selecting only those spinal cord preparations, which demonstrated the polysynaptic reflexes of above 2 mV in amplitude in the Mg^{2+} (1 mM)-containing, TTX-free saline, the perfusing solution was replaced by normal Mg^{2+}-free and TTX-containing solution. Excitatory amino acids and other test compounds were applied to the preparation either by perfusion or by brief pulse injection into the perfusion system. The temperature of the perfusing fluid was kept at 27°C.

Drugs

Eight stereoisomers of α-(carboxycyclopropyl)glycine (CCG) [the (2S, 3S, 4S) isomer (L-CCG-I), the (2S, 3R, 4R) isomer (L-CCG-II), the (2S, 3S, 4R) isomer (L-CCG-III), the (2S, 3R, 4S) isomer (L-CCG-IV), the (2R, 3R, 4R) isomer (D-CCG-I: m.p. 240–242°C, $[\alpha]^{25}D$ −94.7° (c 0.50, H_2O)), the (2R, 3S, 4S) isomer (D-CCG-II: m.p. 254–258°C, $[\alpha]^{25}D$ +21.6° (c 0.50, H_2O)), the (2R, 3R, 4S) isomer (D-CCG-III: m.p. 202–203°C, $[\alpha]^{25}D$ −15.6° (c 0.50, H_2O)) and the (2R, 3S, 4R) isomer (D-CCG-IV: m.p. 172–180°C, $[\alpha]^{25}D$ −86.7° (c 0.50, H_2O))]. D-CCG isomers were newly synthesized according to the previously reported procedure with (2R)-2-amino-3-butenol used as the starting material (Yamanoi *et al.*, 1988). Spectroscopic data of D-CCGs were completely identical with those of the respective corresponding L-CCGs. Enantiometric purity of these compounds found to be >98% was ascertained by high performance liquid chromatography (h.p.l.c.) analysis using an optically active column (Daicel Chemical Industries Ltd., CROWNPACK CR(+), pH 2.0 aqueous $HClO_4$ as an eluent).

The following compounds were used: (±)-2-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA, Tocris), D-(−)-2-amino-5-phosphonovaleric acid (D-APV, Tocris), D- and L-aspartic acid (Nacalai Tesque), 3-[(±)-2-carboxypiperazin-4-yl]propyl-1-phosphonic acid (CPP, Tocris), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, Tocris), L-cysteic acid (Sigma), dihydrokainic acid (Tocris), 6,7-dinitro-quinoxaline-2,3-dione (DNQX, Tocris), D-glutamic acid (Nacalai Tesque), sodium L-glutamate mono (Wako), L-homocysteic acid (Sigma), D-(+)- and L-(−)-*threo*-3-hydroxyaspartic acid (Tocris), L-α-kainic acid (Sigma), N-methyl-D-aspartic acid (NMDA, Sigma), 5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate (MK-801, Merck), picrotoxin (Tokyo Kasei), L-quisqualic acid (purity >99.7%) isolated from the plant, *Quisqualis indica L*, and strychnine hemisulphate salt (Sigma).

Results

Various depolarizing activities of CCGs

When eight CCG stereoisomers used in the present study (see Figure 1) were added to the bathing solution for various times, all CCG isomers demonstrated a depolarization of the spinal motoneurones with a large variation in their depolarizing activity (Figure 2). When these compounds were added to the perfusing fluid for a period of more than 30 s, the amplitude of responses to L-CCG-I, IV and D-CCG-IV was not maintained but gradually declined despite the presence of these agonists (Figure 2a). This decrease in amplitudes of responses to these CCGs during their application appears to be due to the desensitization of receptors for excitatory amino acids. The decline was observed even in small amplitudes of the responses in spite of the fact that small amplitudes of responses to L-glutamate did not decline during its application. The depolarizing responses were generally reproducible in any preparations and isomers and would be roughly divided into two groups showing fast and slow responses, which was evident in the case of a short period application (Figure 2b). NMDA, L-CCG-I, D-CCG-I, II and III showed slow responses (the prolonged time course of action), while L-CCG-II, III, IV and D-CCG-IV gave fast responses. In particular, L-CCG-I had a very long time course of action. It may be noted, in passing, that kainate produces slow responses, but quisqualate and L-glutamate produce fast responses in this preparation (Shinozaki *et al.*, 1989). D-CCG-II seemed to be the most potent among the CCG isomers when compared in terms of

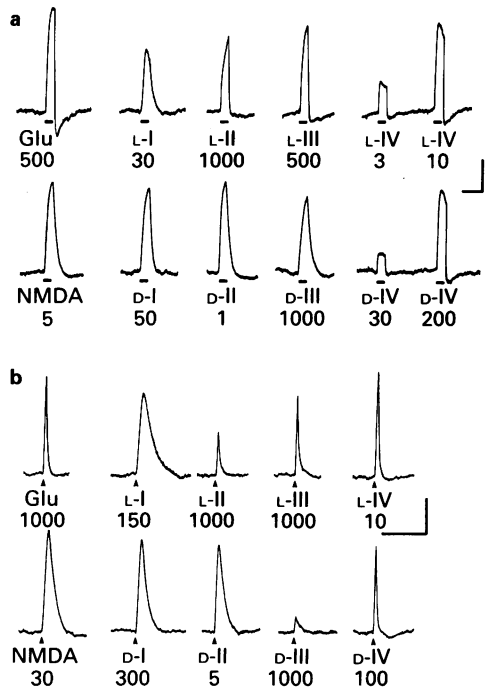

Figure 2 Sample records of responses to eight α-(carboxycyclopropyl)glycine (CCG) isomers, L-glutamate (Glu) and N-methyl-D-aspartate (NMDA). Responses to each excitatory amino acid were recorded from the ventral root extracellularly in Mg^{2+}-free, tetrodotoxin (0.5 μM)-containing solution. All test samples were added to the bathing solution for a period of 2 min (a) or 10 s (b). Numerals under each trace represent the concentrations (μM) of test compounds. All records in each group were obtained from the respective preparation. L-CCG-I and D-CCG-I are abbreviated to L-I and D-I, respectively, and so forth. Scale: 0.5 mV, 5 min.

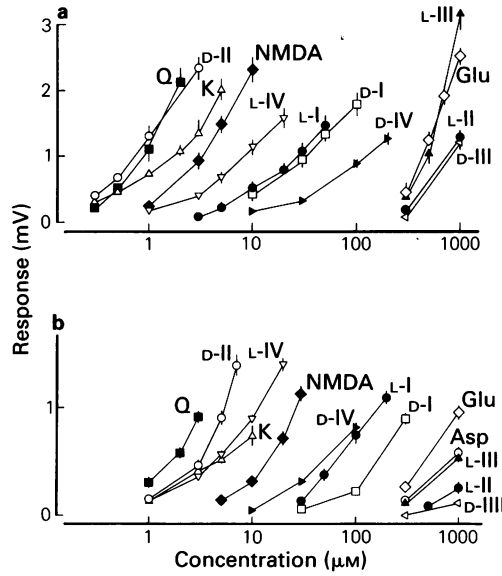

Figure 3 The concentration-response curves of eight stereoisomers of α-(carboxycyclopropyl)glycine (CCG) and representative excitatory amino acids. Experimental conditions were similar to those in Figure 2. Peak amplitudes of depolarization (mV) are plotted against the concentrations (μM): (a) 2 min application, (b) 10 s application. Glu: L-glutamate, Asp: L-aspartate, K: kainate, Q: quisqualate. L-CCG-I and D-CCG-I are abbreviated to L-I and D-I, respectively, and so forth. Vertical bars represent s.e.mean (*n* at least 4).

peak amplitudes of their responses. As shown in our previous paper (Shinozaki *et al.*, 1989), L-CCG-IV also demonstrated a high depolarizing activity, but it was apparently less potent than D-CCG-II.

Several factors, such as desensitization of receptors, equilibrium constants of agonist-receptor complexes and differences in rates of uptake of agonists, would affect the peak amplitude of depolarizing responses, which sometimes varied in accordance with the duration of application of agonists. Therefore, it seemed to be technically difficult to determine exactly the relative potency of excitation induced by CCGs in terms of the peak amplitude. For the experiment shown in Figure 3, peak amplitudes of responses were plotted against the concentrations of excitatory agonists, including representative excitatory amino acids, which were added to the perfusing fluid for a period of 10 s or 2 min. It was noted that the order of depolarizing activities of CCGs in these two cases did not vary much except for L-CCG-IV and D-CCG-IV, which produced fast desensitizing responses (Figure 2a), that appeared relatively less potent from 2 min applications.

Pharmacological classification of responses to CCGs

When the Mg^{2+}-free solution was changed to one containing 1 mM Mg^{2+} to test the NMDA-type depolarizing activity of all CCGs (all 10 s applications), responses to L-CCG-IV and all stereoisomers of D-CCG were markedly reduced, those to L-CCG-I (0.1 mM) were insensitive to 1 mM Mg^{2+} ions and those to L-CCG-III (1 mM) were slightly reduced. After the preparation was exposed to MK-801 (2 μM) for more than 30 min (Wong *et al.*, 1986), repetitive responses to NMDA (20 μM), L-CCG-IV (10 μM) and D-CCG-II (5 μM) gradually decreased in a use-dependent manner, while the responses to kainate (3 μM) or quisqualate (2 μM)

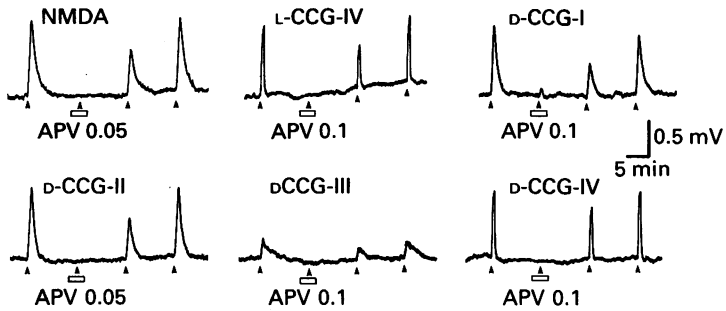

Figure 4 Sample records of depolarizing responses to N-methyl-D-aspartase (NMDA), L-CCG-IV and all D-isomers in the absence and presence of a considerably high concentration of D-(−)-2-amino-5-phosphorovaleric acid (D-APV). All responses were almost completely abolished after application of D-APV (0.05–0.1 mM) and the effect of the antagonists was fully reversible upon washout. Agonists (NMDA: 10 μM, L-CCG-IV: 10 μM, D-CCG-I: 0.1 mM, D-CCG-II: 2 μM, D-CCG-III: 1 mM, D-CCG-IV: 0.2 mM) were added to the perfusing fluid for 30 s. D-APV was added to the perfusing fluid 90 s before the administration of agonists for 3 min, as indicated by rectangles beneath trace. Numerals under each trace represent concentrations of D-APV in mM.

remained unchanged and those to L-CCG-I (0.1 mM) were only slightly reduced or unchanged. Amplitudes of responses to L-glutamate (1 mM) were reduced by about 85% of the control. Neither strychnine (10 μM) nor picrotoxin (10 μM) affected the depolarizations induced by D-CCG-II and L-CCG-IV.

The above experiments using non-competitive NMDA blockers suggest that L-CCG-IV and all stereoisomers of D-CCG preferentially activate NMDA-type receptors. Next, effects of competitive NMDA blockers were examined (Davies *et al.*, 1981; 1986). The blockers were added from 90 s before the application of agonists to the end of the agonist actions in a wide range of concentrations to block NMDA responses fully. When responses to all CCGs were examined in the presence of CPP (1–100 μM) and D-APV (10–100 μM), both blockers reduced responses to L-CCG-IV and all D-CCGs in a dose-dependent manner. Figure 4 shows that high concentrations of D-APV almost completely abolish responses to NMDA, L-CCG-IV and all D-isomers of CCG. Responses to D-CCG-II (2 μM) were completely abolished by 50 μM D-APV. Likewise, responses to L-CCG-IV (10 μM), D-CCG-III (1 mM) and D-CCG-IV (0.2 mM) were also completely abolished. Residual amplitudes of responses to L-CCG-I and II were about 70% of the control in the presence of these NMDA blockers (see below). Responses to L-CCG-III (1 mM) were reduced by about 50% in the presence of 10 μM D-APV, and by about 30% or less in the presence of 30–100 μM CPP or 100 μM D-APV, but residual amplitudes were still observed even in higher concentrations of NMDA blockers. The effects of these NMDA competitive blockers were fully reversible upon washout. Figure 5 represents the relationship between the agonist concentration and the peak amplitude of responses in the absence and presence of CPP (2, 5, 10 μM). This figure demonstrates that CPP shifts the dose-response curve of D-CCG-II, L-CCG-IV and NMDA in an almost parallel manner. The pA_2 values were 5.99 ± 0.08 ($n = 4$), 5.90 ± 0.03 ($n = 4$) and 5.86 ± 0.04 ($n = 4$) for CPP versus D-CCG-II, L-CCG-IV and NMDA, respectively. In all cases the pA_2 values were not significantly different from 6.0.

As mentioned above, L-CCG-IV and all D-isomers demonstrated almost exclusive preference for NMDA-type receptors. Here, the preference of remaining CCGs (L-CCG-I, II and III) for each receptor subtype was examined by a single or combined application of various blockers for excitatory amino acids. CNQX (10–300 μM) and DNQX (10–300 μM) were used for these experiments in addition to D-APV and CPP (0.1 mM). CNQX and DNQX have been characterised as a broad spectrum excitatory amino acid antagonist. They are potent competitive antagonists at non-NMDA receptors and have non-competitive actions at NMDA receptors, although they do not distinguish between the receptors for quisqualate and kainate (Birch *et al.*, 1988; Honore *et al.*, 1988). The blockers were added from 90 s before the application of agonists for a period of 3 min. At a concentration of 10 μM, CNQX and DNQX considerably reduced responses to L-glutamate (0.3–0.7 mM), kainate (2–5 μM), NMDA (5–10 μM), quisqualate (0.3–2 mM) and AMPA (2–5 μM) and at 0.1 mM they completely abolished responses to kainate (2–5 μM), NMDA (5–10 μM) and AMPA (2–5 μM) (Figure 6e, f, h). However, responses to L-glutamate (0.3–0.7 mM) and quisqualate (0.3–2 μM) were still observed even in the presence of CNQX or DNQX (up to 0.3 mM) and D-APV (0.1 mM) (Figure

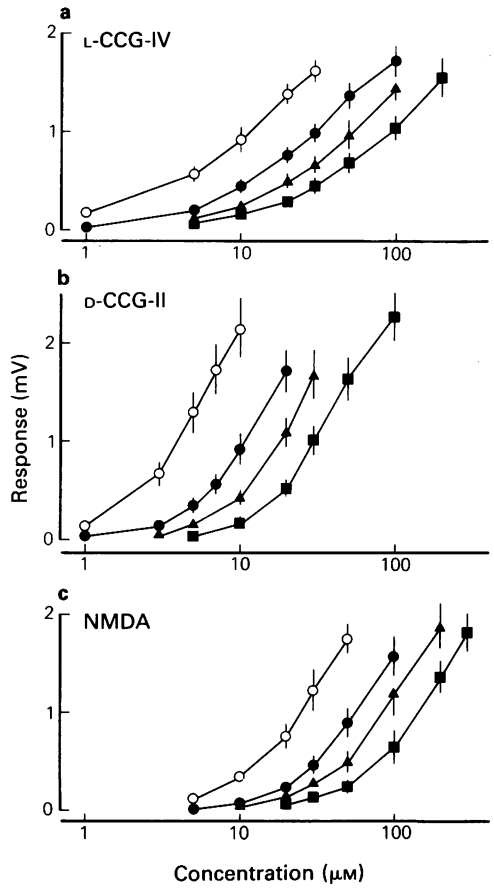

Figure 5 Dose-response curves of (a) L-CCG-IV, (b) D-CCG-II and (c) N-methyl-D-aspartate (NMDA) in the absence and presence of 3-[(±)-2-carboxypiperazin-4-yl]propyl-1-phosphonic acid (CPP). Agonists were added to the perfusing fluid for a period of 10 s. CPP was added to the bathing solution in concentrations of 2 (●), 5 (▲), 10 (■) μM from 90 s prior to the agonist application and was present to the end of the agonist action; (○) control response. Peak amplitudes of response to excitants were plotted against their logarithmic concentrations. Vertical bars represent s.e.mean (n at least 4).

6d, g, h). Residual amplitudes of responses to L-glutamate (0.5 mM) and quisqualate (1 μM) were 28 ± 2% and 21 ± 1% of the control, respectively ($n = 4$), in the presence of 0.1 mM of both D-APV and CNQX. Responses to L-CCG-I (50 μM) and L-CCG-II (1 mM) were merely decreased slightly by the further addition of non-NMDA blockers (Figure 6a, b). Responses to L-CCG-I (50 μM) were reduced by 76 ± 4% ($n = 4$) in the presence of 0.1 mM D-APV, and by 77 ± 2% ($n = 4$) in the combination of D-APV and CNQX (0.1 mM). It was of great interest that responses to L-CCG-I and L-CCG-II were not reduced by even high concentrations of CNQX in combination with CPP or D-APV. L-CCG-I and L-CCG-II might belong to a new category of excitatory amino acids. Residual components of responses to L-CCG-III in the presence of D-APV or CPP (about 30%) were markedly reduced by CNQX (0.1 mM) (Figure 6c). Similar results were obtained when DNQX (0.1 mM) was applied. The pharmacological property of responses to L-CCG-III will be mentioned in a later paragraph because of potentiation of its own and L-glutamate responses.

Glycine in micromolar concentrations has been reported to augment the excitatory action of NMDA in cultured cortical and diencephalic neurones (Johnson & Ascher, 1987). This potentiation is insensitive to strychnine, and seems to be one of the characteristic properties of the NMDA response in the mammalian central nervous system. Since D-CCG-II and L-CCG-IV were shown in the present study to be potent NMDA-type agonists, we examined the action of glycine on responses to these agonists in the rat isolated spinal cord, but we were unable to demonstrate that superfusion with glycine (1 μM–1 mM) facilitated significantly depolarizations evoked by CCG-isomers in strychnine-containing (10 μM) solution. This is interpreted as meaning that the endogenous levels of glycine were already maximal for this effect in the rat isolated spinal cord. In order to demonstrate the potentiation of the NMDA-type response by glycine in the rat spinal cord, other experimental conditions would be required (Fletcher & Lodge, 1988).

Marked potentiation of glutamate responses by L-CCG-III

As shown above, L-CCG-III caused a depolarization of the rat spinal motoneurone. In addition to its own depolarizing action, it caused marked potentiation of responses to L-glutamate and L-aspartate in the rat isolated spinal cord. This potentiation lasted for a long time. After a single application of L-CCG-III (1 mM, 10–15 s), the preparation was thoroughly washed by the normal bathing solution, and the depolarizing response was restored very quickly. When the periodic addition of L-glutamate to the bathing solution was resumed without simultaneous application of L-CCG-III, the amplitude of their responses was markedly enhanced (Figure 7a, c). When L-glutamate was applied for a long time, a decline in amplitude, probably due to receptor desensitization, was observed during application of L-glutamate (Figure 7c), in striking contrast to the control response. The potentiation of the glutamate response lasted for a period of more than several

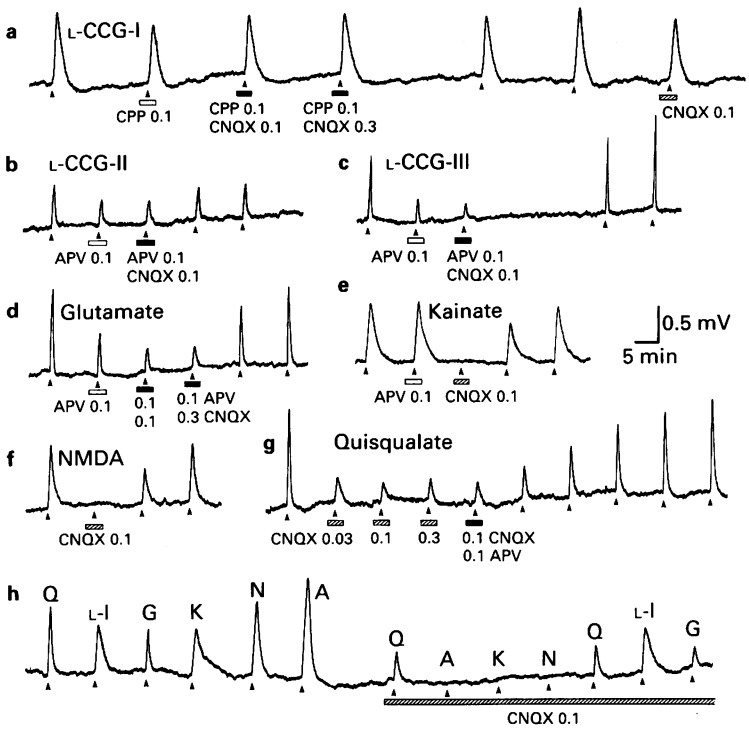

Figure 6 Responses to L-CCG-I, L-CCG-II and L-CCG-III in the presence of high concentrations of 3-[(±)-2-carboxypiperazin-4-yl]propyl-1-phosphonic acid (CPP), D-(−)-2-amino-5-phosphonovaleric acid (D-APV) and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX). Agonists were added to the perfusing fluid for a period of 30 s except L-CCG-III, and antagonists were added for a period indicated by columns in a single or in combination with CNQX. Numerals represent the concentrations of antagonists (mM). (a) L-CCG-I (50 μM); (b) L-CCG-II (1 mM); (c) L-CCG-III (1 mM, 20 s); (d) L-glutamate (0.5 mM); (e) kainate (2 μM); (f) N-methyl-D-aspartate (NMDA) (5 μM); (g) quisqualate (1 μM); (h) Q: quisqualate (1 μM), L-I: L-CCG-I (30 μM), G: L-glutamate (0.5 mM), K: kainate (5 μM); N: NMDA (10 μM), A: 2-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) (5 μM). Note that residual amplitudes of responses to quisqualate are at an almost similar level 90 s and 40 min after the addition of CNQX.

hours without a further application of L-CCG-III (Figure 7a). Responses to L-aspartate were also enhanced by L-CCG-III in a similar manner to L-glutamate, and the potentiation of responses to L-glutamate or L-aspartate was reinforced by repeated application of L-CCG-III (Figure 7b). The above case is one example of the effects observed after a single application of L-CCG-III; however, marked potentiation is seen even when both L-glutamate and L-CCG-III are added simultaneously to the bathing solution (Figure 7d). In this case, the minimum effective concentration of L-CCG-III causing the potentiation seemed to be significantly less than 50 μM. At low concentrations of L-CCG-III, the potentiation did not continue for a long time, in spite of the fact that the response amplitude was considerably augmented at the first stage (Figure 7d). Other isomers of CCG did not demonstrate such potentiation of responses to L-glutamate or L-aspartate.

When L-glutamate or L-aspartate was applied in relatively high or low concentrations, L-CCG-III (1 mM) did not augment significantly responses to them, but in dose-ranges where log–log plots of responses to L-glutamate or L-aspartate against the concentration represent almost a straight line, L-CCG-III significantly augmented these responses, as if the dose-response curve was shifted to the left in a parallel manner (Figure 8).

We examined the responses to a variety of amino acid excitants which were affected by L-CCG-III. Responses to L-glutamate, D- and L-aspartate, L-cysteate and L-CCG-III were potentiated by L-CCG-III (1 mM), but those to kainate, quisqualate, NMDA, D-glutamate, L-CCG-I, IV, D-CCG-I, II and IV were hardly affected by L-CCG-III (1 mM).

As shown above, L-CCG-III seems unlikely to be a pure NMDA-type agonist. To test whether the potentiation of the glutamate response is directly

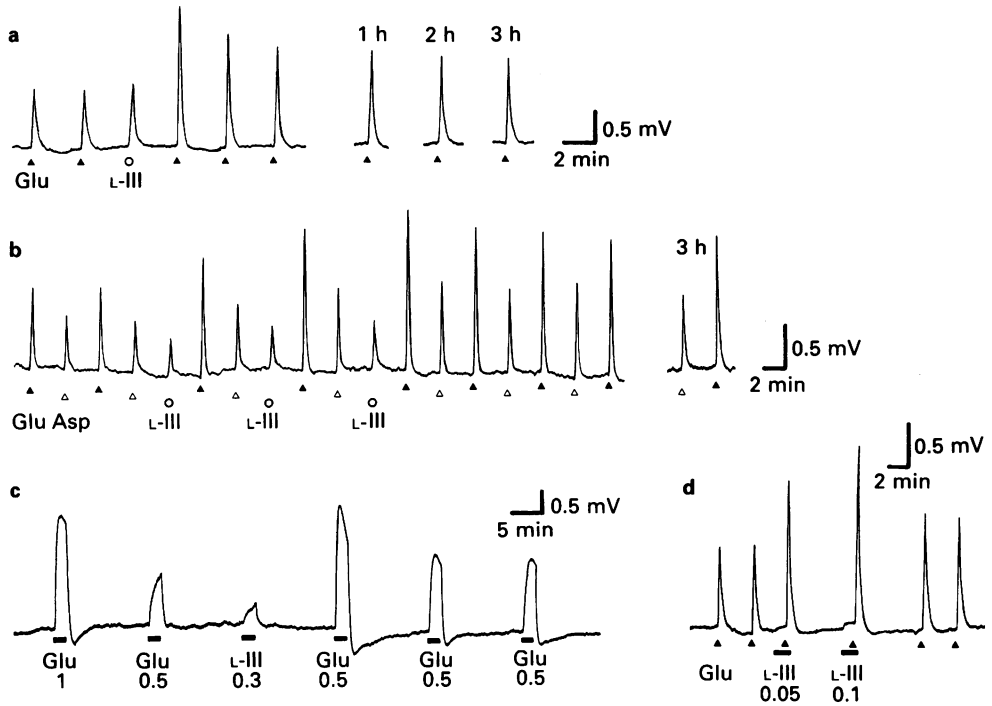

Figure 7 Potentiation of responses to L-glutamate and L-aspartate by L-CCG-III. Glu: L-glutamate, Asp: L-aspartate. (a) After single application of L-CCG-III (○, 1 mM, 15 s), marked potentiation of responses to L-glutamate (▲, 1 mM, 10 s) was observed, and it lasted for a long time. Even 3 h later, a larger response was still observed. (b) Responses to L-aspartate (△) were also augmented by L-CCG-III (○) in a similar manner to those to L-glutamate (▲). (c) Responses evoked by a prolonged application (2 min) of agonists. Numerals represent the concentration (mM) of L-glutamate and L-CCG-III. (d) Simultaneous application of L-CCG-III and L-glutamate. L-CCG-III (0.05 and 0.1 mM) was added with L-glutamate (1 mM, 10 s) for a period indicated by horizontal bars. Relatively rapid restoration was observed.

related to the L-CCG-III-evoked depolarization, the potentiation was examined under conditions where the depolarization was extremely reduced by Mg^{2+}, CPP and DNQX. The responses to L-glutamate (1 mM) were slightly reduced by changing from Mg^{2+}-free to Mg^{2+}-containing normal solution, but, even in the Mg^{2+}-containing solution, the marked potentiation of responses to L-glutamate was still observed after application of L-CCG-III (1 mM). When L-CCG-III (1 mM) was added to the bathing solution in combination with CPP (10 μM) or DNQX (0.1 mM) in order to reduce the peak amplitude of responses to L-CCG-III, the glutamate response was greatly augmented after the application of L-CCG-III. Even when both CPP (10 μM) and DNQX (20 μM) were added simultaneously to the bathing solution in order to reduce further the L-CCG-III response, the marked potentiation was still observed, and the duration of potentiation remained unchanged.

Uptake inhibitors and L-*CCG-III*

As a possible mechanism for the potentiation, it is reasonable to assume that L-CCG-III blocks uptake of excitatory amino acids from the vicinity of their receptors by the surrounding neurone and glia (Watkins & Evans, 1981). Some amino acids, such as D-(+)- and L-(−)-*threo*-3-hydroxyaspartate, dihydrokainate and L-cysteate, have been known to inhibit the uptake of glutamate and aspartate in the mammalian central nervous system (Balcar & Johnston, 1972; Johnston *et al.*, 1979; 1980). So, we compared the actions of L-CCG-III and these uptake inhibitors on the potentiation of the glutamate response. Both D-(+)- and L-(−)-*threo*-3-hydroxyaspartate caused a slight depolarization of the rat spinal motoneurone to a similar degree, but the L-(−)-isomer was superior to the D-(+)-isomer in causing the potentiation of the glutamate response. L-(−)-*threo*-3-hydroxyaspartate (1 mM) augmented

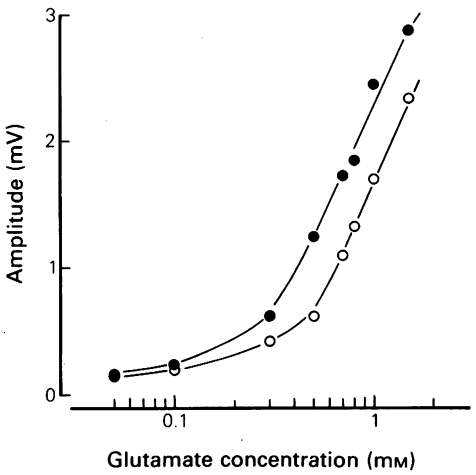

Figure 8 Dose-response curves of L-glutamate before and after application of L-CCG-III. L-Glutamate (10 s) was added to the bathing solution in various concentrations before (○) and after (●) three single applications of L-CCG-III (1 mM, 15 s). Ordinate scale: amplitude of the glutamate response (mV). Abscissa scale: logarithmic concentrations of L-glutamate.

the response to L-glutamate (1 mM) in a very similar manner to L-CCG-III. However, the duration of its action was considerably shorter than that of L-CCG-III (1 mM), demonstrating that L-CCG-III was more potent than L-(−)-*threo*-3-hydroxyaspartate (Figure 9). In the case of *threo*-3-hydroxyaspartate, both L- and D-isomers demonstrate an inhibitory effect on uptake of L-glutamate or L-aspartate, although the L-isomer is approximately twice as potent as the D-isomer with respect to the uptake of either L-glutamate or L-aspartate (Balcar *et al.*, 1977). However, in contrast to L-CCG-III, D-CCG-III hardly affected the responses to these excitatory amino acids. Dihydrokainate was a weak agonist in the rat isolated spinal cord, and caused a slow depolarization of the motoneurones. Dihydrokainate did not affect the response to L-glutamate or L-aspartate in the rat isolated spinal cord. L-Cysteate caused a depolarization of the motoneurone to a similar degree to L-aspartate, but did not enhance the response to L-glutamate.

The spinal reflex evoked by electrical stimulation (duration 100 μs, 10 V, at intervals of about 1 min) of dorsal root (L_4 or L_5) was hardly affected by the one-shot application of L-CCG-III (1 mM) or L-(−)-*threo*-3-hydroxyaspartate (1 mM). When L-CCG-III was added to the bathing solution for a period of 3 min in a concentration of 0.1 mM, potentiation of the spinal reflex was not observed.

Discussion

NMDA-like actions

The isomers of L- and D-CCG were synthesized in order to gain some insight into possible conformations of L-glutamate molecules during activation of each glutamate receptor subtype. As a result, it was found that all D-isomers of CCG and a folded form of L-CCG (L-CCG-IV) activated selectively the NMDA-type receptor. Binding assays of CCG

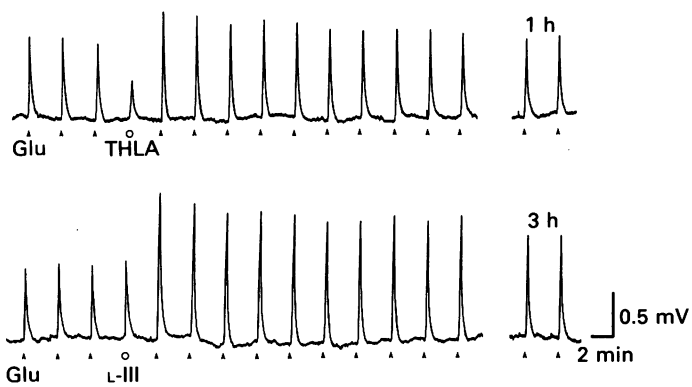

Figure 9 Comparison of the potentiation of the glutamate response induced by L-CCG-III and L-(−)-*threo*-3-hydroxyaspartate. L-Glutamate was added to the bathing solution for a period of 10 s in a concentration of 1 mM. The potentiating effect of L-CCG-III (L-III, ○, 1 mM, 15 s) was more pronounced and lasted for a longer time than that of L-(−)-*threo*-3-hydroxyaspartate (THLA, ○, 1 mM, 15 s).

isomers using labelled CPP in the rat hippocampus neurone have supported our present results (Kawai & Ishihara, unpublished observation). The structure of L-CCG-IV, as mentioned above, mimics very closely the folded conformation of L-glutamate, therefore, the present results suggest that an active conformation of the L-glutamate at the NMDA-type receptor is a folded form. It is of great interest that all D-isomers of CCG, which include both the folded and extended forms, cause a depolarization due to the selective activation of the NMDA-type receptor in the rat isolated spinal cord. Among D-isomers, D-CCG-II demonstrated an extremely potent depolarizing activity. Although the data concerning the depolarizing activity of other NMDA-type agonists were not shown in Figure 3, the depolarizing activity of other NMDA-type agonists was not always superior to that of NMDA. The depolarizing activity of L-homocysteate was almost similar to that of NMDA in the rat isolated spinal cord preparation, L-cysteate being less potent than NMDA. Thus, of those compounds we examined on the rat isolated spinal cord, D-CCG-II is one of the most potent NMDA-type agonists. While D-CCG-II and L-CCG-IV are markedly potent NMDA-type agonists, there is no apparent structural similarity between them. As shown in the Figure 1, the cyclopropyl ring of CCGs fixes its carbon chains in extended or folded forms close to those adopted by L-glutamate. L-CCG-IV corresponds to a folded form of L-glutamate from the point of view of its configuration as well as conformation, on the other hand, D-CCG-II corresponds to an extended form of glutamate in its conformation but not in configuration. The polar functional groups of D-CCG-II and L-CCG-IV, which are probably binding sites with the receptors, never accurately superimpose. The carbon chain of aspartate is one atom shorter than that of glutamate and the ionizable groups of aspartate and NMDA cannot match those of glutamate. The NMDA-type receptor might have high affinity for substrates with the D-configuration by unknown mechanisms. The conformational arrangement of the D-isomer when it interacts with receptors remains to be solved (Watkins & Olverman, 1987; Magnuson *et al.*, 1988).

A novel agonist, L-CCG-I

L-CCG-I is more potent than L-glutamate in causing a depolarization in the rat isolated spinal cord. Responses to L-CCG-I and II were merely decreased slightly by Mg^{2+} and competitive NMDA blockers. Residual amplitudes of responses to these CCGs in the presence of competitive NMDA antagonists were almost insensitive to CNQX or DNQX. Their conformation is the extended form. At the crayfish neuromuscular junction, ibotenic acid demonstrated an increase in Cl^- permeability which was blocked by picrotoxin (Shinozaki & Ishida, 1980), likewise, L-CCG-I showed a quite similar action to ibotenic acid at this junction (unpublished observation). It is of great interest to examine the similarity between L-CCG-I and ibotenic acid in detail from the chemical viewpoint. Our results demonstrate a clear preference of L-CCG-I for non-NMDA, non-kainate and non-quisqualate-type receptors. Similar results were obtained in the case of L-CCG-II. Such results may suggest that L-glutamate has an additional mechanism for exciting spinal neurones (Honore *et al.*, 1988). The development of this new and potent agonist opens the possibility for extensive studies of the glutamate receptor subtypes. Further studies will be required to clarify the receptor subtype to which L-CCG-I binds.

Potentiation of glutamate responses

Only L-CCG-III was found to potentiate markedly the response to L-glutamate and D- and L-aspartate. In contrast to L-CCG-IV, which possessed potent depolarizing activity, L-CCG-III showed considerably lower depolarizing activity despite the fact that it has a similar folded structure to L-CCG-IV, and the configurations at β- and γ-carbon atoms are the only difference between them. Activation of the NMDA-type receptor and the potentiation of the response to L-glutamate are certainly differentiated by the steric role of the cyclopropyl group of CCG. In spite of the fact that the depolarization evoked by L-CCG-III is mainly due to the activation of the NMDA-type receptor, the potentiation of response to L-glutamate does not seem to be directly related to the activation of NMDA-type receptors.

One may consider some possibilities for the mechanism underlying the potentiation of the glutamate response, such as inhibition of uptake of agonists from the neuronal environment, an increase in transmitter release, changes of properties of receptors which might be related to glutamate neurotoxicity, and so on. It is also worth considering whether the potentiation in our case is similar to the glycine-induced potentiation of the NMDA response (Johnson & Ascher, 1987). However, according to binding assays on hippocampal neurones, it seems unlikely that L-CCG-III binds to these glycine receptors (Kawai & Ishihara, unpublished observation). It would seem more plausible that L-CCG-III blocks the uptake process for excitatory amino acids. According to Johnston *et al.* (1980), under conditions where *threo*-3-hydroxyaspartate had little or no effect on the spontaneous firing rate of neurones in the cat spinal cord, excitation induced by the administration of L-glutamate was clearly potentiated. In the present study, spinal reflexes were not affected by

L-CCG-III and neither dihydrokainate nor L-cysteate potentiated responses to L-glutamate or L-aspartate, but only *threo*-3-hydroxyaspartate and L-CCG-III enhanced them. Responses to kainate, quisqualate, NMDA and D-glutamate were hardly affected by L-CCG-III. L-Glutamate, D-aspartate and L-cysteate are able to utilize the glutamate transport system efficiently, but D-glutamate, NMDA, and kainate are poor substrates for the glutamate transport system (Stallcup *et al.*, 1979). It was of great interest that the response to several compounds, which were believed to be taken up from the vicinity of their receptors, was often enhanced by L-CCG-III and/or *threo*-3-hydroxyaspartate in the rat isolated spinal cord. Although the potentiation of the glutamate response by L-CCG-III might be due to the inhibition of the uptake process, the extremely long duration of the potentiation is a question to be solved. Further investigations will be required in order to validate or negate such a relationship between the potentiation of the glutamate response and the inhibition of the uptake of excitatory amino acids.

Neuronal damage is supposed to be aggravated by the potentiation of responses to L-glutamate (McBean & Roberts, 1985), and may be related to the activation of NMDA-type receptors (Rothman & Olney, 1987; Finkbeiner & Stevens, 1988). Since L-CCG-III, a NMDA-preferring agonist, significantly potentiates the glutamate response for a long time, it is possible that L-CCG-III may provide useful information about glutamate neurotoxicity. In addition, the long-term potentiation (LTP) in hippocampal neurones has captured the imagination of neuroscientists as a model with which to probe the cellular mechanisms of learning and memory in the mammalian brain. Many researchers are interested in the relationship between NMDA-type receptors and LTP (Collingridge & Bliss, 1987). Although the present data were obtained in the rat spinal cord, the actions of CCG-isomers are expected to be similar in other regions of the mammalian central nervous system. Whatever the mechanism for the potentiation of the glutamate response, L-CCG-III is expected to be very useful as a pharmacological tool for analyzing the mechanisms underlying glutamate's transmitter functions.

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Regional haemodynamic effects of human α- and β-calcitonin gene-related peptide in conscious Wistar rats

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1 A comparative study was carried out to assess the regional haemodynamic effects of intravenous infusion of human α- and β-calcitonin gene-related peptide (CGRP; 0.006, 0.06 and 0.6 nmol h^{-1}) in conscious, unrestrained Wistar rats.

2 With human α-CGRP, tachycardia was always accompanied by a fall in mean arterial blood pressure (MBP), but human β-CGRP at the middle dose caused tachycardia with no significant hypotension.

3 Human α- and β-CGRP both caused dose-dependent falls in MBP accompanied by common carotid and hindquarters vasodilatations and increased flows. The highest dose of α- and β-CGRP caused mesenteric vasoconstriction and renal vasodilatation, but the latter was not associated with an increase in flow.

4 With the intermediate dose, human α-CGRP caused more significant haemodynamic changes than did human β-CGRP. In addition, comparison with previous experiments involving infusion of rat α-CGRP indicated that human α-CGRP had more potent haemodynamic effects than the former.

Introduction

In a previous study we found that intravenous infusion of rat α-calcitonin gene-related peptide (CGRP) in conscious Wistar rats caused differential, dose-dependent, regional haemodynamic effects (Gardiner *et al.*, 1989). There were vasodilatations in renal, hindquarters and common carotid vascular beds, although only in the latter two regional circulations were flows increased above baseline, and the effect in the common carotid vascular bed was about double that in the hindquarters. In spite of direct evidence for a vasodilator effect of CGRP on the superior mesenteric vascular bed *in vitro* (Marshall *et al.*, 1986a), hypotensive doses of rat α-CGRP caused clear-cut superior mesenteric vasoconstriction, with the increase in vascular resistance being associated with a significant reduction in flow below baseline. The magnitude of the heart rate (HR) responses to rat α-CGRP indicated a possible direct cardiac effect of the peptide in addition to baroreflex-mediated influences.

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These results are consistent with rat α-CGRP having potent cerebral vasodilator properties (McCulloch *et al.*, 1986; Uddman *et al.*, 1986; Edvinsson *et al.*, 1987) and positive chronotropic effects (Marshall *et al.*, 1986b; Sigrist *et al.*, 1986). One possible explanation of the lack of mesenteric vasodilatation with infusion of CGRP is that reflex effects override the dilator actions of CGRP. If this were the case, then low doses of CGRP, that were without effects on mean arterial blood pressure (MBP), might reveal a primary mesenteric vasodilator action. In the present study we addressed this possibility and also assessed the cardiac effects of the same manoeuvre.

Since there are several molecular forms of CGRP (see Zaidi *et al.*, 1987) it is theoretically possible that the haemodynamic profiles of the various peptides might differ such that there was a relative enhancement of a particular feature of the cardiovascular actions of one of the species. We investigated this possibility by carrying out a systematic comparison of the regional haemodynamic effects of human α-

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and β-CGRP in conscious rats. The dose-range chosen was based on our previous findings, but included infusion of CGRP at 0.006 nmol h^{-1}, i.e., 10 fold lower than previously used.

Our overall objectives can be stated formally as the following questions: (1) Does infusion of human α- or β-CGRP at a low dose produce positive chronotropic effects independent of changes in MBP? (2) Does infusion of human α- or β-CGRP at a low dose produce superior mesenteric vasodilatation? (3) Do infusions of a range of doses of human α- or β-CGRP have similar regional haemodynamic effects?

By reference to our previous investigations (Gardiner *et al.*, 1989) we were also in a position to consider the possibility that rat and human α-CGRP have different haemodynamic profiles.

Methods

Male, Wistar rats (from Bantin and Kingman) were used in this study. At the time of operation animals weighed between 330–420 g. Details of the methods used have been described elsewhere (Gardiner *et al.*, 1989). Under anaesthesia (sodium methohexitone 60 mg kg^{-1} i.p., supplemented) pulsed Doppler probes (Haywood *et al.*, 1981) were implanted around left renal and superior mesenteric arteries and the distal abdominal aorta (below the level of the ileocaecal artery) or, in other animals, around both common carotid arteries. At least 1 week after operation all animals that were growing normally, grooming, etc., and with acceptable signals from all 3 probes (signal : noise ratio > 20 : 1) were briefly re-anaesthetized (sodium methohexitone 40 mg kg^{-1}) and had intravascular catheters implanted (in the abdominal aorta via the caudal artery and in the right jugular vein) and the probe wires soldered into a microconnector clamped in a harness fitted to the rat.

No measurements were made until the following day when, at 06 h 15 min, continuous recordings of MBP, instantaneous HR, and renal, mesenteric and hindquarters, or bilateral common carotid Doppler shift signals were begun. Measurements were made whilst the animals were unrestrained in their home cages. Phasic Doppler shift signals were monitored to ensure their acceptability and mean Doppler shift signals were obtained from them electronically. Changes in Doppler shift signal give a reliable index of changes in volume flow (Haywood *et al.*, 1981; Wright *et al.*, 1987) and these were calculated as % of the baseline level. From the MBP and mean Doppler shift, % changes in vascular resistances were calculated relative to the baseline level (Haywood *et al.*, 1981).

The experimental protocol ran over 2 days and animals were randomized to receive human α- or β-CGRP on the first experimental day. All animals received human α-CGRP on one day and human β-CGRP on the other day, both peptides being given as 60 min infusions at 3 different doses (0.006, 0.06 and 0.6 nmol h^{-1}; 0.3 ml h^{-1}). The infusate consisted of CGRP dissolved in isotonic saline containing 1% bovine serum albumin and 0.05% lactose. The amount of peptide needed to achieve the desired concentration was calculated from the stated peptide content of the lyophylizate. CGRP was given in increasing doses since the effects of higher dose infusions are persistent (Gardiner *et al.*, 1989). Each infusion was separated by at least 1 h such that variables had returned to baseline levels for a minimum of 30 min before any subsequent infusion of CGRP was begun. The infusion rate was 0.3 ml h^{-1} for all doses of both peptides. Infusion of vehicle at the same rate had no consistent effects. One-hour infusions of human α-CGRP at repeated intervals of 1 h cause reproducible cardiovascular effects in our model (Gardiner, S.M., Compton, A.M. & Bennett, T., unpublished observations).

Measurements were made at 0, 5, 10, 20, 30, 40, 50, 60, 65, 70, 80, 90, 100, 110 and 120 min, with the infusion running between 0 and 60 min. Data were analysed by two-way, non-parametric analysis of variance (Friedman's test). In the text where effects were referred to, then $P < 0.05$ indicates a significant result.

Peptides: human α-CGRP was obtained from Celltech Ltd., (Lot No. 05543GT00-02) and human β-CGRP was obtained through Celltech Ltd., (manufactured by Peninsula Labs).

Results

Human α- or β-CGRP infusion at 0.006 nmol h^{-1}

Animals with renal, mesenteric and hindquarters probes α-CGRP had no significant effect on MBP or HR. During the infusion, there was a slight, transient reduction (at 30 min) in mesenteric flow, but no significant changes in renal or hindquarters flows. Following infusion there were small reductions in renal (65–120 min) and mesenteric (70–120 min) flows, associated with vasoconstrictions (renal, 70–120 min; mesenteric, 60–120 min); there were no changes in hindquarters haemodynamics. Measurements made in animals not receiving CGRP but made during the same times of day as those receiving the low dose of α-CGRP, showed a similar drift in renal flow and resistance, indicating these changes were probably a consequence of the light-dark transition (the latter ending at 5 h 00 min). A similar drift

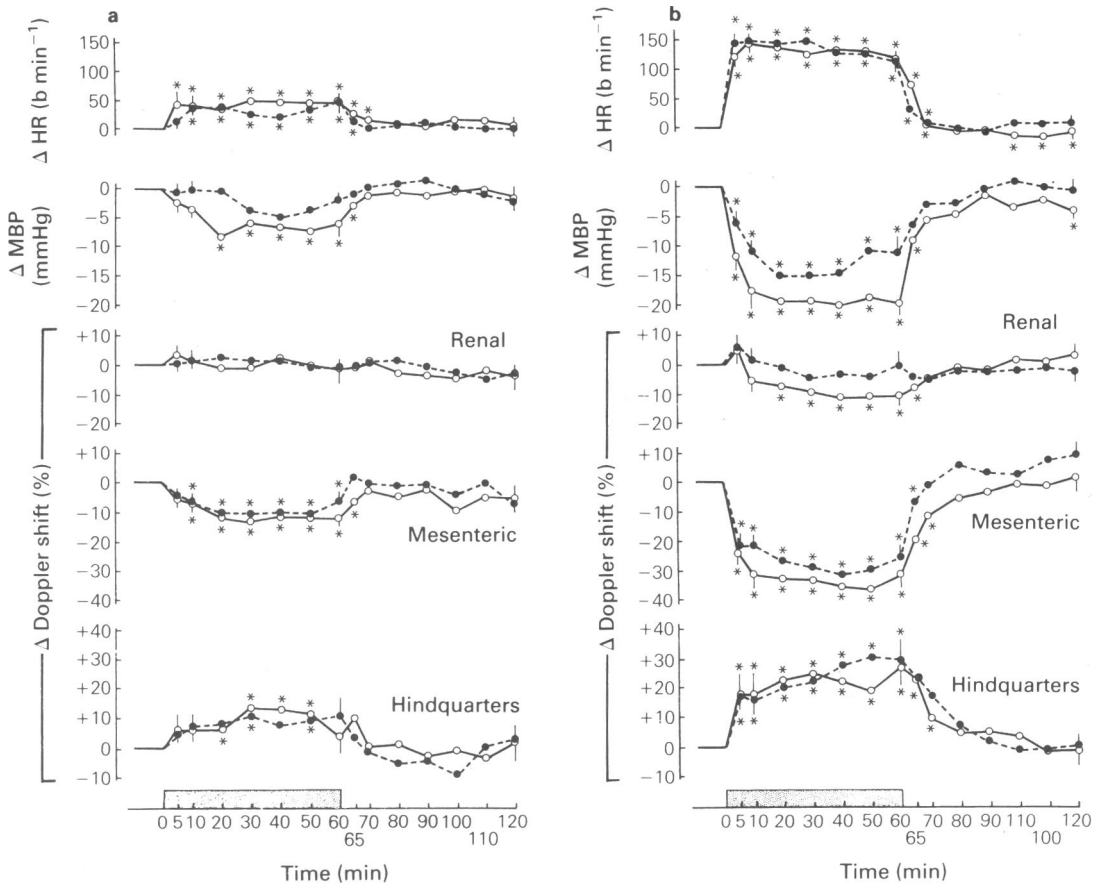

Figure 1 Cardiovascular variables during and after infusion (indicated by stippled horizontal bar) of human α-calcitonin gene-related peptide (α-CGRP) (○) or human β-CGRP (●) at 0.06 nmol h^{-1} (a) or 0.6 nmol h^{-1} (b). The values show the group mean ($n = 8$) with representative s.e.means. The time points at which values were significantly different from baseline are indicated by asterisks.

was seen in renal haemodynamics in animals receiving β-CGRP, but no other significant changes occurred during or after infusion of this peptide.

Animals with bilateral common carotid probes There were no significant changes in any measured variable during or after infusion of α-CGRP.

During infusion of β-CGRP, there were no changes in MBP or HR, but there were small reductions in left (20 min) and right (60 min) common carotid flows, associated with vasoconstrictions (left 20, 60 min; right 50, 60 min). Since, after infusion of β-CGRP, there were significant reductions in common carotid flows (left, 65, 70, 80, 90, 110, 120 min; right 65, 70, 80, 120 min) and increases in vascular resistances (left 65, 70, 80, 110, 120 min; right, 65, 70, 80 min) that were not seen in the same animals given α-CGRP at the same time of day, it is possible these effects were related to the administration of β-CGRP.

Human α- or β-CGRP infusion at 0.06 nmol h^{-1}

Animals with renal, mesenteric and hindquarters probes Infusion of α-CGRP was associated with a fall in MBP and a tachycardia (Figure 1). Renal blood flow was unchanged, but there was a reduction in mesenteric and an increase in hindquarters flow (Figure 1). Calculated renal vascular resistance fell and there was a hindquarters vasodilatation but no significant change in mesenteric vascular resistance (Figure 2).

Infusion of β-CGRP had variable effects on MBP such that, overall, there was no significant change,

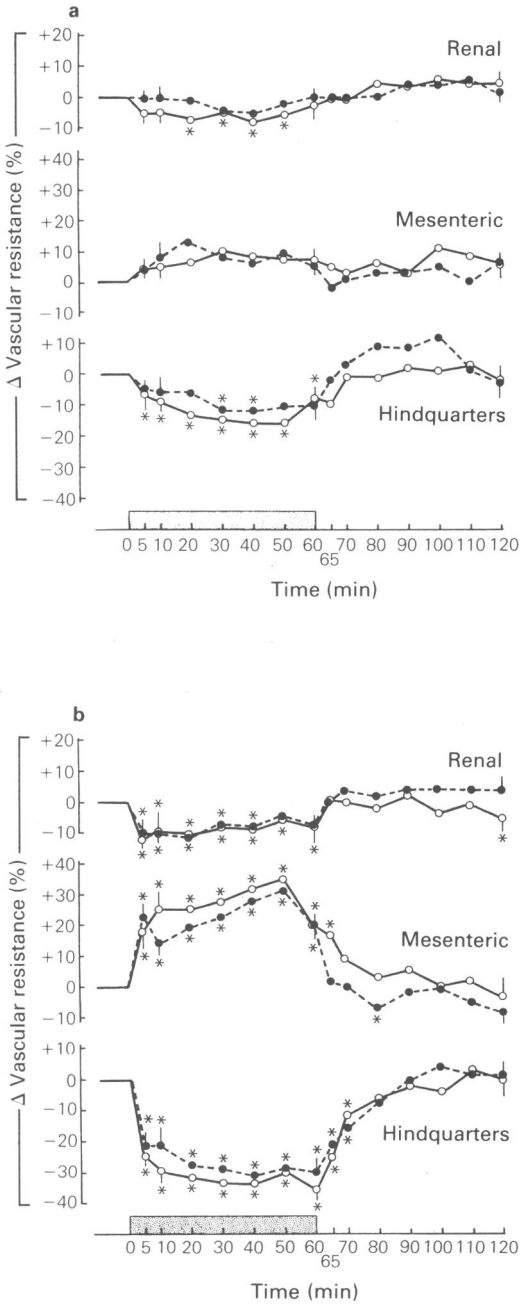

Figure 2 Calculated vascular resistances during and after infusion (indicated by stippled horizontal bar) of human α-calcitonin gene-related peptide (α-CGRP) (○) or human β-CGRP (●) at 0.06 nmol h^{-1} (a) or 0.6 nmol h^{-1} (b). The values show the group mean ($n = 8$) with representative s.e.means. The time points at which values were significantly different from baseline are indicated by asterisks.

although there was a tachycardia (Figure 1). β-CGRP had no effect on renal flow (Figure 1) or resistance (Figure 2), and the reduction in mesenteric blood flow it produced (Figure 1) was not accompanied by a significant change in resistance (Figure 2). However, during infusion of β-CGRP there was an increase in hindquarters flow (Figure 1) that was accompanied by vasodilatation (Figure 2). Following infusion of β-CGRP there was a reduction in hindquarters flow (Figure 1) associated with vasoconstriction (Figure 2).

Thus, the effects of α- and β-CGRP were similar in the mesenteric vascular bed where the fall in flow was associated only with a tendency towards vasoconstriction. However, it is notable that the lack of significant effect of β-CGRP on MBP was accompanied by a less sustained hindquarters vasodilatation than was seen with α-CGRP. Furthermore, there was a post-infusion hindquarters vasoconstriction with β-CGRP that was not seen with α-CGRP (Figure 2).

Animals with bilateral common carotid probes Administration of α-CGRP was accompanied by a reduction in MBP and an increase in HR (Figure 3), similar to those seen in animals with renal, mesenteric and hindquarters probes (Figure 1). These changes were accompanied by increases in common carotid flows (Figure 3) associated with decreases in vascular resistances (Figure 4).

Although the tachycardias induced by human β-CGRP were similar in magnitude to those induced by human α-CGRP, the former did not have a sustained hypotensive effect (Figure 3). The increases in common carotid flow were not as persistent as those seen with human α-CGRP (Figure 3), and this was reflected in the truncated common carotid vasodilatations seen with β-CGRP (Figure 4).

Human α- or β-CGRP infusion at 0.6 nmol h^{-1}

Animals with renal, mesenteric and hindquarters probes Infusion of α-CGRP was accompanied by hypotension and tachycardia (Figure 1). Renal and mesenteric flows were reduced and hindquarters flow was increased (Figure 1). There were renal and hindquarters vasodilatations (Figure 2), and a mesenteric vasoconstriction. After infusion, there was a significant bradycardia and a secondary fall in MBP accompanied by a renal vasodilatation, but no other changes (Figures 1 and 2).

Infusion of β-CGRP caused a tachycardia similar in magnitude to that seen with α-CGRP (Figure 1), but the fall in MBP was less marked and persistent (Figure 1). Renal flow was not significantly affected by β-CGRP, and the reduction in mesenteric flow was not as prolonged as with α-CGRP (Figure 1).

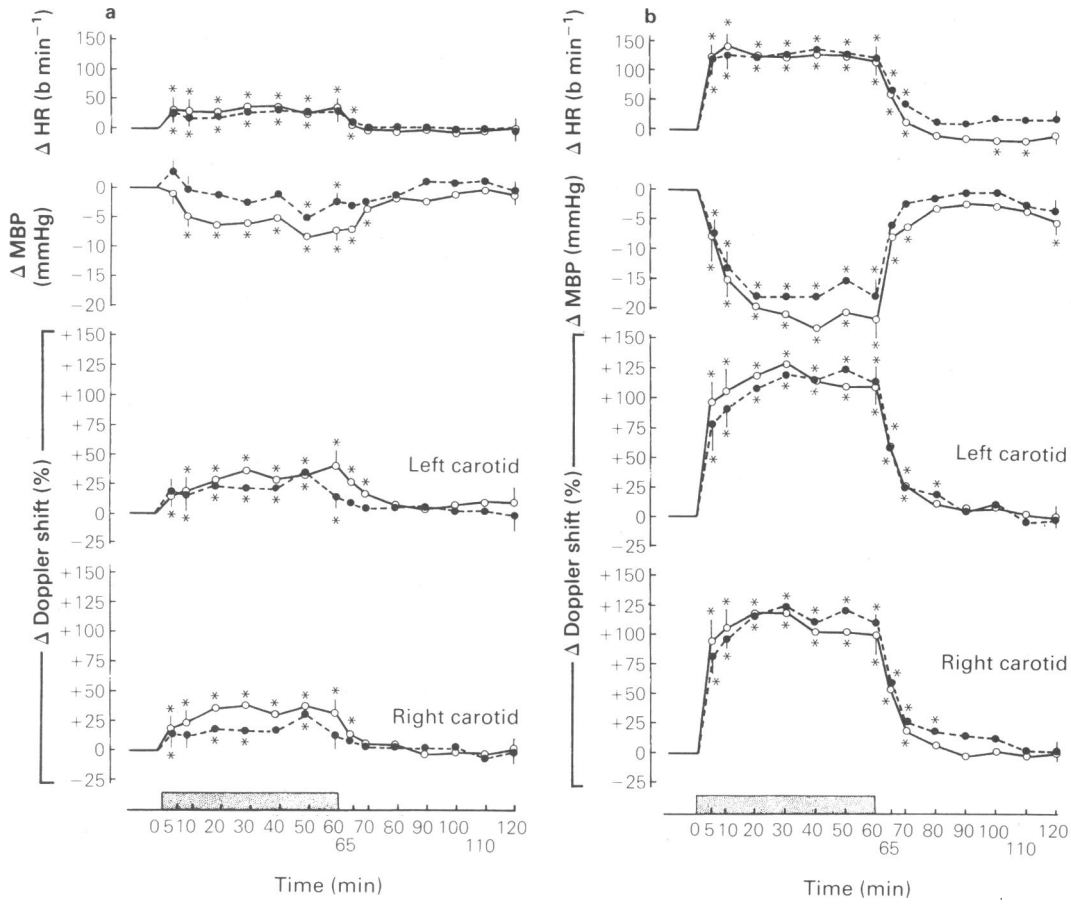

Figure 3 Cardiovascular variables during and after infusion (indicated by stippled horizontal bar) of human α-calcitonin gene-related peptide (α-CGRP) (○) or human β-CGRP (●) at 0.06 nmol h^{-1} (a) or 0.6 nmol h^{-1} (b). The values show the group mean ($n = 8$) with representative s.e.means. The time points at which values were significantly different from baseline are indicated by asterisks.

However, the increase in hindquarters flow with β-CGRP was similar to that seen with α-CGRP (Figure 1). Infusion of β-CGRP was associated with renal and hindquarters vasodilatations not different from those seen with α-CGRP (Figure 2). However, the mesenteric vasoconstriction was more rapid in offset and followed by a brief vasodilatation (Figure 2). Furthermore, there was no bradycardia following β-CGRP infusion (Figure 1), and no secondary fall in MBP (Figures 1 and 2).

Animals with bilateral common carotid probes Infusion of α-CGRP produced a reduction in MBP associated with a tachycardia (Figure 3). There were marked increases in common carotid flow associated with vasodilatations (Figures 3 and 4). After infusion, carotid haemodynamics returned to baseline levels, but there was a post-infusion bradycardia and secondary fall in MBP (Figures 3 and 4), similar to the picture seen in the animals with intra-abdominal probes (Figure 1).

Infusion of β-CGRP produced falls in MBP, increases in HR, increases in common carotid flow and decreases in resistance, similar to those seen with α-CGRP (Figures 3 and 4). However, as with the animals bearing intra-abdominal probes, there was no bradycardia or secondary fall in MBP following infusion of β-CGRP.

Discussion

It is clear that the present study did not demonstrate a positive chronotropic effect of human α-CGRP

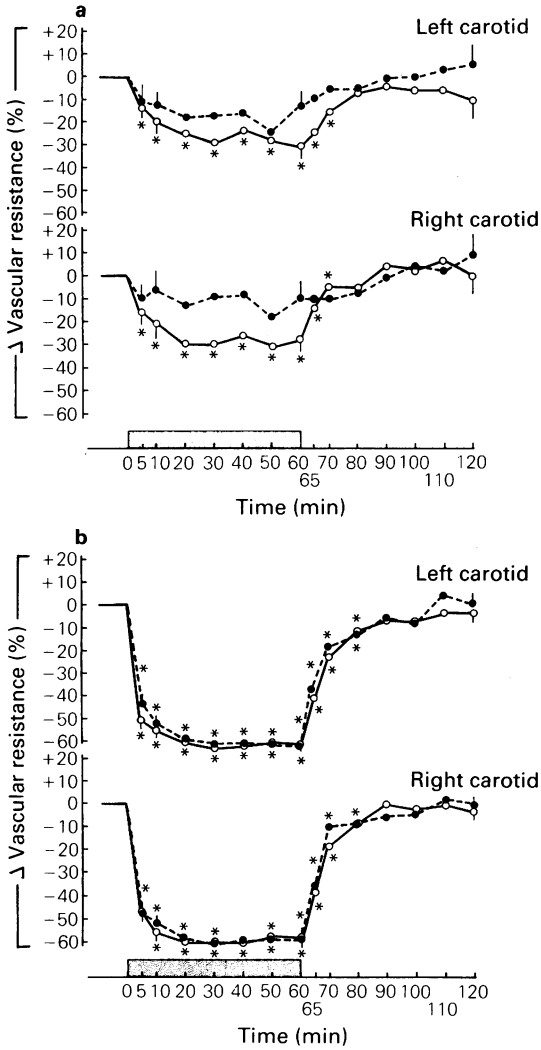

Figure 4 Calculated regional vascular resistances during and after infusion (indicated by stippled horizontal bar) of human α-calcitonin gene-related peptide (α-CGRP) (○) or human β-CGRP (●) at 0.06 nmol h^{-1} (a) or 0.6 nmol h^{-1} (b). The values show the group mean ($n = 8$) with representative s.e.means. The time points at which values were significantly different from baseline are indicated by asterisks.

independently of any change in MBP, although human β-CGRP at a dose of 0.06 nmol h^{-1} was without significant effects on MBP at a time when it caused an increase in HR. Moreover, in some animals, administration of human β-CGRP at 0.6 nmol h^{-1} caused an increase in HR unaccompanied, initially, by any detectable change in MBP. However, without detailed information about central haemodynamics and the transmural pressures experienced by the baroreceptors, it is not possible to exclude a change in afferent signalling as contributing to the changes in HR. In fact, it is feasible that the cardiac effects of human β-CGRP acted to offset any fall in MBP due to vasodilatation. While it could be argued that *in vitro* results support the belief that changes in HR with CGRP *in vivo* may be due to direct actions (Sigrist *et al.*, 1986; Marshall *et al.*, 1986b; Asimakis *et al.*, 1987), the doses of CGRP used in the *in vitro* studies are usually much higher than those that cause tachycardia *in vivo*. In addition, although a recent investigation by Howden *et al.*, (1988) showed that bolus doses of human α-CGRP in conscious human volunteers caused increases in HR with no statistically significant effect on systolic or diastolic BP, the data indicate that some subjects probably did show a fall, particularly in diastolic, BP. Struthers *et al.*, (1986) found that infusion of human α-CGRP did cause falls in diastolic BP and increases in HR in volunteers. Furthermore, their analysis of the change in BP and HR with CGRP, compared to other vasodilators, did not indicate an unexpected tachycardia with this peptide. While the original descriptions of the cardiovascular actions of CGRP in the rat commented that changes in HR could occur in the absence of falls in MBP (Fisher *et al.*, 1983) a more recent investigation (Lappe *et al.*, 1987) showed that falls in calculated total peripheral resistance and increases in cardiac output occurred with doses of CGRP that did not increase HR significantly. However, higher doses of CGRP, with substantial hypotensive effects, caused tachycardia that was reduced, but not abolished, by propranolol (Lappe *et al.*, 1987). On balance, then, it seems likely that the direct chronotropic effects of CGRP will be difficult to separate from its hypotensive and reflex cardiac effects *in vivo*, but it is an intriguing possibility that the inotropic and afterload-reducing actions of CGRP infusions may be exerted with little influence on MBP or HR *in vivo* (Lappe *et al.*, 1987). Such a profile of action might be particularly useful in the treatment of problems such as congestive heart failure. It remains to be determined whether or not human α- or β-CGRP is more effective in this regard.

We were unable to show that infusion of human α- or β-CGRP at a low dose (0.006 nmol h^{-1}) caused mesenteric vasodilatation. On the contrary, human α-CGRP at this dose, caused a slight but significant reduction in mesenteric flow. It may be that the mesenteric vascular bed is a primary target for baroreflex-mediated changes in sympathetic efferent outflow and such effects obscure any local vasodilator effects of CGRP on the mesenteric vasculature (Marshall *et al.*, 1986a; Kawasaki *et al.*, 1988). This conclusion is consistent with the finding that mesen-

teric vasodilatation can be elicited by bolus administration of rat α-CGRP (Gardiner *et al.*, 1988) where the rapidity of onset of vasodilator effect may outstrip 'on-line' baroreflex compensation.

The present study has shown that human α- and β-CGRP may differ in some of their regional haemodynamic effects, and that these differences may be dose-dependent. As mentioned above, the low dose of human α-CGRP appeared to cause a significant reduction in mesenteric blood flow, an effect not seen with human β-CGRP. Furthermore, the low dose of the latter appeared to cause a selective reduction in common carotid flow (associated with vasoconstriction) that did not occur with administration of human α-CGRP. This pattern of effects, in circumstances in which the actions of human α- and β-CGRP were compared in the same animals, makes it unlikely that the differences were attributable to a lack of comparability in the effective doses of the two peptides administered to the animals, due to differences in peptide activity, or to metabolism (Brown & Morice, 1987), for example. This assertion is supported by the finding that the pattern of change in HR and mesenteric flow were similar with both peptides given at 0.06 nmol h^{-1}, but at this dose the change in MBP was less significant for human β- than for human α-CGRP, consistent with the lesser hindquarters and carotid vasodilator effects of the former peptide.

Our finding of more marked vasodilator and hypotensive effects of human α- than of human β-CGRP is in agreement with some observations made *in vivo* (Franco-Cereceda *et al.*, 1987; Brown & Morice, 1987), but not with others (Brain *et al.*, 1986; Williams *et al.*, 1988). Differential metabolism, (due to the presence of a methionine residue in β-CGRP), could explain a lesser bioactivity of human β- than of α-CGRP given intravenously, and the finding that β-CGRP may be more potent than α-CGRP *in vitro* (Holman *et al.*, 1986), although recent studies have not found this (Mulderry *et al.*, 1988). However, it is difficult to reconcile the differences between the results of studies in which human α- and β-CGRP were given locally (Brown & Morice, 1987; Williams *et al.*, 1988), unless the actual amounts of biologically active α- and β-CGRP delivered to the tissues were different.

Infusions of human α- and β-CGRP at 0.6 nmol h^{-1} produced similar regional haemodynamic effects; although the increases in HR were the same, the fall in MBP tended to be less with human β- than with human α-CGRP. As mentioned above, it is possible these two peptides have different effects on cardiac output and this should be investigated formally.

Comparison of the present results with our previous findings (Gardiner *et al.*, 1989) highlights some differences in the haemodynamic effects of rat and human α-CGRP. With infusion of rat α-CGRP at 0.06 nmol h^{-1} there were renal, mesenteric, hindquarters and common carotid vasodilatations, although only in the latter vascular bed was there an increase in blood flow (Gardiner *et al.*, 1989). In the present study, infusion of human α-CGRP at the same rate caused changes in carotid haemodynamics similar to those seen with rat α-CGRP, but unlike the latter, human α-CGRP also caused an increase in hindquarters and a slight decrease in mesenteric flow. These differences are consistent with the slightly greater hypotensive and tachycardiac effects of human, compared to rat, α-CGRP (Gardiner *et al.*, 1989). However, the similarity in the changes in carotid haemodynamics with the two peptides argues against differences in amounts of active peptide administered explaining the difference in profiles of effect. This proposition is supported by the findings that with a higher infusion rate (0.6 nmol h^{-1}) the changes in hindquarters haemodynamics were similar with rat and human α-CGRP, but the carotid hyperaemia and the mesenteric vasoconstriction were greater with human than with rat α-CGRP (Gardiner *et al.*, 1989). A greater overall dilator effect of human compared to rat α-CGRP would be in line with the finding that the tachycardic and hypotensive actions of the former were more marked than the latter at a dose of 0.6 nmol h^{-1} (Gardiner *et al.*, 1989). However, *in vitro* studies indicate that rat α-CGRP has more potent cardiac effects than human α-CGRP (Marshall *et al.*, 1986b; Holman *et al.*, 1986).

In summary, from the present, and previous results it appears that, *in vivo*, human α-CGRP may be a more potent carotid and hindquarters vasodilator than either human β- or rat α-CGRP. It is feasible these peptides also have differential effects on cardiac haemodynamics, but specific experiments are needed to address this possibility.

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Decrease in internal H^+ and positive inotropic effect of heptaminol hydrochloride: a ^{31}P n.m.r. spectroscopy study in rat isolated heart

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1 The cardiotonic effect of heptaminol hydrochloride (Hept-a-myl, Delalande) was studied using ^{31}P-nuclear magnetic resonance (n.m.r.) spectroscopy and left ventricular pressure (LVP) measurements in rat isolated hearts. The possibility of this effect being mediated by an intracellular realkalinisation was tested.

2 Isolated hearts were perfused at 10 ml min^{-1} by the Langendorff method with Krebs-Henseleit solution at 37°C and stimulated at 5 Hz. Mechanical activity was measured as variations of left ventricular pressure (LVP). ^{31}P-n.m.r. spectra were recorded every 2 min. Changes in cardiac adenosine triphosphate (ATP), phosphocreatine (PCr) and inorganic phosphate (Pi) were followed and intracellular pH (pHi) was estimated from the chemical shift of Pi.

3 The effects of heptaminol were tested in different conditions: normoxia, moderate ischaemia, severe ischaemia, and moderate ischaemia in the presence of amiloride or guanidinium chloride as inhibitors of the Na-H exchange.

4 In normoxia, heptaminol induced a cyclic increase of systolic LVP, associated with an increase in Pi. No significant effect on pHi was observed. In changing from normoxia to moderate ischaemia, PCr and systolic LVP decreased; a mild intracellular acidification (pHi 6.96) was obtained. Heptaminol induced a restoration of pHi and increased LVP. In severe ischaemia, the realkalinization effect and the restoration of LVP induced by heptaminol were no longer observed. During moderate ischaemia, Na-H exchange inhibitors decreased pHi and LVP. Heptaminol applied in the presence of these inhibitors was unable to restore pHi and LVP.

5. These results suggest that the positive inotropic effect of heptaminol during moderate ischaemia could be related to a restoration of internal pH, possibly mediated by a stimulation of the Na-H exchange.

Introduction

Heptaminol (6-amino-2-methyl-2-heptanol) hydrochloride (Hept-a-myl) is a cardiotonic drug used in Europe since 1953. It is well established that this cardiotonic agent causes a positive inotropic action in a variety of *in vitro* and *in vivo* systems (Loubatières, 1951; Coraboeuf & Boistel, 1953; Muller *et al.*, 1964; Pourrias *et al.*, 1969). As a general rule, heptaminol produces a large increase in the contraction of ischaemic preparations and restores electrical activity in hypoxic cardiac tissues while in normoxic preparations heptaminol effects are negligible (Coraboeuf & Boistel, 1953).

A hypothesis to explain these results, would be that heptaminol has an effect on the contractility of the myocardium that is brought about by an intracellular alkalosis related to the chemical structure of the drug. Heptaminol indeed possesses alcohol and amine groups ($(CH_3)_2$-COH-$(CH_2)_3$-$CHNH_2$-CH_3) that are able to modify the contractile properties of the myocardium. In fact some alcohols (such as 1-hexanol) increase the calcium sensitivity of contractile proteins (Ventura-Clapier, personal

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communication). Furthermore, it has been demonstrated that the presence of amine and alcohol groups induces an intracellular alkalosis (Vassort *et al.*, 1986) and thus increases the calcium sensitivity of myofilaments (Fabiato & Fabiato, 1978). In addition, in chemically-skinned (Triton X-100 method) rat papillary muscles, heptaminol does not directly modify the calcium sensitivity of myofibrils (Ventura-Clapier, personal communication).

An alternative hypothesis would be a direct stimulation of the Na-H exchange by heptaminol. This will also induce changes in intracellular pH with the consequent effect on contractility. In the present study, we have tested the effect of heptaminol on contractility and intracellular pH. The intracellular pH, together with the level of high-energy phosphate metabolites, was measured in isolated Langendorff-perfused rat hearts, by ^{31}P-nuclear magnetic resonance (^{31}P-n.m.r.) spectroscopy, while left ventricular pressure was monitored continuously.

The results indicate that the positive inotropic effect of heptaminol, observed during moderate ischaemia, could be related to a restoration of pHi. The absence of this effect in the presence of an inhibitor of Na-H exchange, suggests that the effect of heptaminol may be mediated by a Na-H exchange stimulation.

Methods

Experiments were performed on rat (Wistar, weighing 300–400 g) isolated hearts. Animals were heparinized and anaesthetized with intraperitoneal injections of sodium pentobarbitone (50 mg kg^{-1}).

Hearts were retrogradely perfused (Langendorff) at 37°C with a constant flow of 10 ml min^{-1}. The perfusion solution was Krebs-Henseleit (KH) without phosphate, of the following composition (mM): NaCl 120, KCl 5.6, $CaCl_2 \cdot 2H_2O$ 2.4, $MgCl_2 \cdot 6H_2O$ 1.2 and glucose 11. Solutions were equilibrated with 95% O_2, 5% CO_2. At the heart level, Po_2 and Pco_2 were respectively 60 mmHg and 50 mmHg. pH 7.4 was obtained by addition of $NaHCO_3$. Heptaminol hydrochloride (Delande) 10^{-3} M, amiloride (Sigma) 10^{-3} M and guanidinium chloride (B.D.H.) 30 mM were added when necessary. When guanidinium chloride was used, NaCl concentration was reduced to 90 mM. Hearts were placed in an n.m.r. probe of 20 mm diameter. The volume of the bath was kept constant with a peristaltic pump in a reverse mode (12 ml min^{-1}). Each heart was stimulated at just above the threshold voltage, at a rate (5 Hz) slightly higher than its intrinsic frequency, via a catheter filled with agar-KCl (3 M), positioned in the right ventricle (Hoerter *et al.*, 1986).

A catheter introduced in the left ventricle and connected to a Statham pressure transducer was used to monitor continuously systolic and diastolic pressure on a Gould pen recorder. Thus, the pressure developed by the left ventricle (LVP) was used as an indicator of the contractile function. The LVP was expressed as percentage of the initial pressure. In some experiments a latex balloon was introduced instead of a catheter and progressively inflated until a peak in developed pressure was observed. No significant differences were observed between these two techniques.

After 30 min of perfusion with KH, until all the recorded parameters were stable (Lavanchy *et al.*, 1985), the following experimental procedures were used: (1) perfusion with KH solution (10 ml min^{-1}) for 2 h to test the validity of our experimental conditions; (2) perfusion with KH solution added with heptaminol for 8 min and then washing; (3) 22 min of reduction of flow rate to induce ischaemia. Flow rates of 6 ml min^{-1} and 3 ml min^{-1} were considered as moderate and severe ischaemia respectively. After 8 min of ischaemia, heptaminol was admitted in the solution for 8 min. Procedure (4): after 4 min of moderate ischaemia, amiloride or guanidinium chloride was added for 16 min; 4 min after amiloride addition, a supplementary pulse of heptaminol was then applied for 8 min.

Nuclear magnetic resonance measurements

^{31}P-n.m.r. spectra were recorded at 81.015 MHz with a Bruker AM 200 (4.7 Tesla) spectrometer. Using a 1 s recycle delay and 90°, pulse spectra corresponding to 100 pulses were recorded every 2 min. Areas under peak signals for ATP, phosphocreatine (PCr) and inorganic phosphate (Pi) were measured by integration. Changes in ATP, PCr and Pi concentrations were expressed as percentage of initial values of peak areas.

Intracellular pH (pHi) was measured from the chemical shift of the intracellular Pi peak (Moon & Richards, 1973) by use of a titration curve obtained from a solution containing ATP (10 mM), PCr (10 mM), Pi (10 mM), NADPH (10 mM), glucose-6 P (10 mM) and $MgCl_2$ (10 mM). This method allowed a resolution of 0.05 pH unit using the following equation:

$$pHi = pKa + \log (\delta_A - \delta_{obs})/(\delta_{obs} - \delta_B)$$

with pKa = 6.90, δ_A = 3.30 p.p.m. and δ_B = 5.85 p.p.m. The PCr resonance frequency (independent of pH within the physiological range of pH values) served as reference (Jacobus *et al.*, 1982).

A capillary containing a methylene diphosphonate (MDP) solution was placed next to the heart to control the stability of the n.m.r. parameters.

Results

Validity of experimental conditions

In our experimental conditions, ($n = 15$) the PCr/Pi ratio was 5.2 ± 0.5, pHi was 7.07 ± 0.02 and the systolic LVP was 80 ± 10 mmHg. These values are comparable to those obtained by others (Hoerter *et al.*, 1988). During 2 h of perfusion these values showed non significant variations, indicating a functional and metabolic stability of the heart during experiments lasting this length of time.

Effect of heptaminol in normal KH solution

Figure 1c shows the effect of heptaminol on the LVP of a heart stimulated at a frequency of 5 Hz in KH solution. A cyclic modulation of LVP every 6 to 7 beats was consistently observed without arrhythmia. The systolic pressure increased to about 127% of the normal pressure. The inset of Figure 1 shows that the pacing frequency was not changed. In Figure 1a and b, the metabolic parameters from 5 similar experiments are given. The average PCr decreased to $78 \pm 3.5\%$ and Pi correspondently increased to $141 \pm 4.5\%$. The ATP and pHi were not significantly modified. The cyclic modulation of LVP was obtained in all preparations. After washing, all the described effects were fully reversed.

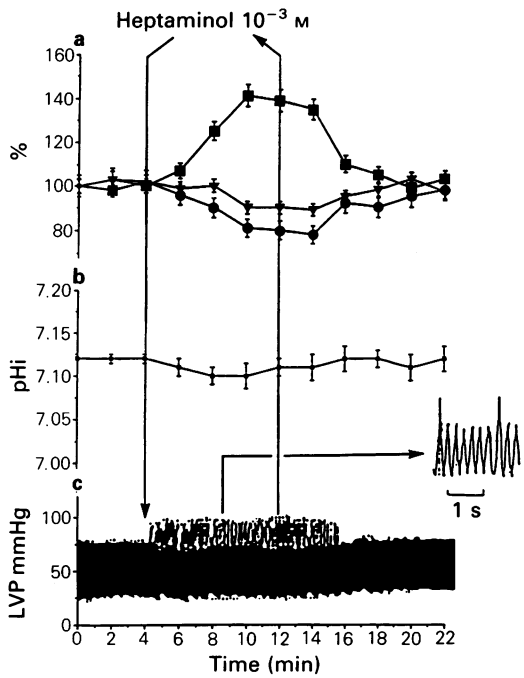

Figure 1 Time course of phosphocreatine (PCr), adenosine triphosphate (ATP), inorganic phosphate (Pi), intracellular pH (pHi) and left ventricular pressure (LVP) in stimulated (5 Hz) rat isolated hearts: effects of heptaminol 10^{-3} M. (a) Mean values of metabolic parameters from 5 experiments: PCr (●), ATP (▼) and Pi (■) are expressed as percentage of initial value. (b) Mean values of pHi from the same 5 experiments. In (a) and (b) vertical bars show s.e.mean. (c) LVP (mmHg) record from a representative experiment. The inset shows, on an expanded time scale, the cyclic modulation of LVP every 6 to 7 beats.

Experiments in ischaemic hearts

Moderate ischaemia A representative series of n.m.r. spectra from an isolated rat heart initially perfused in control conditions (KH) is shown in Figure 2. The peak intensities of phosphorylated metabolites appeared to be quite similar to those previously described in normoxia (Lavanchy *et al.*, 1985; Hoerter *et al.*, 1986). When moderate ischaemia was applied, the spectrum reflected the typical consequences of the metabolic disturbances. Heptaminol was added after 8 min of ischaemia, the main effect being a restoration of internal pH from pHi 6.96 to 7.05. Figure 3 illustrates the time course of these phenomena. A representative record of LVP (Figure 3c) shows that a moderate ischaemia induces a decrease in systolic LVP with a steady-state level reached after 2 to 3 min. When heptaminol was added during moderate ischaemia, the decrease of systolic LVP was reversed and a positive inotropic effect developed. In 5 experiments (not shown) LVP decreased during ischaemia to $72 \pm 7\%$ and heptaminol increased LVP to $113 \pm 15\%$.

Figure 3 also shows the modifications of the metabolic parameters in the same 5 experiments. During moderate ischaemia, a decrease of PCr to $70 \pm 4\%$ was observed and was associated with a $148 \pm 5\%$ increase in Pi. Intracellular pH varied from 7.10 ± 0.01 to 6.92 ± 0.02. ATP levels were slightly decreased. All these effects were reversed when ischaemic conditions were suppressed. When heptaminol was added during moderate ischaemia, a full restoration of intracellular pH was obtained. This effect was accompanied by a slight decrease of PCr and a consequent increase in Pi, while ATP variations were not significant. When heptaminol was washed out during ischaemia all its effects were reversed.

Severe ischaemia In 5 experiments in which severe ischaemia was induced, a drastic negative inotropic effect can be seen, with a typical decrease of PCr and ATP to $41 \pm 7.5\%$ and $77 \pm 4.5\%$, respectively (Figure 4); a corresponding increase in Pi to $173 \pm 10\%$ was observed. Under these conditions, the magnitude of the intracellular acidification was

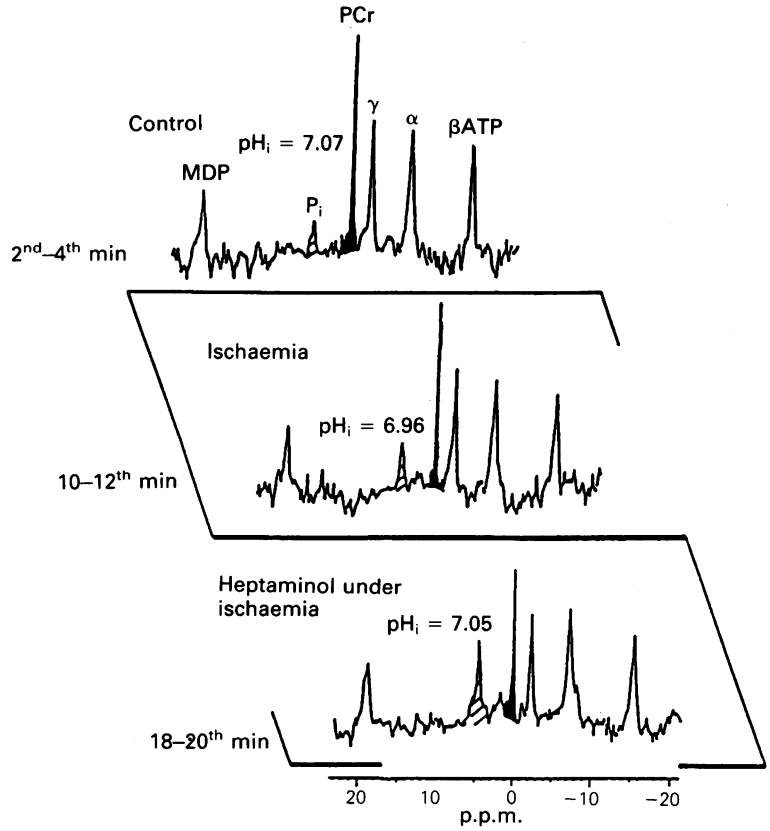

Figure 2 Series of ^{31}P-n.m.r. spectra obtained on perfused isolated heart of rat in control conditions, then exposed to moderate ischaemia and treated with heptaminol 10^{-3} M; 2 min spectra. Scale in parts per million with reference to the phosphocreatine (PCr) frequency. Upper spectrum obtained (2nd to 4th min) in control conditions (10 ml min^{-1}; 37°C, pH 7.4), middle spectrum obtained (10th to 12th min) under moderate ischaemia (6 ml min^{-1}). Lower spectrum also obtained under ischaemia (18th to 20th min) but in the presence of heptaminol 10^{-3} M.

greater (pHi 6.82) than that obtained in moderated ischaemia (pHi 6.95). In contrast with the restitution of contraction and intracellular pH induced by heptaminol in moderate ischaemia, no effect with the drug was observed in severe ischaemic conditions. The negative inotropic effect was always obtained in all experiments, with a decrease of LVP to 39 ± 10%. After washing in normoxic conditions all these variations were fully reversed.

Perfusion of Na-H exchange inhibitors during moderate ischaemia Amiloride induces an intracellular acidosis by inhibition of the Na-H exchange (Deitmer & Ellis, 1980, for more references see Frelin *et al.*, 1987). Figure 5 shows that in 4 experiments during ischaemia, amiloride decreases pHi from 6.95 ± 0.02 to 6.85 ± 0.02, while the systolic pressure change was biphasic, an initial increase followed by a slight, persistent decrease. No significant changes in metabolites were observed. When heptaminol was added in the presence of amiloride, the typical changes in LVP, pHi and metabolites, observed in moderated ischaemia, were no longer obtained. When we returned to normoxic conditions all the parameters recovered to the initial values. Similar results were obtained using guanidinium chloride as Na-H exchange blocker (Frelin *et al.*, 1986).

Effect of intracellular alkalosis during moderate ischaemia To assess further the effect of intracellular alkalosis alone on the contractile performance of the heart we performed the following experiment during moderate ischaemia. Ammonium chloride (20 mM) (Thomas, 1984) was added to the perfusion medium to induce an intracellular realkalinization from pHi 6.95 to pHi 7.08. This realkalinization was equivalent to that produced by heptaminol (see Figure 3) and induced the same increase in contraction (30% increase). In severe ischaemia, heptaminol had no effect. This could not be attributed to irre-

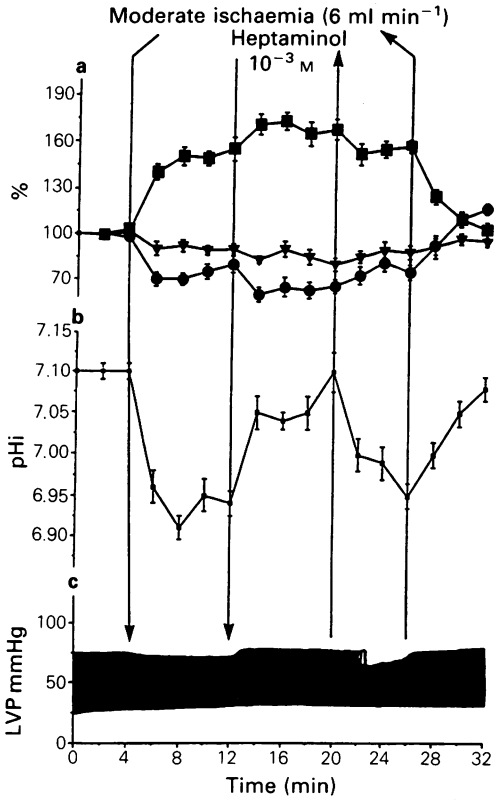

Figure 3 Time course of phosphocreatine (PCr), adenosine triphosphate (ATP), inorganic phosphate (Pi), intracellular pH (pHi) and left ventricular pressure (LVP) of stimulated (5 Hz) rat isolated hearts exposed to moderate ischaemia (6 ml min^{-1}) after equilibration (30 min) in control conditions (10 ml min^{-1}). Effects of heptaminol 10^{-3} M. (a) Mean values of metabolic parameters from 5 experiments: PCr (●), ATP (▼) and Pi (■) expressed as percentage of initial value. (b) Mean values of pHi from same 5 experiments. In (a) and (b) vertical bars show s.e.mean. (c) LVP (mmHg) record from a representative experiment. Note the simultaneous evolution of pHi (realkalinization) and LVP (positive inotropic effect) as soon as heptaminol was added. After washing in normoxic conditions all parameters were fully recovered.

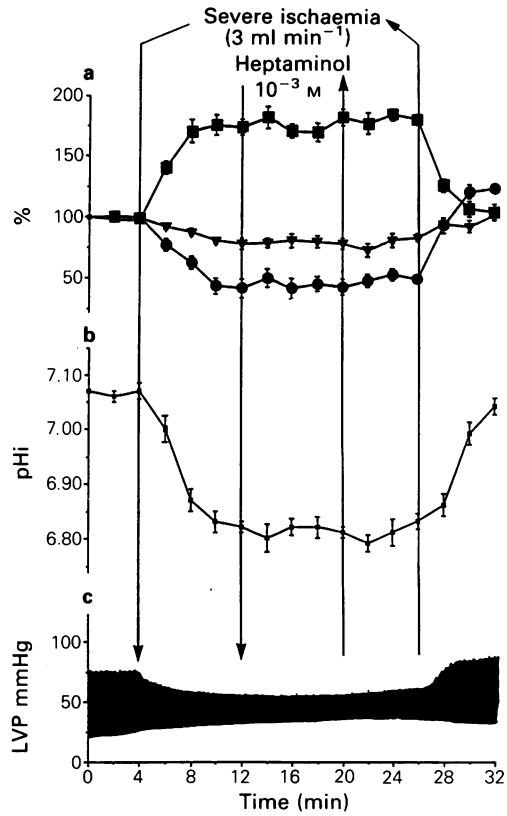

Figure 4 Time course of phosphocreatine (PCr), adenosine triphosphate (ATP), inorganic phosphate (Pi), intracellular pH (pHi) and left ventricular pressure (LVP) of stimulated (5 Hz) rat isolated hearts exposed to severe ischaemia (3 ml min^{-1}) after equilibration (30 min) in control conditions (10 ml min^{-1}) and treated with heptaminol 10^{-3} M. (a) Mean values of metabolic parameters from 5 experiments: PCr (●), ATP (▲) and Pi (■) expressed as percentage of initial value. (b) Mean values of pHi from same 5 experiments. In (a) and (b) vertical bars show s.e.mean. (c) LVP (mmHg) record from a representative experiment. Note the absence of effect of heptaminol on all the parameters.

versible damage, since the values of the metabolic parameters were fully recovered after suppression of ischaemia (see Figure 4).

Discussion

We have found that in hearts stimulated in normoxic conditions (rate flow 10 ml min^{-1}) heptaminol had no positive inotropic effect. However, a cyclic increase in systolic pressure was observed every 6 to 7 beats without arrhythmia. A similar cyclic variation of the contraction was obtained by Lab & Lee (1988) in ferret ventricular muscle. They showed that the spontaneous alternations in contraction are associated with a modulation of intracellular calcium cycling, involving the sarcoplasmic reticulum.

During moderate ischaemia, a positive inotropic effect of heptaminol was observed associated with normalization of pHi. This result cannot be interpreted as a consequence of catecholamine release, since an inotropic effect of heptaminol during moderate ischaemia was also obtained (result not shown;

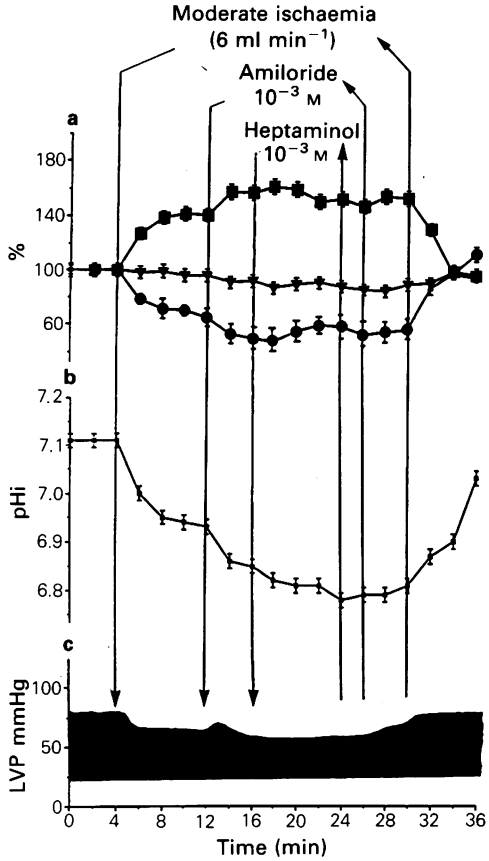

Figure 5 Time course of phosphocreatine (PCr), adenosine triphosphate (ATP), inorganic phosphate (Pi), intracellular pH (pHi) and left ventricular pressure (LVP) in stimulated rat isolated hearts exposed to moderate ischaemia (6 ml min^{-1}) and treated with amiloride (10^{-3} M. (a) Mean values of metabolic parameters from 4 experiments: PCr (●), ATP (▼) and Pi (■) expressed as percentage of initial values. (b) Mean values of pHi from same 4 experiments. In (a) and (b) vertical bars show s.e.mean. (c) LVP (mmHg) record from a representative experiment. In the presence of amiloride, the typical effect of heptaminol 10^{-3} M (realkalinization and positive inotropic effect) is no longer observed.

$n = 4$) in the presence of a β-adrenoceptor antagonist, pindolol (10^{-7} M). This inotropic effect could be related to an intracellular alkalosis. The mechanism of depression of the myocardial contractility by acidosis has been extensively examined (Steenbergen *et al.*, 1977; Vogel & Sperelakis, 1977; Poole-Wilson & Langer, 1979; Philipson *et al.*, 1982). Several possibilities have been postulated: intracellular acidosis could (1) decrease inward calcium current (Chesnais *et al.*, 1975; Kohlhardt *et al.*, 1976; Vogel & Sperelakis, 1977; Irisawa & Sato, 1986); (2) modulate calcium-induced calcium release from the sarcoplasmic reticulum (Fabiato & Fabiato, 1978; Fabiato, 1985; Orchard, 1987); (3) change the concentration of calcium stored in the sarcoplasmic reticulum (Fabiato, 1985) and (4) modify the calcium activation of the myofilaments by way of competition for the calcium binding sites on troponin by intracellular hydrogen ions (Williamson *et al.*, 1975; Blanchard & Solaro, 1984; Chapman, 1983).

In our experimental conditions, the intracellular acidosis induced by moderate ischaemia was associated with a negative inotropism; acidosis was always inhibited by heptaminol and realkalinization was always associated with a positive inotropic effect.

Two possibilities must be taken into consideration in speculating on the mechanism of action of heptaminol: (1) *The cell membrane is not permeable to heptaminol.* In this case, the realkalinization induced by heptaminol could be explained by a stimulation of the Na-H exchange. This would be in agreement with the results showing that in the presence of amiloride or guanidinium chloride (potent blockers of Na-H exchange) and under moderate ischaemia, heptaminol was unable to restore internal pH and inotropism. In the absence of these blockers, heptaminol induced a realkalinization which was always associated with a positive inotropic effect. In rat ventricular myocytes, it has been found that intracellular alkalosis is accompanied by an increase in the force of contraction. Furthermore, alkalosis produced a larger increase of contraction in cells which were initially contracting more weakly (Eisner *et al.*, 1987). (2) *The cell membrane is permeable to heptaminol.* In this case, the inotropic effect could be due to the presence of the alcohol function in the heptaminol molecule. It has been shown that alcohols which are able to interact with the membrane, can increase the inward calcium current in cardiac cells (Mongo, 1985) and the intracellular free calcium associated with a decrease in internal H^+ in squid axon (Vassort *et al.*, 1986).

In this connection, we have performed some experiments with 6-amino-2-methyl-heptane 10^{-5} M (Tuamine). This drug has a similar molecular structure to that of heptaminol but it lacks the alcohol groups. A negative inotropic effect was obtained with Tuamine, which suggests that the alcohol function is essential for the positive inotropism found with heptaminol.

However, this hypothesis conflicts with our results using Na-H exchange blockers which show an absence of effect of heptaminol.

The main mechanism of stimulation of the Na-H exchange is mediated by protein kinase C (PKC) (Castagna *et al.*, 1982). Thus the effect of heptaminol could be associated with a stimulation of PKC either directly or indirectly through phospholipase C

(PLC). The indirect effect could explain some of our results. In normoxia the turnover of Na-H exchange is reduced (Lazdunski *et al.*, 1985; L'Allemain & Pouyssegur, 1987) and this explains the absence of effect of heptaminol on intracellular pH. However, the stimulation of PLC will simultaneously induce an increase in inositol triphosphate (IP_3) which is known to activate calcium release from the sarcoplasmic reticulum. This would explain the cyclic variation of LVP induced by heptaminol in normoxia.

It should be pointed out that these cyclic variations of LVP, were also obtained in some preparations subjected to moderate ischaemia when the Na-H exchange had been blocked by amiloride or guanidinium chloride.

In moderate ischaemia without blockers, the Na-H exchange would be activated, allowing the restoration of the intracellular pH by heptaminol. The positive inotropic effect will be a consequence of this realkalinization and of IP_3 increase. Also, a stimulation of Na-H exchange will increase internal sodium which would stimulate the Na-Ca exchange and increase calcium entry.

In conclusion, our results show that a positive inotropic effect of heptaminol can be obtained under moderate ischaemic conditions. This effect appears to be concomitant with a restoration of intracellular pH, suggesting that it could be related to a stimulation of the Na-H exchange by heptaminol, mediated by PKC. This hypothesis is strengthened by the absence of any inotropic effect when, in moderate ischaemia, heptaminol was given with Na-H exchange blockers.

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Differences in the adenosine receptors modulating inositol phosphates and cyclic AMP accumulation in mammalian cerebral cortex

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1 2-Chloroadenosine stimulated adenosine 3':5'-cyclic monophosphate (cyclic AMP) accumulation and potentiated (guinea-pig) or inhibited (mouse) the histamine H_1-receptor-stimulated hydrolysis of inositol phospholipids in slices of guinea-pig and mouse cerebral cortex.

2 Two xanthine-based adenosine receptor antagonists were identified which were one order of magnitude more potent at the adenosine receptor mediating augmentation of the histamine-stimulated inositol phospholipid hydrolysis than at the receptor linked to cyclic AMP formation in guinea-pig cerebral cortical slices.

3 These compounds, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) and 7-benzyl-3-(2-methylpropyl)xanthine (BMPX) retained their selectivity under near-identical incubation conditions in both assays.

4 These compounds also showed similar affinities and selectivity for the adenosine receptor mediating inhibition of histamine-stimulated inositol phospholipid hydrolysis in mouse cerebral cortical slices.

5 Inclusion of agents which give rise to, or mimic, high levels of cyclic AMP (forskolin (1 μM), 8-bromo-cyclic AMP (1 mM) and vasoactive intestinal polypeptide (1 μM)) in the incubation medium failed to mimic the action of 2-chloroadenosine on inositol phospholipid turnover in cerebral cortical slices from either species.

6 These data suggest that the adenosine receptor modulating the hydrolysis of inositol phospholipid in mouse and guinea-pig cerebral cortical slices is different from the adenosine receptor linked to cyclic AMP formation, and, furthermore, that the modulation of inositol phospholipid metabolism in either species is not mediated via alterations in cyclic AMP levels.

Introduction

It has been shown recently that adenosine can modulate histamine-induced inositol phospholipid hydrolysis in mammalian cerebral cortical slices (Hollingsworth *et al.*, 1986; Hill & Kendall, 1987; Kendall & Hill, 1988). This effect of adenosine on inositol phospholipid hydrolysis is manifested as an augmentation of the response to histamine H_1-receptor stimulation in guinea-pig cerebral cortex (Hollingsworth *et al.*, 1986; Hill & Kendall 1987), but as an inhibition of the H_1 response in mouse (Kendall & Hill, 1988) and human (Kendall & Firth, 1989) cerebral cortex. In all cases, the modulatory effect of adenosine appears to be confined to histamine H_1-receptor responses, since the inositol phospholipid hydrolysis induced by noradrenaline, carbachol, 5-hydroxytryptamine or KCl is unchanged by adenosine receptor agonists (Hill & Kendall, 1987; Kendall & Hill, 1988). The identity of the adenosine receptor involved in these actions on inositol phospholipid is unclear. The rank order of agonist potencies is suggestive of the involvement of an A_1-type receptor in both guinea-pig and mouse brain (Hollingsworth *et al.*, 1986; Hill & Kendall, 1987; Kendall & Hill, 1988). However, the EC_{50} values for these agonists are all in the micromolar range and lie within a very narrow band, i.e. the potencies of N^6-cyclopentyladenosine and 5'-N-ethylcarboxamidoadenosine differ by only a factor of two in the guinea-pig, and between four and five in the mouse (Hill & Kendall, 1987; Kendall & Hill, 1988).

An interaction between adenosine and histamine on cyclic AMP accumulation is also seen in cerebral cortical slices from mouse and guinea-pig brain

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(Daly, 1977; Hill & Kendall, 1989). In this case, histamine H_1-receptor stimulation (and also stimulation of certain other calcium-mobilising receptors) leads to a potentiation of the cyclic AMP accumulation induced by adenosine analogues through an adenosine A_2-receptor (Daum *et al.*, 1982; Daly, 1977). This augmentation is presumably the result of some indirect action of H_1-receptors since there is no direct effect of H_1-receptor activation on cyclic AMP synthesis (Donaldson *et al.*, 1988b). A relationship between raised cyclic AMP levels and modulation of agonist-induced inositol phospholipid hydrolysis has been observed in a number of tissues, including airway smooth muscle (Hall *et al.*, 1989), platelets (Takai *et al.*, 1982; Watson *et al.*, 1984) and gastric mucosal cells (Puurunen *et al.*, 1987). This raises the possibility that the large accumulation of cellular cyclic AMP that occurs in the presence of both histamine and adenosine may mediate one or both of the actions of adenosine (augmentation or inhibition) on inositol phospholipid hydrolysis.

In the present study we have examined whether xanthine-based antagonists can be used to distinguish between the receptors responsible for the actions of adenosine on cyclic AMP and inositol phosphate accumulation in mammalian brain and whether there is a level of 'cross-talk' between the cyclic AMP and inositol phospholipid second messenger systems similar to that seen in peripheral tissues. A preliminary account of this work has been presented to the British Pharmacological Society (Alexander *et al.*, 1989).

Methods

[^3H]-cyclic AMP accumulation

The formation of ^{3}H-labelled cyclic AMP in [^{3}H]-adenine-prelabelled slices of mouse or guinea-pig cerebral cortical slices was assayed essentially as described previously (Donaldson *et al.*, 1988a). Briefly, after 60 min equilibration at 37°C with Krebs Henseleit buffer gassed with O_2:CO_2 (95:5), cross-chopped slices of cerebral cortex (350 × 350 μm) from mouse brain (black C57 strain; 25–40 g; either sex) or guinea-pig brain (Hartley strain; 200–600 g; either sex) were further incubated with 1.5 MBq of [^{3}H]-adenine for 40 min. After washing three times with Krebs-Henseleit buffer, gravity-packed slices (25 μl for mouse or 50 μl for guinea-pig) were then distributed into flat-bottomed vials containing antagonist where appropriate, and incubated for 20–30 min. Agonists were then added in 10 μl of medium to give a total volume of 300 μl. The incubation was terminated after 10 min (unless otherwise specified) with 200 μl of ice-cold 1 M HCl. [^{3}H]-cyclic AMP was then isolated by sequential Dowex-alumina chromatography as described previously (Donaldson *et al.*, 1988b).

Accumulation of ^{3}H-labelled inositol phosphates

The accumulation of ^{3}H-labelled inositol phosphates was assayed essentially as previously described (Brown *et al.*, 1984). Briefly, cross-chopped slices were equilibrated for 60 min as described above and then distributed as aliquots (25 μl for mouse or 50 μl for guinea-pig) into flat-bottomed vials for incorporation of [^{3}H]-inositol (4–12 kBq) in Krebs-Henseleit buffer containing 5 mM LiCl for 40 min. Where appropriate, antagonist drugs were added during this incubation. Agonist drugs were then added in 10 μl of medium to give a final volume of 300 μl, and the incubation was terminated after 45 min by the addition of 100 μl of ice-cold 10% (w/v) perchloric acid. After neutralisation with ice-cold 0.15 M KOH and centrifugation at 3000 g for 10 min, 0.75 ml aliquots of the supernatant layer were diluted to 3 ml with 50 mM Tris, pH 7.0, and added to columns of Dowex 1 anion exchange resin (X8, 100–200 mesh, chloride form). [^{3}H]-inositol was removed with 20 ml distilled water, and the total ^{3}H-labelled inositol phosphates were eluted with 3 ml 1 M HCl. Radioactivity was quantified by scintillation counting in the gel phase (Opti Phase, LKB).

Data analysis

Antagonist equilibrium dissociation constants (K_d) were estimated by two methods. Firstly, concentration-response curves to 2-chloroadenosine were generated in the absence and presence of a fixed concentration of antagonist. The K_d was then calculated from the parallel log concentration-response curves from the relationship:

$$K_d = A/(DR - 1)$$

where A is the antagonist concentration, and DR represents the ratio of the concentrations of 2-chloroadenosine required to give a specified response in the presence and absence of antagonist.

Second, increasing concentrations of 2-chloroadenosine were used to generate dose-response curves, and a concentration (C) of 2-chloroadenosine was chosen which gave a response greater than 50% of the maximal agonist response. The concentration of antagonist (A_{50}) required to reduce the response elicited by this concentration of agonist (C) to 50% of the maximal response elicited in the absence of antagonist was then determined and the K_d was then

calculated from the relationship (Lazareno & Roberts, 1987):

$$K_d = A_{50}/(C/EC_{50} - 1)$$

Chemicals

Cimetidine and 8-cyclopropyl-1,3-dimethylxanthine (8-cyclopropyltheophylline) were generous gifts from Smith, Kline & French and Abbott Laboratories, respectively. 7-Benzyl-3-(2-methylpropyl)xanthine (BMPX) and 1,3-diethyl-8-phenylxanthine were synthesized by Dr R.J. Grout in the Department of Pharmaceutical Sciences, University of Nottingham. Adenosine deaminase (183 u mg^{-1}) and 1,3-dimethyl-8-phenylxanthine (8-phenyltheophylline) were purchased from Sigma. 8-Cyclopentyl-1,3- dipropylxanthine (DPCPX) was purchased from Research Biochemicals Incorporated. [2-^{3}H]-inositol (529 GBq mmol^{-1}) and [8-^{14}C]-cyclic AMP (1.6 GBq mmol^{-1}) were obtained from New England Nuclear. [8-^{3}H]-adenine (888 GBq mmol^{-1}) was obtained from Amersham International.

Results

Augmentation of inositol phosphates accumulation in guinea-pig cerebral cortex

Despite having no effect alone, 2-chloroadenosine potentiated the accumulation of [^{3}H]-inositol phosphates induced by histamine (0.3 mM) over 40–45 min in guinea-pig cerebral cortex slices, as previously described (Hill & Kendall, 1987). The 2-chloroadenosine-mediated augmentation of the histamine response was antagonized by five xanthine-based compounds with K_d values varying from 10 nM to 5 μM (Table 1). 8-Cyclopentyl-1,3-dipropylxanthine (DPCPX) was the most potent antagonist (K_d = 0.010 ± 0.002 μM), while the least potent compound tested was 7-benzyl-3-(2-methylpropyl)xanthine (BMPX) with a K_d value of 5.0 ± 1.6 μM (Table 1). None of the five antagonists showed a significant alteration in the histamine-induced inositol phosphate response at the concentrations employed (e.g. Figure 3).

Stimulation of cyclic AMP formation in guinea-pig cerebral cortex

2-Chloroadenosine stimulated the formation of [^{3}H]-cyclic AMP in guinea-pig cerebral cortical slices (10 min agonist incubation time) in a concentration-dependent manner, as previously described (Daly, 1977). DPCPX was the most potent of the antagonists studied with a K_d value (0.082 ± 0.008 μM, Table 1) which was significantly greater ($P < 0.05$, Student's t test) than that obtained from studies of inositol phosphate accumulation ($n = 3$, Table 1). Two of the other four compounds examined in this study had K_d values which were not significantly different for the two assay systems, however BMPX and 8-cyclopropyltheophylline were also able to discriminate between the two responses to 2-chloroadenosine (Table 1). At the concentrations used, all of the antagonists had little effect on basal cyclic AMP formation (e.g. see legend to Figure 2).

The determination of K_d values for the two most discriminatory compounds, DPCPX and BMPX, as antagonists for the adenosine receptor-mediated cyclic AMP and inositol phosphates responses were subsequently carried out under near-identical conditions. The agonist incubation period for the assay of [^{3}H]-cyclic AMP was extended to 40–45 min to match that of the inositol phosphate assay. This led to no alteration in the concentration-response profile for 2-chloroadenosine (data not shown), in keeping with our previous observations that the steady state

Table 1 Antagonism of 2-chloroadenosine-mediated augmentation of inositol phosphate and cyclic AMP accumulation in guinea-pig cerebral cortical slices

	K_d (μM)		
Compound	*Cyclic AMP* (A)	*Inositol phosphates* (B)	*Ratio* (A/B)
DPCPX	0.082 ± 0.008	0.010 ± 0.002	8.2*
1,3-Diethyl-8-phenylxanthine	0.20 ± 0.03	0.60 ± 0.20	0.33
8-Phenyltheophylline	0.40 ± 0.20	0.80 ± 0.10	0.50
8-Cyclopropyltheophylline	2.3 ± 0.2	0.50 ± 0.10	4.6*
BMPX	40.0 ± 8.0	5.0 ± 1.6	8.0*

DPCPX = 8-cyclopentyl-1,3-dipropylxanthine; BMPX = 7-benzyl-3-(2-methylpropyl)xanthine.
Equilibrium dissociation constants (K_d) for xanthine derivatives for antagonism of 2-chloroadenosine-stimulated [^{3}H]-cyclic AMP formation (agonist incubation time 10 min) and 2-chloroadenosine-potentiated histamine-induced [^{3}H]-IP accumulation (agonist incubation time 40–45 min) in guinea-pig cerebral cortical slices. Values are mean ± s.e.mean of 3–4 separate experiments carried out with 3–4 replicates.
* $P < 0.05$; Student's t test; inositol phosphate K_d versus cyclic AMP K_d.

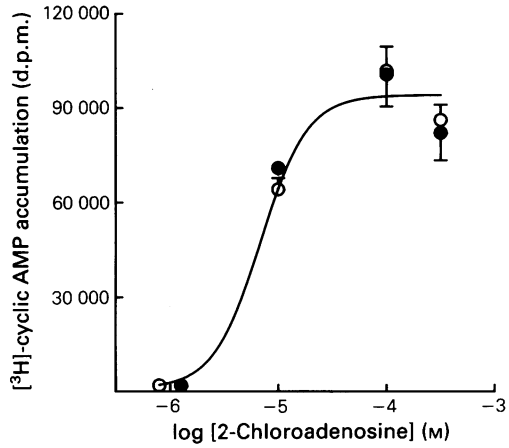

Figure 1 The effect of LiCl (5 mM) on 2-chloroadenosine-stimulated [^{3}H]-cyclic AMP accumulation in guinea-pig cerebral cortical slices. Agonist incubations were for 40–45 min in the presence of histamine (0.3 mM), cimetidine (0.3 mM) and adenosine deaminase (1 u ml^{-1}). Data represent mean of triplicate determinations obtained in the absence (○) and presence of LiCl (●) in a single experiment; vertical bars show s.e.mean. Where no error bars are shown, the error is less than the symbol size. Basal [^{3}H]-cyclic AMP formation was 864 ± 79 d.p.m. and 830 ± 47 d.p.m. in the absence and presence of LiCl, respectively.

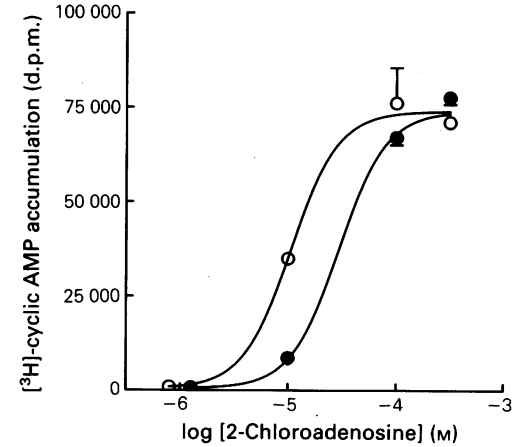

Figure 2 Antagonism by 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) of 2-chloroadenosine-stimulated [^{3}H]-cyclic AMP formation in guinea-pig cerebral cortical slices. Incubations were for 40 min in the presence of histamine (0.3 mM), cimetidine (0.3 mM), adenosine deaminase (1 u ml^{-1}), and LiCl (5 mM). Measurements were made in the absence (○) and presence of 500 nM DPCPX (●). Data represent mean of triplicate determinations in a single experiment; vertical bars show s.e.mean. Similar results were obtained in two further experiments. Basal [^{3}H]-cyclic AMP formation was 457 ± 5 and 492 ± 4 d.p.m. in the absence and presence of DPCPX, respectively.

levels of cyclic AMP stimulated by 2-chloroadenosine are well maintained in this tissue (Donaldson *et al.*, 1988b). Histamine (0.3 mM) and LiCl (5 mM) were also added to the incubation medium for the measurement of [^{3}H]-cyclic AMP. The incubations also contained cimetidine (0.3 mM) to preclude direct stimulation of [^{3}H]-cyclic AMP formation via histamine H_2-receptors (Hegstrand *et al.*, 1976), and adenosine deaminase (1 u ml^{-1}) to negate any effects of endogenous adenosine.

In the presence of adenosine deaminase and cimetidine, histamine decreased the EC_{50} for 2-chloroadenosine from 45.0 ± 4.2 μM ($n = 5$) to 22.6 ± 2.2 μM ($n = 7$), while markedly increasing the maximal response of this compound from 15,660 ± 75 d.p.m. ($n = 4$) to 50,360 ± 160 d.p.m. ($n = 7$). This confirms the results of previous findings (Daum *et al.*, 1982; Hollingsworth & Daly, 1985; Hollingsworth *et al.*, 1986; Donaldson *et al.*, 1988b). The further inclusion of LiCl (5 mM) in the [^{3}H]-cyclic AMP accumulation assay led to no significant alteration in either the EC_{50} or the maximal response of 2-chloroadenosine (Figure 1). Under these near-identical incubation conditions, BMPX and DPCPX retained their ability to discriminate between the two responses to 2-chloroadenosine (Table 2). Thus, as illustrated in Figure 2, 500 nM DPCPX induced a small rightward shift in the 2-chloroadenosine stimulatory response for cyclic AMP levels, while a lower concentration (100 nM) produced a much larger shift in the 2-chloroadenosine concentration-response curve for augmentation of the histamine H_1-receptor-mediated inositol phosphates response (Figure 3). In the case of DPCPX, which proved to be approximately ten fold more potent as an antagonist of the adenosine receptor potentiating inositol phospholipid hydrolysis, K_d values were calculated by both of the methods described under Methods with similar results (Table 2).

Mouse cerebral cortical slices

In mouse cerebral cortical slices, the maximal response to 2-chloroadenosine with regard to cyclic AMP formation was low and often poorly defined. In the mouse, as in the guinea-pig, histamine was found to potentiate the maximal response of cyclic AMP, and to give better defined concentration-response curves. For example, in one experiment, the response to 0.3 mM 2-chloroadenosine was increased from 2802 ± 271 d.p.m. to 7692 ± 499 d.p.m. by the addition of 0.3 mM histamine (similar data were

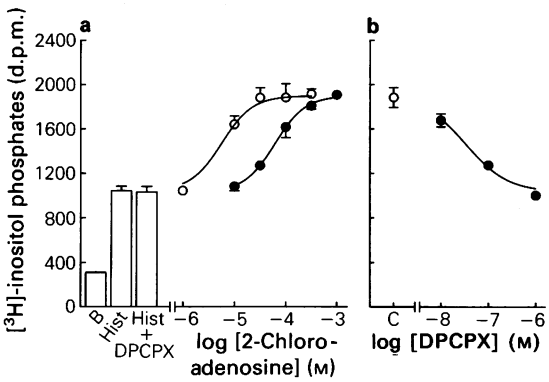

Figure 3 Antagonism by 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) of the 2-chloroadenosine augmentation of histamine-induced [^{3}H]-inositol phosphates accumulation in guinea-pig cerebral cortical slices. Incubation times were 45 min in the presence of cimetidine (0.3 mM), adenosine deaminase (1 u ml^{-1}) and LiCl (5 mM). Data represent means of triplicate determinations in a single experiment; vertical bars show s.e.mean. Similar data were obtained from two further experiments. (a) Basal (B) and histamine-stimulated (0.3 mM) [^{3}H]-inositol phosphate accumulation in the absence (Hist) and presence of 100 nM DPCPX (Hist + DPCPX) are shown by the columns. Concentration-response curves to 2-chloroadenosine (in the constant presence of 0.3 mM histamine) were obtained in the absence (○) and presence of 100 nM DPCPX (●). (b) Inhibition of the augmented accumulation of [^{3}H]-inositol phosphates induced by 30 μM 2-chloroadenosine (○) by increasing concentrations of DPCPX (●). Histamine (0.3 mM) was present in all incubations.

obtained in two other experiments). Consequently, measurements of antagonist K_d values were only performed in the presence of H_1-receptor stimulation (Table 2).

In mouse cerebral cortical slices adenosine analogues inhibit histamine-stimulated inositol phospholipid hydrolysis (Figure 4; Kendall & Hill, 1988). However, the K_d values obtained for BMPX and DPCPX were similar to those obtained from antagonism of the potentiation of histamine-induced inositol phospholipid hydrolysis in guinea-pig brain (Table 2). Furthermore, BMPX and DPCPX were respectively 19 and 10 fold more potent at the receptor mediating inhibition of inositol phospholipid metabolism in the mouse than at the receptor involved in the stimulation of cyclic AMP levels (Table 2).

Effect of cyclic AMP stimulants

Agents that stimulate an increase in cyclic AMP levels (vasoactive intestinal polypeptide and forskolin), or that simulate high cyclic AMP levels (8-bromo-cyclic AMP) were unable to mimic the effects of adenosine analogues on histamine-stimulated inositol phospholipid hydrolysis in either guinea-pig or mouse cerebral cortex (Figure 5).

Discussion

Initial experiments using guinea-pig cerebral cortical slices identified two adenosine receptor antagonists, DPCPX and BMPX, which appeared to distinguish between two second messenger responses to 2-

Table 2 Antagonism of 2-chloroadenosine-mediated stimulation of cyclic AMP and augmentation or inhibition of inositol phosphates accumulation in guinea-pig and mouse cerebral cortical slices

	K_d (μM)		
	Cyclic AMP (− histamine)	*Cyclic AMP* (+ histamine)	*Inositol phosphates* (+ histamine)
Guinea-pig			
DPCPX[1]	0.073 ± 0.006 (3)	0.131 ± 0.026 (4)	0.012 ± 0.004* (3)
DPCPX[2]	NA	0.149 ± 0.036 (3)	0.008 ± 0.002* (4)
BMPX[1]	77.0 ± 6.3 (3)	53.0 ± 1.0 (3)	10.7 ± 0.7* (3)
Mouse			
DPCPX[1]	NA	0.113 ± 0.013 (5)	0.010 ± 0.003* (4)
DPCPX[2]	NA	0.220 ± 0.052 (3)	0.024 ± 0.004* (5)
BMPX[1]	NA	150.0 ± 21.0 (3)	8.0 ± 3.2* (3)

DPCPX = 8-cyclopentyl-1,3-dipropylxanthine; BMPX = 7-benzyl-3-(2-methylpropyl)xanthine.
Equilibrium dissociation constants (K_d) were determined in the presence or absence of 0.3 mM histamine. 5 mM LiCl, 0.3 mM cimetidine and 1 u ml^{-1} adenosine deaminase were present in all incubations. Agonist incubation times were 40–45 min throughout. Values are means ± s.e.mean of triplicate determinations from the number of separate experiments indicated in parentheses. K_d values were determined using either a fixed antagonist concentration[1] or by the method of Lazareno & Roberts (1987)[2] as described under *Methods*. NA = not available.
* $P < 0.05$; Student's t test inositol phosphates K_d versus cyclic AMP K_d in the presence of histamine.

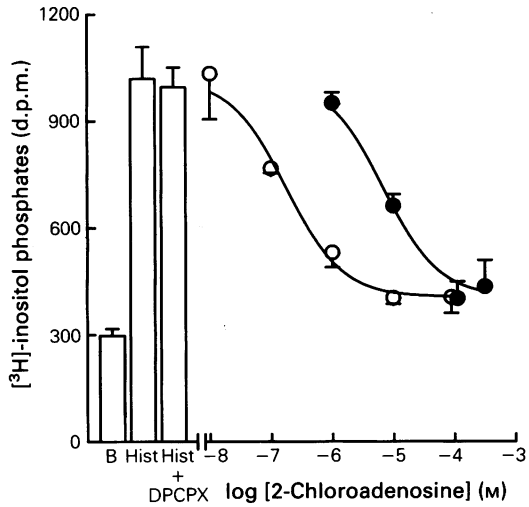

Figure 4 Antagonism by 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) of the 2-chloroadenosine inhibition of histamine-induced [^{3}H]-inositol phosphates accumulation in mouse cerebral cortical slices. Incubations were for 45 min and contained cimetidine (0.3 mM), adenosine deaminase (1 u ml^{-1}), and LiCl (5 mM). Data represent mean of triplicate determinations in a single experiment; vertical bars show s.e.mean. Similar data were obtained in two further experiments. Basal (B), histamine-stimulated (0.3 mM) (Hist) and histamine-stimulated [^{3}H]-inositol phosphates accumulation in the presence of 100 nM DPCPX (Hist + DPCPX) are shown by the columns. Concentration-response curves to 2-chloroadenosine (in the constant presence of 0.3 mM histamine) were obtained in the absence (○) and presence of 100 nM DPCPX (●).

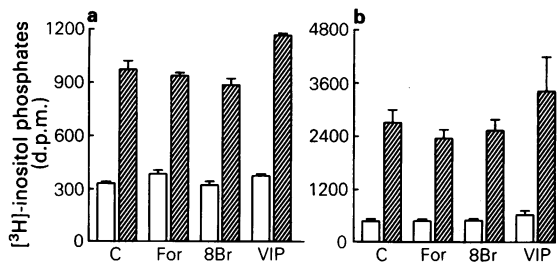

Figure 5 The effect of cyclic AMP stimulants or a cyclic AMP analogue on [^{3}H]-inositol phosphates accumulation in (a) guinea-pig or (b) mouse cerebral cortical slices. Forskolin (For, 1 μM), 8-bromo-cyclic AMP (8 Br, 1 mM) and vasoactive intestinal polypeptide (VIP, 1 μM) were present during the determination of triplicate determinations of basal (open bars) and histamine-stimulated (0.3 mM, shaded bars) [^{3}H]-inositol phosphates accumulation. Values represent means ± s.e.mean from three separate experiments conducted in quadruplicate.

chloroadenosine. That is, they were approximately 10 fold more potent as antagonists of the receptor augmenting histamine H_1-receptor-induced inositol phospholipid turnover compared with their potencies as antagonists of the receptor which stimulates cyclic AMP accumulation. However, the standard protocols for the two assays differ in certain aspects: the [^{3}H]-cyclic AMP assay is carried out in the absence of lithium ions for 10 min, whereas the [^{3}H]-inositol phosphate assay includes histamine and lithium in the incubation medium, with agonist incubation times of 45 min. Since it could be argued that the apparent differences in antagonist affinities are artifacts resulting from these procedural differences, the assays were standardized to common conditions.

Histamine is able to increase the formation of cyclic AMP in guinea-pig brain slices by both direct, via H_2-receptors (Hegstrand *et al.*, 1976; Palacios *et al.*, 1978), and indirect, via H_1-receptors, mechanisms (Daum *et al.*, 1982; Donaldson *et al.*, 1988b; Hill & Kendall, 1989). Consequently, cimetidine, a selective H_2-receptor antagonist (Green *et al.*, 1977), was included in the assays to prevent the direct H_2-receptor-mediated stimulation of cyclic AMP formation. Adenosine deaminase was also included to preclude any effects of endogenous adenosine. Neither cimetidine nor adenosine deaminase altered the affinities of DPCPX or BMPX for the adenosine receptor which augments the H_1-receptor inositol phospholipid response (Tables 1 and 2). In the assay for cyclic AMP, histamine H_1-receptor stimulation increased the apparent efficacy of 2-chloroadenosine as expected (Daly, 1977; Hollingsworth & Daly, 1985; Donaldson *et al.*, 1988b), and also decreased the EC_{50} for the agonist, although we are as yet unable to explain this latter effect.

The ability of DPCPX and BMPX to discriminate between the two adenosine receptor-mediated responses was retained under virtually identical assay conditions in guinea-pig cerebral cortical slices (Table 2). Similarly, in mouse cerebral cortical slices, DPCPX and BMPX were between 9 and 19 fold more potent at the adenosine receptor modulating inositol phospholipid turnover than at the cyclic AMP-stimulating receptor. From this it seems reasonable to conclude that the adenosine receptor which modulates the histamine H_1-receptor inositol phospholipid response is different from the A_2-receptor positively linked to cyclic AMP formation in both species. It also seems possible that the adenosine receptors augmenting inositol phospholipid metabolism in guinea-pig and inhibiting the same histamine response in mouse are very similar, if not identical.

The data obtained in this study further suggest that cyclic AMP does not mediate the effects of 2-

chloroadenosine on inositol phospholipid hydrolysis in cerebral cortical slices. The literature suggests some species or tissue differences in the effects of elevated cyclic AMP levels on inositol phospholipid metabolism. In platelets, lymphocytes and neutrophils, cyclic AMP appears to decrease receptor-induced inositol phospholipid hydrolysis (Kaibuchi *et al.*, 1982; Watson *et al.*, 1984; Della Bianca *et al.*, 1986; Jakobs *et al.*, 1986). In contrast, in rat vas deferens, in which adenosine augments α_1-adrenoceptor-induced inositol phospholipid hydrolysis (Häggblad & Fredholm, 1987), the elevation of cyclic AMP levels or the use of cyclic AMP analogues failed to alter phenylephrine-stimulated inositol phospholipid breakdown. Our results using forskolin, vasoactive intestinal polypeptide, and 8-bromo-cyclic AMP indicate that accumulation of [^{3}H]-inositol phosphates in both guinea-pig and mouse cerebral cortical slices is unaffected by either raised cyclic AMP levels or the presence of cell-permeant cyclic AMP analogues. This is in accord with the results of Hollingsworth & Daly (1985) who reported no effect of forskolin on basal inositol phospholipid hydrolysis, or on that induced by noradrenaline, carbachol or histamine plus 2-chloroadenosine in guinea-pig cerebral cortical slices.

In this study, we have been able to distinguish by pharmacological means the adenosine receptor which modulates histamine-stimulated inositol phospholipid hydrolysis from the A_2-receptor which stimulates cyclic AMP accumulation. We have previously been able to show that the intracellular P-site is not involved (Hill & Kendall, 1987; Kendall & Hill, 1988), since methylxanthines are ineffective at this latter adenosine 'receptor'. The rank order of agonist potencies for the receptor modulating inositol phospholipid turnover is similar to that for the A_1-receptor, but, as previously noted, the absolute potencies of agonists are considerably lower than those published for the A_1-receptor in other systems (Hill & Kendall, 1987). Since DPCPX shows a greater than 700 fold selectivity for the A_1-receptor compared to the A_2-receptor (in both radioligand binding and adenylate cyclase studies; Bruns *et al.*, 1987; Lohse *et al.*, 1987) it was hoped that this compound would be useful in the identification of the inositol phospholipid-modulating adenosine receptor. However, the affinities of DPCPX at this receptor (K_d 8–24 nM, Tables 1 and 2) are inconsistent with the involvement of either A_1- ($K_d \leqslant 0.5$ nM) or A_2- (K_d 330–340 nM) receptors (Bruns *et al.*, 1987; Lohse *et al.*, 1987), although it is possible that these differences arise from differences in the tissue preparations employed, since the hydrophobic nature of the antagonists used may well lead to sequestration in structures not found in the disrupted cell.

In summary, the adenosine receptor responsible for the positive modulation of histamine-stimulated inositol phospholipid breakdown in guinea-pig cerebral cortical slices cannot be distinguished from that which inhibits the same response in mouse brain. This receptor is, however, different from the adenosine receptor which mediates a stimulation in cyclic AMP formation; a contention supported by the lack of effect of elevated levels of cyclic AMP or cyclic AMP analogues on the inositol phospholipid response in either species. It seems likely that the advent of more selective adenosine receptor antagonists or the application of molecular biological techniques will be necessary to classify positively the adenosine receptor which modulates CNS inositol phospholipid metabolism.

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The spontaneous electrical and mechanical activity of human bronchial smooth muscle: its modulation by drugs

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1 Tissue taken at operation was used to study the electrical and mechanical properties of human bronchial smooth muscle with intracellular microelectrodes and isometric recording of tension changes.

2 Over 90% of the muscle strips exhibited spontaneous tone and 70% produced spontaneous phasic contractions. The resting membrane potential of the smooth muscle cells ranged between -40 to -50 mV with a mean value of -44.9 ± 5.2 mV ($n = 92 \pm$ s.d.). Spontaneous oscillations of the membrane potential (slow waves) were observed in 90% of the cells examined.

3 The electrical slow waves, phasic contractions and spontaneous tone were greatly reduced by FPL 55712 (10^{-6}–10^{-5} M). Indomethacin ($1–5 \times 10^{-5}$ M), atropine (10^{-6} M) or a 5-lipoxygenase inhibitor, AA 861 (5×10^{-6} M) each reduced spontaneous mechanical tone. Indomethacin and atropine each caused minor reduction in the amplitude of electrical slow waves.

4 Leukotriene C_4 (10^{-8} M), physostigmine (10^{-6} M) and K^+-rich physiological salt-solution (containing atropine 10^{-6} M) each caused tone development in tissue treated with AA 861 (5×10^{-6} M). In the case of leukotriene C_4 and physostigmine, phasic contractions were superimposed on the developed tone.

5 Electrical field stimulation evoked an excitatory junction potential (e.j.p.) followed by a small group of slow waves. Repetitive field stimulation (2–20 stimuli at 20 Hz) markedly enhanced the amplitude of oscillatory slow waves. FPL 55712 (1.9×10^{-6} M) abolished the oscillatory slow waves following the e.j.p., and physostigmine (10^{-6} M) enhanced the amplitude of the e.j.p. and slow waves.

6 These results indicate that, under *in vitro* conditions, the electrical activity of human bronchial smooth muscle comprises slow wave discharge which may be related to the spontaneous contractions and generation of basal tone.

Introduction

Smooth muscle taken from the large airways of several mammalian species has a stable resting membrane potential and lacks spontaneous mechanical activity (Widdicombe, 1963; Small & Foster, 1987). However, spontaneous mechanical activity has been recorded from guinea-pig trachea *in vivo* and *in vitro* (Souhrada & Dickey, 1976). Furthermore, spontaneous oscillations of the membrane potential (slow waves) have been recorded from guinea-pig and bovine trachea (McCaig & Souhrada, 1980; Kirkpatrick, 1981; Small, 1982; Allen *et al.*, 1986).

It has been observed that spontaneous mechanical activity occurs in roughly 10% of human tracheal or bronchial muscle specimens examined *in vitro* (Davis *et al.*, 1982). A more recent report showed that spontaneous contraction developed in 4 out of 5 human tissues, within 25 min of the start of tissue incubation in an organ bath (Chideckel *et al.*, 1987). However, in these two studies, the human tracheal tissue originated mainly from cadavers. Therefore it is conceivable that the observed spontaneous phasic mechanical activity was a consequence of anoxic tissue damage.

The spontaneous basal mechanical tone of airway smooth muscle seems to vary greatly between different species. In canine isolated tracheal muscle, for example, the basal tone appears to be very small (Stephens *et al.*, 1975; Kannan & Daniel, 1980). On the other hand, guinea-pig isolated trachealis develops substantial tone which is abolished by

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Table 1 Clinical and pulmonary function data of subjects from whom samples of bronchial smooth muscle were obtained

Number of patients	*Age* ± s.d.	*Smoking status* (B.I.) ± s.d.	*VC* ± s.d.	$FEV_{1.0}$% ± s.d.
Males 32	60.9 ± 10.3	687 ± 598	98.7 ± 14.4	79.1 ± 9.3
Females 10	(32–86)	(0–2040)	(63–125)	(39–95)
TLC ± s.d.	Pao_2 ± s.d.	$Paco_2$ ± s.d.	ΔN_2 ± s.d.	D_{LCO} ± s.d.
102.3 ± 11.8	86.5 ± 7.0	39.4 ± 3.9	1.4 ± 1.2	103.3 ± 16.2
(80–126)	(72.4–100)	(27–44)	(0.45–4.7)	(67–129)

Data indicate mean ± s.d. (upper row) and range (lower row) of values for age, smoking status (B.I.), VC (vital capacity), $FEV_{1.0}$% (forced expiratory volume in 1 s), TLC (total lung capacity), Pao_2, $Paco_2$, ΔN_2 (percentage change in N_2 concentration per litre during phase 3 of the single-breath N_2 washout curve) and D_{LCO} (diffusion capacity) of 42 patients.

indomethacin, suggesting that tone is prostanoid-induced (Farmer *et al.*, 1974; Orehek *et al.*, 1975; Brink *et al.*, 1981; Kawanishi *et al.*, 1984). Spontaneous mechanical tone has also been observed in human tracheal and bronchial smooth muscles (Brink *et al.*, 1980; Huta's *et al.*, 1981; Davis *et al.*, 1982; Ito *et al.*, 1985). However, the effects of indomethacin on the spontaneous tone of human airways smooth muscle are the subject of some debate. Some workers have shown that indomethacin does not significantly change the basal tone of either fresh or stored human muscle (Brink *et al.*, 1980; Davis *et al.*, 1982). Others have shown that indomethacin increases the basal tone and potentiates the action of histamine or Ca^{2+} (Huta's *et al.*, 1981; Ito *et al.*, 1985). There are also reports indicating that indomethacin suppresses the basal tone of human bronchial smooth muscle strips (Dunlop & Smith, 1975).

Thus, although there have been several pharmacological studies of human tracheal and bronchial smooth muscle, the mechanism underlying spontaneously generated mechanical activity remains to be clarified. The present experiments were carried out to examine the electrical and mechanical properties of human bronchial smooth muscle in tissue taken at operation. They included examination of the actions of cyclo-oxygenase and 5-lipoxygenase inhibitors, leukotriene antagonists and calcium antagonists.

Methods

Tissue preparation and status

Human bronchial strips were obtained by procedures which conformed to the Declaration of Helsinki. At the time of thoracic surgery, macroscopically normal lung tissue was obtained from patients with lung cancer and the tissues were immediately placed in well-oxygenated Krebs solution. After rapid transportation to the laboratory, bronchi (internal diameter 3 to 5 mm) were dissected free of lung parenchyma, and were usually stored overnight in a large volume of cooled (4°C) Krebs solution. This procedure was designed to wash out drug residues and any substances liberated during the dissection. The pH of the solution after overnight storage ranged between 7.2–7.3.

The bronchi were cut open and muscle strips were carefully freed from cartilage and epithelium, under a binocular microscope. The muscle strips (width 1–1.5 or 2–3 mm; length 1–2 or 3–5 mm; thickness 0.2–0.5 mm) were composed of several muscle bundles. The small strips were used for tension recording and the large strips were used for microelectrode experiments.

The range and mean values of age, smoking status (Brinkman's index; Brinkman & Block, 1966), vital capacity (VC), forced expiratory volume in one second ($FEV_{1.0}$%) total lung capacity (TLC), Pao_2, $Paco_2$, percentage change in nitrogen concentration per litre during phase 3 of the single-breath nitrogen wash out curve (ΔN_2) and diffusion capacity (D_{LCO}), of the 42 patients are given in Table 1. None of the patients had a history of chronic obstructive pulmonary disease, including asthma.

Mechanical studies

To measure the mechanical changes, the tissue was mounted in a 1 ml organ bath through which the test solution, at a temperature of 35°C, flowed continuously and the solutions were changed by use of a multi-way tap. The preparation was arranged vertically and the ends were tied with silk thread. One end of the tissue strip was tied to a mechano-

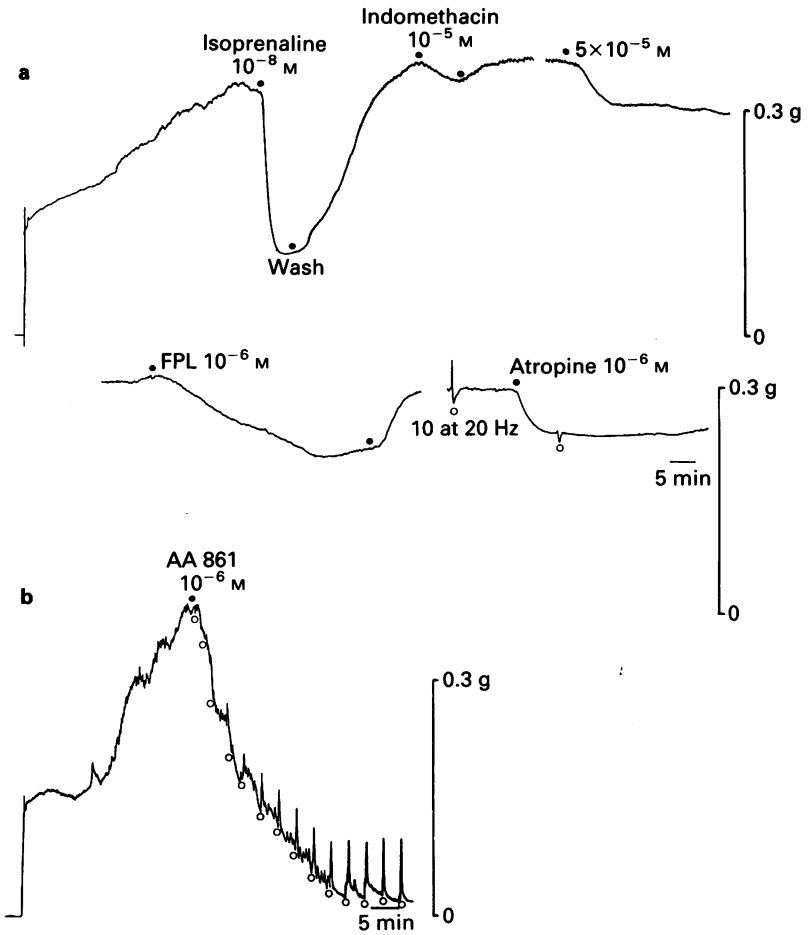

Figure 1 Effects of isoprenaline (10^{-8} M), indomethacin (10^{-5} and 5×10^{-5} M) FPL 55712 (10^{-6} M), atropine (10^{-6} M) and AA 861 (10^{-6} M) on the spontaneously generated muscle tone (isometric recording) of human bronchus. (a) Effects of isoprenaline, indomethacin, FPL and atropine each tested in the same preparation. Indomethacin was washed off before the application of FPL. (b) Effects of AA 861 tested in a different preparation: (●) indicate the application or removal of the drugs, and (○) indicate the application of field stimulation (10 stimuli at 20 Hz). Field stimulation with short duration (500 μs) pulses evoked twitch contractions with (a) or without (b) secondary relaxation.

transducer (Nihon-Kohden Ltd., RCA-5734) and the other end to a hook at the bottom of the bath. The strips were set up under an initial tension of 0.1–0.2 g and were equilibrated for 1 to 3 h with Krebs solution. Preliminary experiments, where the amplitude of twitch tension evoked by field stimulation (5, 10, 20 and 30 stimuli at 20 Hz; 500 μs duration; 10–20 V strength) was used as an indicator of the contractile response, showed that an imposed tension of 0.1–0.2 g resulted in optimal isometric force development in each bronchial muscle strip. The resting tension giving the maximal twitch response was determined and used in each preparation.

Electrophysiological studies

For intracellular recording, the mucosal membrane covering the luminal surface was removed and a microelectrode filled with 3 M KCl (30–50 MΩ electrode resistance; tip potential less than 5 mV) was inserted from the luminal surface of the preparation.

In each preparation 3–10 cellular impalements were obtained; 1–2 preparations from each patient (total of 46 preparations) were used. The chamber in which the muscle preparation was mounted had a volume of 2 ml, and was perfused at a rate of 3 ml min^{-1} and at a temperature of 35–36°C.

Electrical stimulation of intramural nerves

To produce neurogenic responses, field stimulation was applied through a pair of silver plates 5 mm apart and placed so that a current pulse would pass transversely across the tissue. Single and repetitive stimuli at 20 Hz were applied, with a pulse of 500 μs duration and 10–20 V strength.

Drugs and solutions/statistical analysis of data

The muscle strips were bathed in a modified Krebs solution of the following ionic composition (mM): Na^+ 137.4, K^+ 5.9, Mg^{2+} 1.2, Ca^{2+} 2.5, Cl^- 134.0, $H_2PO_4^-$ 1.2, HCO_3^- 15.5 and glucose 11.5. The solution was equilibrated with 97% O_2 and 3% CO_2 and the pH was adjusted to 7.3–7.4. The following drugs were used: indomethacin, atropine sulphate (Sigma), 7-[3-(4-acetyl-3-hydroxy-2-propylphenoxy)-2-hydroxypropoxy]-4-oxo-8-propyl-4H-1-benzopyran-2-carboxylic acid (FPL 55712, Fisons), 2,3,5-trimethyl-6-(12-hydroxy-5,10,dodecadinyl)-1,4-benzoquinone-(AA 861) (Takeda Pharmac. Co.), gallopamil (Sigma) and (−)isoprenaline (+)-bitartrate (Nakai Chemicals).

Na_2CO_3 was used to dissolve indomethacin i.e. equivalent amounts of indomethacin and Na_2CO_3 were dissolved in distilled water at a concentration of 10^{-2} M before addition to the saline. The final concentrations of indomethacin was in the range 5×10^{-5}–10^{-6} M, and at these concentrations the vehicle had no effect on electrical and mechanical activities of the muscle tissues. On the other hand, FPL 55712 and AA 861 were dissolved in ethanol (50%) at a concentration of 10^{-2} M. The final concentration of FPL 55712 and AA 861 ranged between 10^{-5}–10^{-6} M, and at these concentrations ethanol (0.5–0.05%) showed no effect on electrical and mechanical activities of the muscle tissues.

Results (amplitude of contractions and e.j.ps) are expressed as mean ± s.d. and were analyzed for statistical significance by Student's *t* test.

Results

Spontaneous tone and phasic mechanical activity of human bronchial smooth muscle

After mounting the preparations of human bronchus in the organ bath, tension gradually developed during the first 30–60 min. This spontaneous tone reached a value of 0.3–0.5 g. While tone fluctuated somewhat, it was reasonably well maintained.

Since it is known that prostaglandins and leukotrienes play an important role in the regulation of airway smooth muscle tone, it was of interest to observe the effects of cyclo-oxygenase and 5-lipoxygenase inhibitors (indomethacin and AA 861 respectively) and the leukotriene antagonist, FPL 55712.

Figure 1 shows examples of spontaneous tension development and the effects of isoprenaline, indomethacin, FPL 55712, atropine and a 5-lipoxygenase inhibitor, AA 861 (Yoshimoto *et al.*, 1982). The preparations were initially subjected to imposed tension of approximately 0.1–0.2 g (see Methods), and allowed to develop spontaneous tone. Tone fluctuated and a steady level was not obtained. After equilibration for 45 min (Figure 1a), isoprenaline (10^{-8} M) was applied to relax the tissue, and the amplitude of relaxation was defined as a relative relaxation of 1.0.

When indomethacin (10^{-5} or 5×10^{-5} M) was applied, tone was consistently reduced and the amplitude of the relaxation was in the range 0.1 to 0.5 (Figure 1a). Similarly, FPL 55712 (10^{-6} M), AA 861 (10^{-6} M), or atropine (10^{-6} M) each reduced the muscle tone, and in the presence of these drugs a steady basal tone was maintained. Combined application of indomethacin (10^{-5} M) and FPL 55712 (10^{-5} M) markedly suppressed tone. The mean relaxation was 0.82 ± 0.10 (±s.d., $n = 10$), and a stable basal tone without fluctuations was obtained. Figure 2 summarizes the effects of indomethacin, FPL 55712, atropine, AA 861 and gallopamil on the spontaneously generated muscle tone of human bronchus. These results indicate that the metabolites of cyclo-oxygenase as well as 5-lipoxygenase pathways of the arachidonic acid cascade, and acetylcholine contribute to generation of the basal tone of human bronchus.

Figure 1a and b also show the effects of field stimulation of short duration (500 μs, 10 stimuli at 20 Hz) on the muscle tone. Field stimulation evoked contraction with or without following muscle relaxation (cf. Figure 1a and b). The contraction and relaxation were abolished by tetrodotoxin (10^{-7} M; data not shown) and atropine (10^{-7} M) selectively blocked the contraction (Figure 1a), thereby indicating that the mechanical responses were of neurogenic origin.

During and after the development of spontaneous basal tone, phasic and rhythmic fluctuations of the muscle tone were observed in roughly 70% of the examined strips. From a total of 96 strips from 42 lungs, 65 strips from 34 lungs showed spontaneous contractions. These include 5 muscle specimens pre-

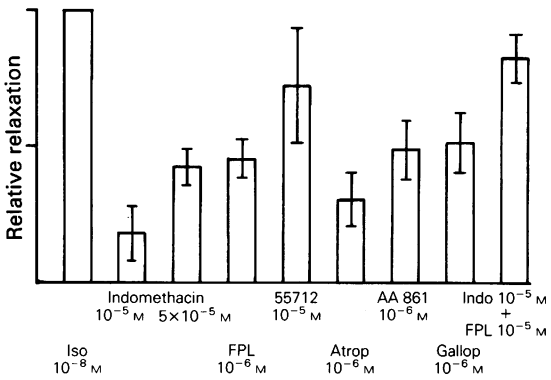

Figure 2 Effects of indomethacin (10^{-5} and 5×10^{-5} M), atropine (Atrop, 10^{-6} M), FPL 55712 (10^{-6} and 10^{-5} M), AA 861 (10^{-6} M), and gallopamil (Gallop, 10^{-6} M), and combined application of indomethacin (Indo, 10^{-5} M) nd FPL 55712 (10^{-5} M) on the spontaneous tone of human bronchus. The amplitude of muscle relaxation evoked by 10^{-8} M isoprenaline (Iso) was defined as a relative amplitude of 1.0. Each value indicates the mean of five to thirteen experiments, and bars are ±s.d.

pared from two non-smokers. The Brinkman's index ranged between 0 to 2040 (mean = 745 ± 630 (±s.d.), $n = 24$) for patients providing tissue with spontaneous activity or 0 to 870 (mean = 210 ± 329 (±s.d.), $n = 8$) for patients providing tissue without spontaneous activity. Spontaneous contractions could be classified into two types, (1) regular phasic contractions with relatively high frequency ranging between 14 and 20 per min (mean value 17.5 ± 2.1, $n = 26$ ± s.d.) with or without slow fluctuations in the basal tension (Figure 3a and b) and (2) relatively irregular phasic contractions at frequencies of 3 to 6 per min (mean value 4.3 ± 1.1, $n = 15$, ±s.d.). The age distribution of the subjects providing tissues exhibiting spontaneous contraction (39–83 years) did not differ from those providing quiescent tissue (42–86 years).

To investigate the properties of spontaneous phasic tension waves, we examined the effects of indomethacin, FPL 55712, AA 861 and leukotriene C_4. As shown in Figure 3b, indomethacin (10^{-5} M) gradually reduced the basal tone to a new level, abolished the slow fluctuations in the basal tone and reduced the amplitude of rhythmic spontaneous contractions to 0.2–0.3 times the control value. However this agent did not abolish the spontaneous contractions. Figure 3 also shows the effects of field stimulation (1–3 stimuli at 20 Hz). Application of single or repetitive field stimulation evoked phasic contractions that were sensitive to tetrodotoxin (10^{-7} M) or atropine (10^{-6} M). After application of indomethacin, the amplitude of the initial phasic contraction evoked by a train of 3 pulses was markedly enhanced (Figure 3). Although the amplitude of the spontaneous contractions was markedly reduced in the presence of indomethacin, field stimulation evoked several phasic contractions superimposed on a longer-lasting tension wave (Figure 3c).

Figure 4 shows the effects of the leukotriene antagonist FPL 55712 on the basal tone and spontaneous contractions of the human bronchial muscle. FPL 55712 (10^{-6} M) markedly suppressed the basal tone, and completely abolished the spontaneous contractions. Thus, in the presence of FPL 55712, a stable basal tone without fluctuations was obtained, and single or repetitive field stimulation evoked phasic contraction without following spontaneous contractions (cf. Figure 4a and c).

To investigate further the mechanisms involved in the generation of spontaneous contractions and basal tone, we examined the effects of AA 861 and leukotriene C_4. As shown in Figures 2 and 5, AA 861 (5×10^{-6} M) reduced the muscle tone and abolished the spontaneous contractions. Application of leukotriene C_4 (LTC_4, 10^{-8} M) or physostigmine

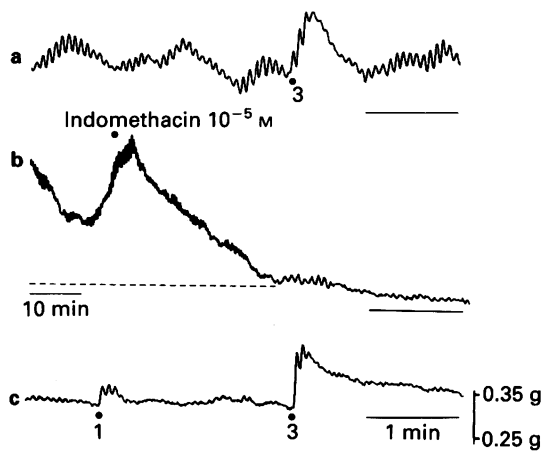

Figure 3 Effects of indomethacin (10^{-5} M) on spontaneous tension waves, basal tone and contractions evoked by field stimulation (1–3 stimuli at 20 Hz) of the human bronchus. The broken line indicates a period during which chart speed was reduced; (●) indicate application of indomethacin or field stimulation. Numerals indicate the number of stimuli at 20 Hz. Field stimulation with 3 pulses at 20 Hz evoked three phasic contractions which summated to form the rising phase of a large, longer-lasting tension wave (a). In the presence of indomethacin, single or repetitive pulses (3 at 20 Hz) again evoked enhanced phasic contractions. The amplitude of the first phasic contraction evoked by a train of 3 stimuli at 20 Hz was larger than that observed in the absence of indomethacin (c).

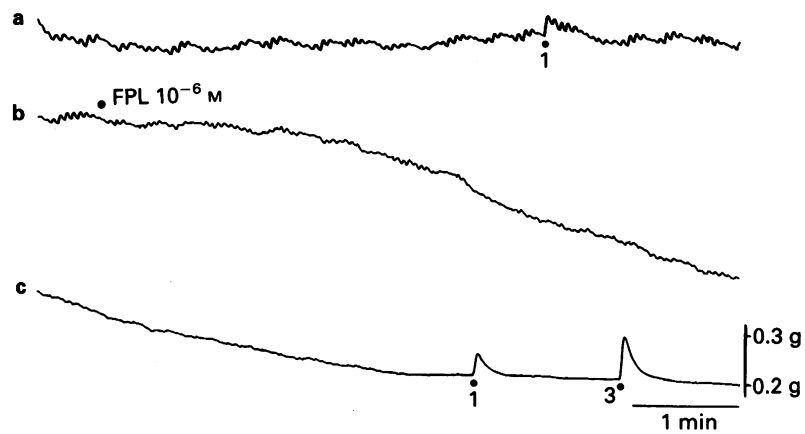

Figure 4 Human bronchial smooth muscle: effects of FPL 55712 (10^{-6} M) on the spontaneous contractions, basal tone and contractions evoked by field stimulation (1–3 stimuli at 20 Hz). Before and after application of FPL 55712, field stimulation with a single pulse evoked twitch contraction with and without subsequent repetitive contractions, respectively.

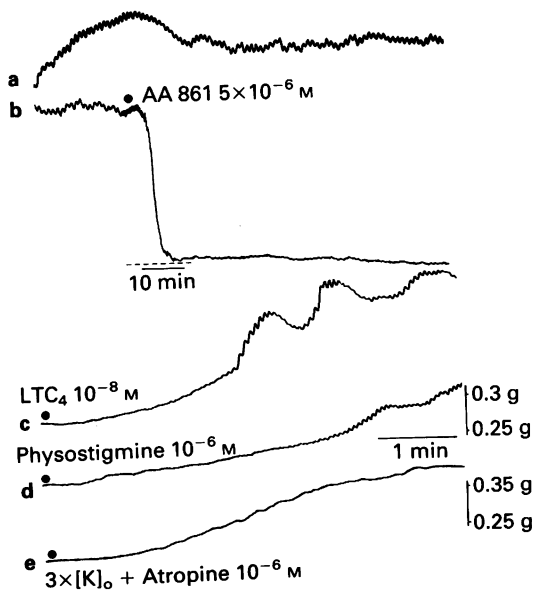

Figure 5 Human bronchial smooth muscle: the effects of some drugs on mechanical activity recorded after tissue treatment with AA 861 (5×10^{-6} M). (a) Control activity; (b) tone suppression induced by AA 861 (5×10^{-6} M); (c), (d) & (e) show the effects of leukotriene C_4 (LTC_4, 10^{-8} M), physostigmine (10^{-6} M) or excess $[K]_o$ solution (17.7 mM) on the mechanical response in the presence of AA 861 (5×10^{-6} M). All recordings are taken from the same preparation.

(10^{-6} M), during the stable resting tone, gradually increased the resting tension and 2–5 min after the application, spontaneous phasic contractions were observed. However, application of K^+-rich (17.7 mM) physiological salt-solution containing atropine (10^{-6} M) did not evoke spontaneous contractions, although this procedure increased the basal tone to roughly the same extent as observed with LTC_4 or physostigmine (Figure 5e).

In order to investigate the properties of the spontaneous contractions and basal tone, we used the calcium antagonist, gallopamil, which is supposed to block the influx of Ca^{2+} (Fleckenstein *et al.*, 1971). Gallopamil (10^{-7} M) reduced the basal tone (Figure 2) and abolished the spontaneous contractions, indicating the contribution of the influx of Ca^{2+} in the generation of spontaneous contraction and basal tone.

Electrical properties of the smooth muscle cells of human bronchus

The resting membrane potential of the human bronchial muscle ranged between −42 mV and −50 mV, with a mean (±s.d.) value of -44.9 ± 5.2 mV ($n = 92$). In 91% of the examined cells (64 out of 70 cells), spontaneous slow and oscillatory potential changes (slow waves) were recorded. The slow waves could be classified into two types: (1) regular rhythm slow waves of relatively small (1–5 mV) or large (5–15 mV) amplitude (Figure 6a and b), and (2) bursts of slow waves alternating with small (1–5 mV) and irregular potential changes (Figure 6c). The slow waves had a duration of 0.5 to 2 s, an amplitude of 1

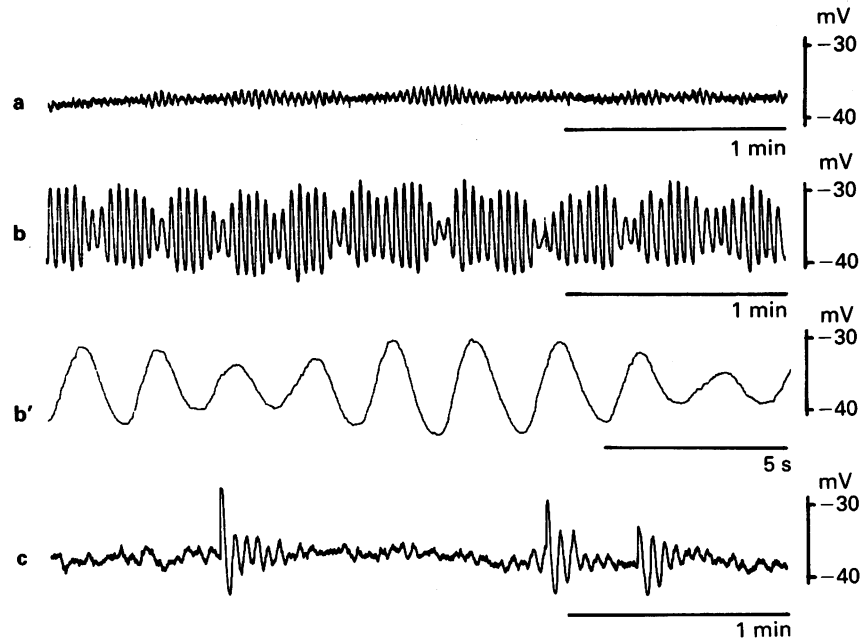

Figure 6 Spontaneous electrical activity of human bronchial smooth muscle cells: (a), (b) & (c) are membrane potential recordings from single smooth muscle cells in different preparations. (a) Slow waves with amplitude of 1–3 mV; (b & b') oscillatory slow waves with larger amplitude (5–10 mV) from the same cell but recorded at different chart speeds; (c) burst type of slow wave discharges.

to 15 mV, and a frequency of 14–36 per min at 35°C (mean value 22.3 ± 6.2 min^{-1}, ±s.d.). Tetrodotoxin (10^{-7} M) had no effects on the amplitude or the frequency of the slow waves indicating their myogenic origin.

Figure 7 shows the effects of the leukotriene antagonist, FPL 55712 (1.9×10^{-6} M) on the slow wave discharge. FPL 55712 hyperpolarized the membrane from −41 to −57 mV in this particular cell, and gradually reduced the amplitude of the oscillatory slow waves. About 30 min after the application, the amplitude of the oscillatory potentials was reduced to less than 5 mV in all of the cells examined ($n = 5$), but they were not abolished.

Atropine (10^{-6} M) or indomethacin (10^{-5} M) each reduced the amplitude of the oscillatory slow potential changes (to 80–90% and 60–70% respectively of the control values), but had no effect on the frequency of the slow waves.

Effects of field stimulation on the membrane potential

When field stimulation (single or multiple pulses at 20 Hz) was applied, excitatory junction potentials (e.j.ps) followed by oscillatory potential changes were recorded. These potential changes were abolished by atropine (Figure 8d and d') or tetrodotoxin (data not shown), thereby indicating their neurogenic origin.

Figure 8 also shows the effects of FPL 55712 and physostigmine (10^{-6} M) on the e.j.p. evoked by 5 or 10 stimuli at 20 Hz. Application of FPL 55712 (1.9×10^{-6} M) hyperpolarized the membrane to −56.6 ± 4.3 mV ($n = 23$), and in the presence of FPL 55712, repetitive field stimulation evoked e.j.ps without oscillatory potential changes (Figure 8b and b'). Application of physostigmine (10^{-6} M) in the presence of FPL 55712 depolarized the membrane to −47.8 ± 3.1 mV ($n = 12$), and e.j.ps followed by oscillatory potential changes were evoked in response to the field stimulation (Figure 8c and c'). Additional application of atropine hyperpolarized the membrane to −56.8 ± 2.0 mV ($n = 10$) and abolished the e.j.p. (Figure 8d and d'). In other words, physostigmine neither enhanced nor prolonged the amplitude or duration of the e.j.p., but enhanced the amplitude of oscillatory slow waves following the e.j.p.

Figure 9 shows the effects of single pulse and repetitive field stimulation on the spontaneous slow waves. When more than 2 stimuli at 20 Hz were applied, the amplitude of e.j.ps was not increased,

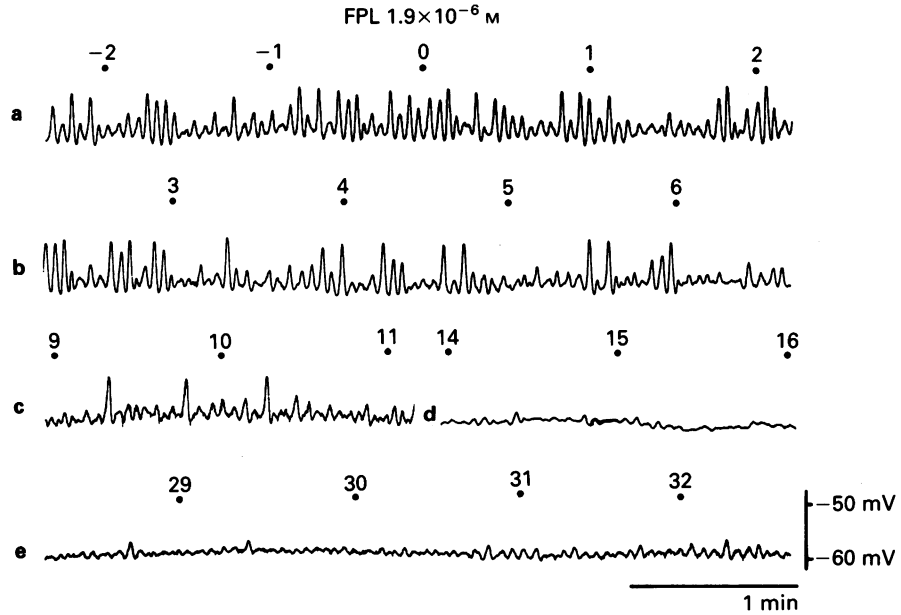

Figure 7 Human bronchial smooth muscle: effects of FPL 55712 (1.9×10^{-6} M) on slow wave discharge: (a–c) recordings of membrane potential from the same cell. Eleven min after the application of FPL 55712, the microelectrode came out, and was re-inserted into another cell. (d & e) Recordings from the second cell; 30 min after the application of FPL 55712, the amplitude of oscillatory slow waves was markedly suppressed.

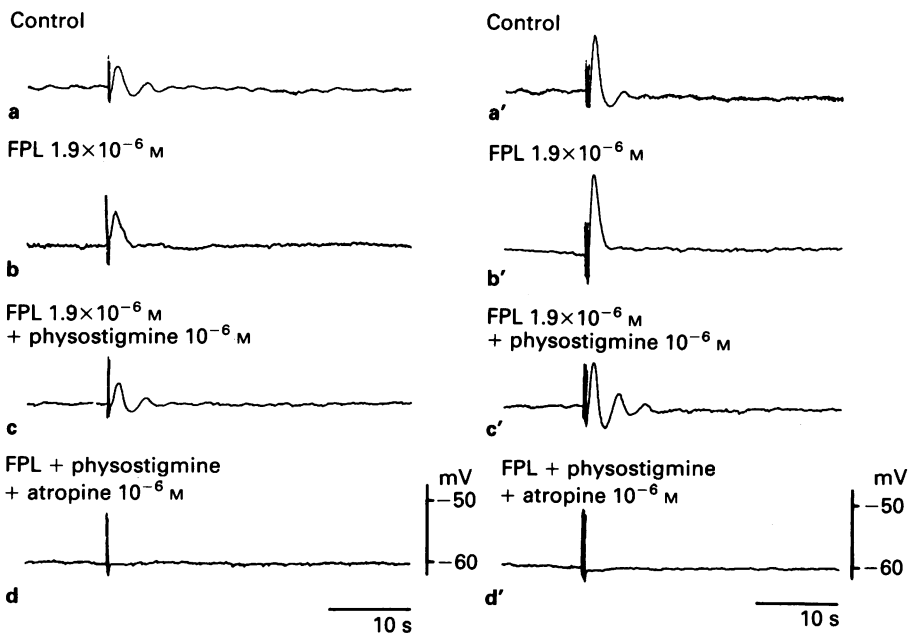

Figure 8 Effects of FPL 55712 (10^{-6} M), physostigmine (10^{-6} M) and atropine (10^{-6} M) on the e.j.p. and membrane potential of human bronchial smooth muscle cells: 5 (a–d) or 10 (a'–d') stimuli at 20 Hz were applied. (a) Control; (b) effects of FPL 55712; (c & d) additional application of physostigmine (10^{-6} M) and atropine (10^{-6} M). All recordings are taken from the same cell.

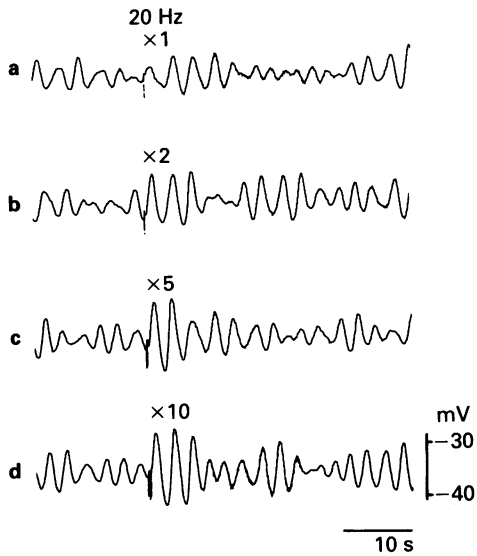

Figure 9 Human bronchial smooth muscle: effects of field stimulation on slow waves. Single or repetitive field stimulation (1–10 stimuli at 20 Hz) was applied. Repetitive stimulation markedly enhanced the amplitude of the oscillatory potential changes rather than the amplitude of the e.j.p.

but the amplitude of subsequently generated slow waves was markedly enhanced. These results indicate that acetylcholine released from the vagus nerve terminal enhanced the amplitude of slow waves.

Discussion

Our *in vitro* studies of human bronchial smooth muscle have demonstrated that in most preparations there is spontaneously generated basal tone and spontaneous phasic contractions occur in roughly 70% of the muscle strips examined. About 90% of the cells that were examined exhibited electrical slow waves. The frequencies of spontaneous phasic contractions and slow waves ranged from 14–20 min^{-1} and 14–36 min^{-1}, respectively. The mean values (17.3 $\pm$ 2.1 and 22.3 $\pm$ 6.2 min^{-1}, $\pm$s.d.) are in good agreement considering that mechanical responses were recorded from relatively large muscle specimens (2 × 1 × 0.5 mm) and electrical membrane phenomena from single smooth muscle cells. The electrical slow waves of the human bronchial smooth muscle observed in the present experiments are essentially similar to those of human trachealis (Honda & Tomita, 1987). In the guinea-pig trachea, generation of slow waves corresponded to phasic contractions in the presence of tetraethylammonium, where the electrical and mechanical activities were recorded simultaneously (Clark & Small, 1979; Allen *et al.*, 1986). Furthermore, slow waves followed by phasic contractions were observed with the double sucrose gap method in the dog trachea treated with indomethacin (Ito & Tajima, 1981b). Thus, each slow depolarization during the oscillatory slow waves probably corresponds to a phasic contraction, and continuous generation of slow waves could explain the generation of the spontaneous basal tone observed in the present experiments. In the guinea-pig trachea, in which considerable tone is produced, continuous slow wave discharge has also been recorded (Small, 1982).

It was first reported in 1975 that electrical and mechancial activities occur in asthmatic patients as measured *in situ* by means of an electromyogram (Akasaka *et al.*, 1975). It was subsequently found that trachealis muscles removed from 3 cadavers exhibited spontaneous mechanical activity (Kneussl & Richardson, 1978). However, these three individuals had suffered from pneumonia or septic shocks and therefore the spontaneous activity was attributed to those pathologies. It has also been reported that spontaneous contractions occurred in roughly 10% of tracheal and bronchial muscle strips examined (Davis *et al.*, 1982), and the authors concluded that the phasic activity was not a function of the preparative techniques, the duration of ischaemia, the age of the subjects or the presence of macroscopically evident pneumonia. Since verapamil abolished the mechanical activity (Davis *et al.*, 1982), the spontaneous contractions were considered to be myogenic in origin and to depend on a voltage-dependent calcium channel. In the present experiments, gallopamil also suppressed the generation of spontaneous contractions and greatly reduced the basal tone. Thus, it may be that Ca^{2+} influx during oscillatory slow waves is responsible for spontaneous contractions and generation of basal tone. The difference in the ratio of occurrence of spontaneous contractions in the present and the previous report (Davis *et al.*, 1982) may be because the human tissue was fresh in this study and obtained mainly from cadavers in the previous report. Thus, the nature of the spontaneous contractions (or phasic contractions) might indicate the *in vivo* status of the trachealis and bronchus in man. However, the muscle specimens with spontaneous contractions were obtained from the patients with relatively high Brinkman's index, and it is possible that the tissues had mild inflammatory changes. Therefore it may be that release of mediators from inflammatory cells is responsible for the generation of spontaneous electrical and mechanical activities, although 5 muscle specimens from 2 non-smokers also showed spontaneous contractions. Further, it is

also conceivable that spontaneous contractions are artifactual due to the mediators released from the tissues by trauma during the preparative procedures. In accord with this, oscillations in membrane potential and subsequent spontaneous contractions have been observed in response to histamine (Kirkpatrick 1975) acetylcholine (Cameron & Kirkpatrick, 1977), 5-hydroxytryptamine (Coburn & Yamaguchi, 1977) or after prolonged treatment with indomethacin (Ito & Tajima, 1981b) in bovine or dog trachea. In the present experiments FPL 55712, indomethacin or atropine suppressed the slow waves and spontaneous contractions. Further investigations should be carried out with non-diseased lung.

Human tracheal muscles are reported to be highly sensitive to leukotriene C_4 (LTC_4) and D_4 (LTD_4), and the contraction produced by 10^{-8} M LTD_4 is almost suppressed by 10^{-6} M FPL 55712 (Jones *et al.*, 1982). LTC_4 and LTD_4 are also known to be 500 times more potent than prostaglandin $F_{2\alpha}$ in contracting human bronchial muscle and this contraction is antagonized by 10^{-6}–10^{-5} M FPL 55712 (Dahléen *et al.*, 1980). In the present experiments, AA 861, a 5-lipoxygenase inhibitor, suppressed the spontaneous contractions and markedly decreased the basal tone, and human bronchial smooth muscle showed much higher sensitivity to AA 861 than to indomethacin. Thus, it may be that in human bronchial tissues, an intrinsic production of leukotrienes is mainly responsible for generation of the phasic contractions and active basal tone. However, the cyclo-oxygenase products of arachidonic acid have also to be considered in the mechanisms involved in the spontaneous contractions and basal tone, since indomethacin consistently reduced the amplitude of phasic contractions and basal tone as reported for guinea-pig trachea (Simonsson *et al.*, 1973; Kawanishi *et al.*, 1984). This observation is in contrast to the findings in human trachealis, in which indomethacin markedly enhanced the basal tone (Ito *et al.*, 1985). This difference might be due to regional differences of the airway smooth muscle or to the source of the human tissues, i.e., fresh tissue or tissue from cadavers. Spontaneous release of acetylcholine from vagus nerve terminals could also contribute to the generation of spontaneous contractions and generation of basal tone, since physostigmine in the presence of FPL 55712 gradually enhanced the basal tone and triggered the generation of spontaneous contractions. Atropine suppressed these contractions and the basal tone.

When the number of repetitive stimuli at a constant stimulus frequency was increased in a step wise manner, the amplitude of the e.j.p. increased within a limited stimulus number (up to 2 or 3 stimuli) and reached a steady level. This is in marked contrast to the findings in the dog trachea, where a linear relationship between the number of stimuli and the amplitude of e.j.p. was observed up to 10–15 stimuli (Ito & Tajima, 1981a; Ito & Itoh, 1984; Korenaga *et al.*, 1984). In man, repetitive stimuli enhanced the amplitude of oscillatory potential changes following the e.j.p. Similarly, in the presence of FPL 55712, physostigmine evoked oscillatory potential changes after each e.j.p. rather than enhancing or prolonging the amplitude or duration of the e.j.p. itself. These differences between man and dog, may be partly due to the difference in the resting membrane potential [the mean values are -44.9 ± 5.2 and -59.5 ± 0.6 mV in man and dog (Ito & Tajima, 1981a) respectively]. However, it seems important to point out that in man, acetylcholine released from the vagus nerve terminal not only evoked e.j.ps, but also triggered or enhanced the amplitude of oscillatory potentials. In other words, activation of muscarinic acetylcholine receptors distributed on the human bronchial smooth muscle cells generates oscillatory membrane potential changes and induces phasic contractions. Similarly LTC_4 (10^{-8} M) in the presence of AA 861 enhanced the basal tone and evoked spontaneous contractions. In contrast, FPL 55712 abolished the oscillatory potential changes following the e.j.p., and repetitive generation of phasic contractions following field stimulation. However, in the presence of atropine, excess $[K]_o$ solution (17.7 mM) depolarized the membrane to a similar extent (about 8 mV) and increased the muscle tone, but did not induce oscillations in the membrane potential or spontaneous contractions. All these data taken together indicate that in man, activation of receptors for various chemical mediators including leukotriene and acetylcholine closely correlates with the initiation of slow waves and spontaneous contractions.

Chemical mediators are also known to initiate contraction of the airway smooth muscle through pharmaco-mechanical coupling without changing the membrane potential (Hashimoto *et al.*, 1985). Thus in man, it is likely that various chemical mediators control the contractility of the airway smooth muscles in two different ways, i.e. through pharmaco-mechanical coupling and membrane potential changes as represented by slow waves.

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Effects of okadaic acid on cytosolic calcium concentrations and on contractions of the porcine coronary artery

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1 We investigated the effects of okadaic acid (OA), a phosphatase inhibitor derived from a 38-carbon fatty acid and isolated from the black sponge, genus *Halichondria*, on cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) and tension developed in porcine coronary arterial strips loaded with fura-2.

2 Both in the presence (1.25 mM) and absence of extracellular Ca^{2+}, OA (over 10^{-6} M) induced a concentration-dependent, slow and progressive increase in tension. Calcium removal had no effect on the maximum level of tension, time between application of the drug and the onset of tension, or the time required to reach the maximum tension. However, there was a slight concentration-dependent increase in $[Ca^{2+}]_i$, only in the presence of extracellular Ca^{2+}.

3 At a lower concentration that did not cause contraction or increase $[Ca^{2+}]_i$, OA (10^{-6} M) inhibited tension development but not the Ca^{2+} transient on readmission of Ca^{2+} in 118 mM K^+-depolarizing solution. OA inhibited the maximum levels of the developed tension, without affecting the K_D value (598 ± 204 nM for control vs 678 ± 464 nM after OA treatment) or the Hill coefficient (1.78 ± 0.10 for control vs 1.98 ± 0.47 for OA treatment).

4 It is concluded that high concentrations of OA induce a contraction independent of extracellular Ca^{2+} and without any changes in $[Ca^{2+}]_i$. Lower concentrations of OA inhibit the Ca^{2+}-dependent contractions. The lack of effect on K_D values suggests that the $[Ca^{2+}]_i$-sensitivity of the contractile apparatus is not affected by this inhibition of contraction.

Introduction

Reversible phosphorylation of myosin light chain plays a central role in contraction and relaxation of vascular smooth muscle. An increase in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) and the resultant activation of myosin light chain kinase induce phosphorylation of myosin light chain, which is dephosphorylated by protein phosphatases. The related mechanisms are complex and not well understood (Di Salvo *et al.*, 1983; Kamm & Stull, 1985). Okadaic acid (OA) is a unique exogenous inhibitor of protein phosphatases (type 2A and 1) which modulates the regulatory mechanisms of contraction and relaxation and, thus, can serve as a useful tool for research on vascular smooth muscle (Bailojan & Takai, 1988). OA was isolated from an extract of common black sponges, genus *Halichondria*, found along the Pacific coast of Japan and at the Florida Keys. It is a polyether derivative of a 38-carbon fatty acid (Tachibana *et al.*, 1981). Shibata *et al.* (1982) showed that OA caused a contraction of smooth muscle that was not affected by extracellular Ca^{2+} deficiency, such as in the absence of extracellular Ca^{2+}, with or without ethylene-glycol-bis(β-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA) or in the presence of Ca^{2+} entry blockers. In permeabilized smooth muscles, OA also caused contraction in the absence of Ca^{2+}. In addition, it enhanced the contraction induced by increases in Ca^{2+}, and shifted Ca^{2+}-tension curves to the left (Ozaki *et al.*, 1987a,b; Bialojan *et al.*, 1988). Thus, the role of extracellular Ca^{2+} and cytosolic Ca^{2+} in the OA-induced contraction of the intact vascular smooth muscle required clarification.

We characterized the underlying mechanism for the OA-induced contraction of vascular smooth muscles by simultaneously measuring changes in $[Ca^{2+}]_i$ and tension developed in fura-2-loaded porcine coronary arterial strips, while making use of

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a front-surface fluorometry system. We found that OA induces a contraction independent of both extracellular Ca^{2+} and $[Ca^{2+}]_i$ and that it inhibits the contraction accompanying elevations of $[Ca^{2+}]_i$ induced by K^+-depolarization.

Tissue preparation

Porcine hearts were obtained from a local slaughterhouse, immediately after the animals had been killed, and were transported to our laboratory in ice-cold Krebs-bicarbonate buffer. Left circumflex arteries were dissected and segments 2–3 cm from the origin were excised. The luminal surface was rubbed off with a cotton swab to remove the endothelium (Furchgott & Zawadzki, 1980). After removal of the adventitia, medial preparations were cut into 1×5 mm circular strips of 0.1 mm thickness (length: 6.0 ± 0.8 mm, wet weight: 1.2 ± 0.3 mg ($n = 23$)). The tissue density of the strip was assumed to be $1.05\,g\,cm^{-3}$ (Murphy *et al.*, 1974) and the cross-sectional area of each strip was calculated, from the following equation: Cross-sectional area (m^2) = wet weight (mg)/length (mm)/1.05 ($g\,cm^{-3}$) $\times 10^{-6}$. The mean value of cross-sectional area was $1.94 \pm 0.35 \times 10^{-7}\,m^2$ ($n = 23$).

Fura-2 loading

The vascular strips thus obtained were loaded with the calcium indicator dye, fura-2 by incubation in medium containing 25 μM fura-2/AM (an acetoxymethyl ester form of fura-2) and 5% foetal bovine serum for 3–4 h at 37°C. Subsequently, the strips were washed with physiological saline solution (PSS) containing 1.25 mM Ca^{2+} at 37°C to remove the dye from the extracellular space, and were then incubated with PSS for 1 h before initiation of the measurements. Strips thus treated showed the specific peak of the fluorescence emission spectrum for fura-2 (500 nm) and the specific peak and valley of the fluorescence excitation spectrum for fura-2, 340 nm and 380 nm, respectively, determined with a fluorescence spectrophotometer (model 650-40, Hitachi, Tokyo, Japan). Loading the vascular strips with fura-2 did not alter the time courses and the maximum levels of tension development during 118 mM K^+-depolarization, suggesting that no tissue damage by possible acidification of the cells due to formaldehyde released on AM-ester hydrolysis (Tsien *et al.*, 1982; Rink & Pozzan, 1985) had occurred.

Measurement of tension

Strips were mounted vertically in a quartz organ bath, (strain gauge TB-612T, Nihon Koden, Tokyo, Japan). During a 1 h fura-2 equilibration period, the strips were stimulated with 60 mM K^+-depolarization every 15 min, and the resting tension was increased stepwise. After equilibration, the resting tension was adjusted to 250 mg. The response of each strip to 118 mM K^+-depolarization just before the start of each protocol was $4.9 \pm 1.7 \times 10^4\,N\,m^{-2}$ ($n = 23$). The tension developed was expressed as % assuming the values in PSS (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 during the measurement of the tension. The fluorescence intensity was measured by use of a front-surface fluorometer specially designed for fura-2 fluorometry (CAM-OF-1), with the collaboration of Japan Spectroscopic Co., Tokyo, Japan. In brief, strips were illuminated by guiding the alternating (800 Hz), 340 nm and 380 nm, excitation light from a Xenon light source through quartz optic fibres arranged in a concentric inner circle (diameter = 3 mm). Surface fluorescence of strips was collected by glass optic fibres arranged in an outer circle (diameter = 7 mm) and introduced through a 500 nm band-pass filter (full width at half maximum transmission 10 nm) into a photon-counting photomultiplier. Special care was taken to keep the distance between a strip and the end of the optic fibres constant during each measurement.

The ratio of the fluorescence at 340 nm excitation to that at 380 nm excitation was expressed as %, assuming the values in PSS (5.9 mM K^+) and 118 mM K^+ PSS to be 0% and 100%, respectively. The absolute value of $[Ca^{2+}]_i$ was calculated from the % ratio (R), from the following equation.

$$[Ca^{2+}]_i = K_D(R - R_{min})/(R_{max} - R)$$

where K_D is the dissociation constant and is assumed to be 224 nM (Grynkiewicz *et al.*, 1985). R_{max} and R_{min} were determined by the addition of 25 μM ionomycin (Hesketh *et al.*, 1983) and 2 mM EGTA (Tsien *et al.*, 1982) and were 156.3% ($n = 10$) and −76.1% ($n = 10$), respectively. The mean values of 10 different measurements of $[Ca^{2+}]_i$ at rest (0%) and during 118 mM K^+-depolarization (100%) were 108 ± 27 and 715 ± 103 nM, respectively.

Drugs and solutions

Physiological salt solution (PSS) was of the following composition (in mM): NaCl 123, KCl 4.7, $NaHCO_3$ 15.5, KH_2PO_4 1.2, $MgCl_2$ 1.2, $CaCl_2$ 1.25, and D-glucose 11.5. High potassium PSS was identical, except for an equimolar substitution of KCl for NaCl. PSS was gassed with a mixture of 95% O_2-5% CO_2 (pH adjusted to 7.4 at 37°C). OA was dissolved

in dimethylsulphoxide (DMSO) and diluted with PSS just before use (final concentration of DMSO was 1%). Fura-2/AM was purchased from Molecular Probes (Eugene, OR, U.S.A.).

Statistical analysis

Values are expressed as mean ± s.e. ($n = 3$). Student's t test was used to determine the statistical significance. $[Ca^{2+}]_i$-tension relationship was fitted to the following Hill function by the least squares method.

$$\log(T/(T_{max} - T)) = n(\log[Ca^{2+}]_i - \log K_D)$$

where T represents measured value of developed tension, expressed as % and $[Ca^{2+}]_i$ represents value of cytosolic Ca^{2+} concentration calculated as described for Front-surface fluorometry. n and K_D are constant values and n is the Hill coefficient. T_{max} represents estimated maximum tension development and was calculated from the following equation and least squares method.

$$T/[Ca^{2+}]_0 = (T_{max} - T)/K$$

where $[Ca^{2+}]_0$ represents extracellular Ca^{2+} concentration applied and K is a constant.

Results

Characteristics of contraction induced by okadaic acid

Figure 1 shows the representative time course of changes in tension and $[Ca^{2+}]_i$ induced by 10^{-5} M OA. In the presence of extracellular Ca^{2+} (Figure 1a and c), 10^{-5} M OA caused a slow and progressive increase in $[Ca^{2+}]_i$ and tension after about 10 min. Although there was no difference between the extent of the maximum contraction observed at 50 min after the application of 10^{-5} M OA ($7.5 \pm 2.8 \times 10^4$ N m^{-2}, $n = 3$) and that seen with the 118 mM K^+-depolarization-induced contraction ($6.9 \pm 2.5 \times 10^4$ N m^{-2}, $n = 3$), the $[Ca^{2+}]_i$ corresponding to the maximum tension induced by OA (194 ± 26 nM, $n = 3$) was significantly lower than that induced by 118 mM K^+-depolarization (715 ± 103 nM, $n = 10$, $P < 0.001$). In the absence of extracellular Ca^{2+}, the extent of the OA-induced contraction was similar to that observed in the presence of extracellular Ca^{2+} (Figure 1d). However, there was no elevation of $[Ca^{2+}]_i$ (Figure 1b).

Figure 2 shows the characteristics of the development of tension induced by OA in the presence and absence of extracellular Ca^{2+}. At concentrations over 10^{-6} M, OA induced a dose-dependent contraction of the vascular strips. The maximum values of tension, latent periods between application of OA and onset of observed tension development, and time required to reach the maximum tension (peak time) all depended on the concentration of OA. There were no significant differences between these values in the presence and absence of extracellular Ca^{2+} (Figure 2). Thus OA induced similar changes in tension with a similar time course that depended on the concentration of OA but not on the extracellular Ca^{2+}.

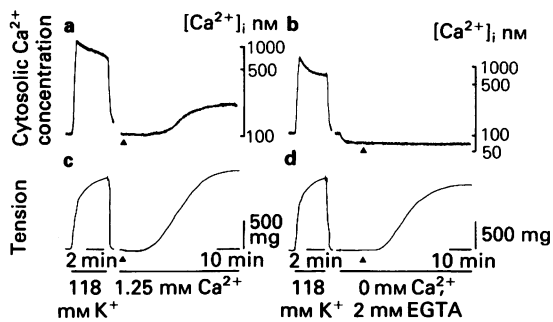

Figure 1 Representative recordings of changes in fluorescence ratio (a and b) and tension (c and d) induced by 10^{-5} M okadaic acid (OA) in the presence (1.25 mM) (a and c) and absence (b and d) of extracellular Ca^{2+}. OA 10^{-5} M was applied at the time indicated by a triangle (▲), after the response to 118 mM K^+-depolarization had been recorded. Note that the time scale for the recordings of the 118 mM K^+-depolarization-induced contraction differ from those of the OA-induced contraction.

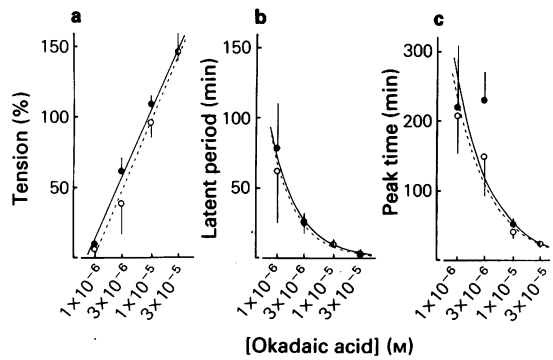

Figure 2 Characteristics of tension development induced by okadaic acid (OA). (a) Dose-response curves for maximum tension. (b) Dose-response curves for latent period (between application of the drug and onset of tension development). (c) Dose-response curves for time required to reach maximum tension: (●——●) indicate values in the presence of extracellular Ca^{2+} and (○---○) values in the absence of extracellular Ca^{2+}. Vertical lines show s.e.mean.

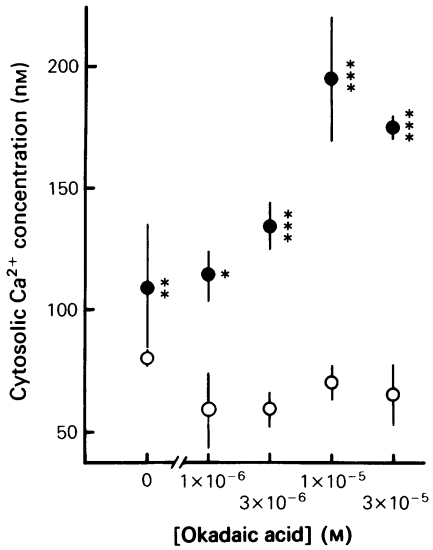

Figure 3 Effects of okadaic acid (OA) on cytosolic Ca^{2+} concentration in the presence and absence of extracellular Ca^{2+}. Cytosolic Ca^{2+} concentrations induced by various concentrations of OA in the presence (●) and absence (○) of extracellular Ca^{2+}. Measurements were made at the time of maximum tension development for each concentration of OA. The asterisks represent significant differences of $[Ca^{2+}]_i$ between the presence and absence of extracellular Ca^{2+} (*** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$). Each point represents the mean ($n = 3$) and vertical lines show s.e.mean.

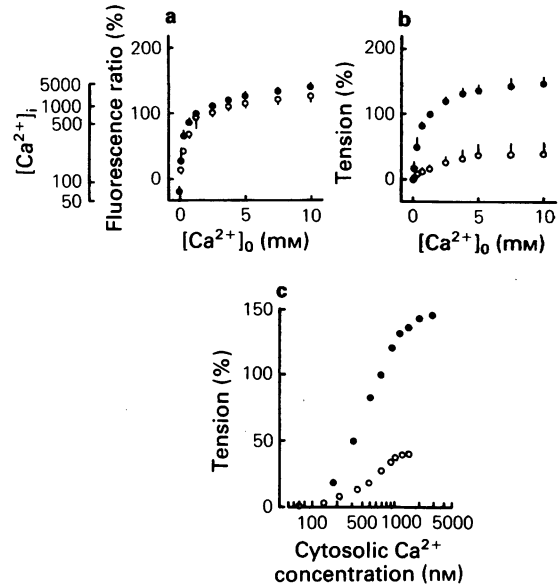

Figure 4 Effects of 10^{-6} M okadaic acid (OA) on contractions induced by cumulative applications of extracellular Ca^{2+} during high K^+-depolarization of vascular strips. Effects of OA on changes in cytosolic Ca^{2+} concentrations and tension development are shown in (a) and (b), respectively. (c) The effects of OA on $[Ca^{2+}]_i$-tension relation, as constructed from data in (a) and (b). Values obtained with (○) or without (●) treatment with 10^{-6} M OA. Fluorescence ratio (%) was determined by assuming values at 5.9 mM K^+ and 118 mM K^+ in PSS to be 0% and 100%, respectively.

The effects of increasing concentrations of OA on $[Ca^{2+}]_i$ are shown in Figure 3. In the presence of extracellular Ca^{2+}, OA elevated $[Ca^{2+}]_i$, in a concentration-dependent manner. The maximum level obtained was 193 nM at a concentration of 10^{-5} M OA. In the absence of extracellular Ca^{2+}, OA did not elevate $[Ca^{2+}]_i$. These results show that while producing no significant differences in maximum tension for a given dose, OA did produce a significantly different $[Ca^{2+}]_i$ at the time of maximum developed tension in the presence and absence of extracellular Ca^{2+}.

Effects of okadaic acid, at subthreshold concentrations, on changes in contraction and $[Ca^{2+}]_i$ of Ca^{2+}-dependent contraction during high K^+-depolarization

After a 10 min incubation in Ca^{2+}-free PSS containing 2 mM EGTA, and then a 5 min incubation in Ca^{2+}-free PSS without EGTA, Ca^{2+}-dependent contractions were induced by cumulative applications of extracellular Ca^{2+}, from 0 mM to 10 mM, in 118 mM K^+-depolarizing solution (Godfraind & Kaba, 1969). Changes in $[Ca^{2+}]_i$ and tension induced by the elevation of extracellular Ca^{2+} are shown in Figure 4a and b, respectively and $[Ca^{2+}]_i$-tension curves are given in Fig. 4c. With no OA treatment, both $[Ca^{2+}]_i$ and the tension increased on elevations of extracellular Ca^{2+} (Figure 4, closed circles). $[Ca^{2+}]_i$ increased from 72 nM at 0 mM extracellular Ca^{2+} to 2900 nM at 10 mM extracellular Ca^{2+}. The tension increased from 0% to 150%. The K_D value and Hill coefficient of the $[Ca^{2+}]_i$-tension relationships for the control contraction were 598 ± 204 nM and 1.78 ± 0.10, respectively. To examine the effects of OA on Ca^{2+}-dependent contractions, we used 10^{-6} M, because at this concentration OA had negligible effects on both $[Ca^{2+}]_i$ and on tension (Figures 2 and 3). Treatment with 10^{-6} M OA for 20 min before and during cumulative applications of extracellular Ca^{2+} in 118 mM K^+-depolarizing solution signifi-

cantly inhibited increases in tension development but had little effect on increases in $[Ca^{2+}]_i$ (Figure 4, open circles). The tension increased from 0% to 40%. The K_D value and Hill coefficient were 678 ± 464 nM and 1.98 ± 0.47, respectively. These values were the same in untreated and OA-treated strips.

Discussion

We have confirmed that the OA-induced concentration-dependent contraction is independent of extracellular Ca^{2+}, as found by Shibata *et al.* (1982). In addition, the present study revealed that not only the extent of maximum tension development but also the latent period and time to peak tension were similar in the presence and absence of extracellular Ca^{2+}. These observations suggest that the contraction induced by OA is completely independent of extracellular Ca^{2+}. As OA elevated $[Ca^{2+}]_i$ only in the presence of extracellular Ca^{2+}, elevations of $[Ca^{2+}]_i$ are probably not linked to the mobilization of stored Ca^{2+}. The elevation of $[Ca^{2+}]_i$ led to no additional tension development in the presence of extracellular Ca^{2+}.

At least two possibilities for the absence of additional tension development, despite the elevation of $[Ca^{2+}]_i$ induced by OA in the presence of extracellular Ca^{2+}, have to be considered. The first is that the extent of elevation of $[Ca^{2+}]_i$ was too small to add significant increases in tension, and the second is that OA, directly or indirectly, had inhibitory effects on the Ca^{2+}-dependent contractile mechanism. The first possibility could be excluded by the finding that 10^{-5} M OA induced an elevation of $[Ca^{2+}]_i$ up to 193 nM; this level was high enough to induce tension development in the endothelium-free porcine coronary artery, as shown in Figure 4c. Exposure of the vascular strips to OA 10^{-6} M, a procedure which did not elevate $[Ca^{2+}]_i$ or tension, strongly inhibited subsequent tension development of the Ca^{2+}-dependent contraction during high K^+-depolarization, reducing the maximum effect without affecting the K_D of the $[Ca^{2+}]_i$-tension relationship. This suggests that OA might have inhibitory effects on Ca^{2+}-dependent contractile mechanisms by decreasing the effectiveness of $[Ca^{2+}]_i$ without affecting the sensitivity of the contractile apparatus to $[Ca^{2+}]_i$.

OA produces a marked increase in the phosphorylated states of various proteins, including myosin light chain in vascular smooth muscle cells (Ozaki *et al.*, 1987a; Erdödi *et al.*, 1988; Bialojan *et al.*, 1988), enzymes related to glucose and lipid metabolism in adipocytes and hepatocytes (Haystead *et al.*, 1989), and nuclear protein in human fibroblasts (Issinger *et al.*, 1988). OA has been found to inhibit protein phosphatase activity, specifically type 2A and type 1 phosphatases (Ingebritsen & Cohen, 1983; Takai *et al.*, 1987; Bialojan & Takai, 1988). Inhibition of phosphatases causes increases in the phosphorylated state of various proteins, including those transiently phosphorylated and undetected in the absence of OA. Slow, progressive, and Ca^{2+}-independent contractions induced by OA and inhibitory effects of OA on Ca^{2+}-dependent contractions might be related to such sustained or abnormal phosphorylations of essential proteins involved in contractile mechanisms. Erdödi *et al.* (1988) demonstrated that OA inhibited dephosphorylation of phosphorylated myosin light chain and that in the presence of OA, both myosin light chain kinase and protein kinase C were involved in phosphorylation of myosin light chain, whereas in the absence of OA, phosphorylation was predominantly due to myosin light chain kinase. Siegman *et al.* (1989) showed that OA inhibited ADP formation of permeabilized vascular smooth muscle, resulting in decreases of crossbridge cycling rates. They suggested that OA might inhibit myosin ATPase activity. Although, they did not define whether the inhibition of ATPase activity was a direct effect of OA on the enzyme, or an indirect effect mediated by phosphorylation of the enzyme due to phosphatase inhibition.

OA enhanced the slow Ca^{2+} inward current in isolated ventricular myocytes (Hescheler *et al.*, 1988; Kodama *et al.*, 1986). In the present study, OA did not affect the elevation of $[Ca^{2+}]_i$ induced by cumulative applications of extracellular Ca^{2+}, under conditions of high K^+-depolarization. Thus, OA may have little effect on currents through the voltage-dependent Ca^{2+} channel in porcine coronary vascular smooth muscle cells.

OA was found to inhibit the Ca^{2+}-dependent contractions by decreasing the effectiveness of $[Ca^{2+}]_i$. The lack of change in the K_D values suggests that the decrease in $[Ca^{2+}]_i$-effectiveness is not due to a reduction in $[Ca^{2+}]_i$-sensitivity of the contractile apparatus. OA itself can induce a slow and progressive contraction, an event which may be related to Ca^{2+}-independent activation of Ca^{2+}-dependent contractile mechanisms or unknown Ca^{2+}-independent contractile mechanisms.

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Modulation of arterial endothelial permeability: studies on an *in vitro* model

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1 An *in vitro* model of the arterial endothelial barrier was established in which transfer of trypan blue-labelled albumin across confluent monolayers of pig aortic endothelial cells grown on polycarbonate membranes was measured.

2 A range of inflammatory mediators, i.e. histamine, bradykinin, platelet activating factor and thrombin, had no effect on the transfer of labelled albumin across aortic endothelial monolayers.

3 Calcium ionophore A23187 and the phorbol ester, phorbol myristate acetate (PMA), each induced concentration-dependent increases in transfer of labelled albumin. These increases were associated with changes in cell shape, consistent with endothelial contraction. Ionophore A23187 caused some detachment of cells.

4 The ability of PMA to increase transfer of labelled albumin probably results from activation of protein kinase C since it was not shared by the inactive analogue, 4α-phorbol 12,13-didecanoate.

5 Neither a combination of superoxide dismutase and catalase nor the cyclo-oxygenase inhibitor, flurbiprofen, affected resting or PMA-induced increases in albumin transfer. Oxygen-derived free radicals and prostaglandins appear not to be involved in the response to PMA.

6 Each of three procedures designed to elevate adenosine 3':5'-cyclic monophosphate (cyclic AMP) levels, i.e. dibutyryl cyclic AMP, forskolin and (±)-isoprenaline, reduced the ability of PMA to promote increased transfer of labelled albumin but had no effect on resting transfer. The effect of (±)-isoprenaline was abolished by the β-adrenoceptor blocking agent, propranolol.

7 Elevation of cyclic GMP content by use of 8 bromo cyclic GMP or atriopeptin II had no effect on resting or PMA-induced transfer of labelled albumin.

8 Arterial endothelial barrier function can be compromised by agents that promote endothelial contraction. Agents that increase endothelial cyclic AMP levels, and so reduce entry of high molecular weight substances into the arterial wall, may warrant evaluation as potential anti-atherogenic drugs.

Introduction

The vascular endothelium plays a central role in regulating the exchange of fluid, solutes, and cells between the blood and the extravascular tissues. The exchange of fluid and solutes can be modulated either by trans-endothelial endocytosis (Palade, 1960), or, following the actions of inflammatory mediators such as histamine, by formation of inter-endothelial gaps (Majno & Palade, 1961). The occurrence of inter-endothelial gaps in response to inflammatory mediators is restricted to the post-capillary venule (Svensjo *et al.*, 1979), and is thought to be the result of active endothelial contraction (Majno *et al.*, 1967); a proposal supported by the discovery of contractile proteins, including actin and myosin (Becker & Nachmann, 1973; Drenckhahn, 1983) in endothelial cells.

The arterial endothelial barrier also is of critical importance since its impairment leads to increased entry of cholesterol-rich low-density lipoproteins into the arterial wall (Ross & Harker, 1976), with the subsequent development of atherosclerosis (Ross, 1986; Munro & Cotran, 1988). Factors which modulate the arterial endothelial permeability barrier are poorly understood: they may include endothelial contraction with the development of inter-endothelial gaps, or endothelial damage resulting

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from the effects of oxygen-derived free radicals or peroxidized lipids (Rosen & Freeman, 1984; Hennig & Chow, 1988). Ideally, studies of arterial endothelial barrier function should be performed *in vivo*, but under these circumstances concentrations of drugs and the possible involvement of cells other than endothelial cells are difficult to control. These disadvantages might be circumvented by establishing an *in vitro* model of arterial permeability. Rotrosen & Gallin (1986) devised a model of the venous endothelial permeability barrier in which transfer of labelled albumin across confluent monolayers of endothelial cells grown on polycarbonate membranes was measured. We wished to establish a similar model of the arterial endothelial permeability barrier in which pig aortic endothelial cells were grown on polycarbonate membranes. In particular, we wished to study the actions of the major intracellular transduction mechanisms involving calcium, cyclic nucleotides, and protein kinase C, on transfer of albumin across aortic endothelial monolayers.

Methods

Endothelial cell culture

Pig aortic endothelial cells were isolated and grown in 75 cm^2 tissue culture flasks as previously described (Martin *et al.*, 1988). When these primary cultures reached confluence (4–7 days) the cells from each flask was detached with trypsin (0.05%) and ethylenediamine tetraacetic acid (0.02%) and seeded into 48 Transwell (Costar) polycarbonate membrane assemblies (6.5 mm diameter, 3 μm pore size). The membrane assemblies were then placed in 1 ml of tissue culture medium in 24-well plates (Costar) and the cells allowed to grow for a further 2–4 days.

Albumin transfer experiments

Membrane assemblies with endothelial cells attached were washed twice by immersion in Krebs solution containing (mM): NaCl 118, KCl 4.8, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 2.4, glucose 11 and HEPES (N-2 hydroxyethylpiperazine-N'-2-ethanesulphonic acid) 5 at 37°C and pH 7.4, and then transferred to 24-well plates. Six hundred μl of Krebs solution was placed in each of the wells which formed the lower chamber and 100 μl of Krebs containing trypan blue-labelled albumin (4%) was placed above the endothelial monolayer. These volumes were chosen so that no hydrostatic gradient was created across the membrane. The preparations were then incubated at 37°C in air with mild, continuous agitation produced by an orbital mixer. After 5 min monolayers that demonstrated leakage of dye visible to the naked eye were discarded, and those remaining were used in transfer studies. Following incubation with drugs for the times indicated in the Results, albumin transfer across endothelial monolayers was quantified by measuring the absorbance of fluid from the lower chamber at 590 nm. The trypan blue-labelled albumin content of the lower chamber is expressed as a percentage of the maximum concentration that would have been achieved at equilibrium.

The trypan blue-labelled albumin complex was prepared by adding trypan blue (180 mg, Sigma) and bovine serum albumin (4 g, fraction V, Sigma) to 100 ml of Krebs solution. Precipitation with trichloroacetic acid (6%) showed that the trypan blue was >98% albumin bound.

Morphological studies

Transwell polycarbonate membranes are opaque which prevents the visualisation of cells by phase-contrast microscopy. Studies of the effects of drugs on cell morphology were performed with cells grown on glass coverslips where visualisation was possible.

Drugs

Atriopeptin II (rat synthetic), bradykinin triacetate, 8 bromo cyclic GMP, catalase (bovine liver), dibutyryl cyclic AMP, histamine dihydrochloride, ionophore A23187, (±)-isoprenaline hydrochloride, 4α-phorbol 12,13-didecanoate (4α-PDD), phorbol 12-myristate 13-acetate (PMA), superoxide dismutase (bovine erythrocyte) and thrombin (bovine plasma) were obtained from Sigma. Forskolin and platelet activating factor were obtained from Calbiochem and sodium flurbiprofen was a generous gift from Dr R.V. Holland, Boots Pure Drug Co. All drugs were dissolved in twice-distilled water except for ionophore A23187, 4α-PDD and PMA which were dissolved in ethanol, and forskolin which was dissolved in dimethyl sulphoxide (DMSO). Concentrations of ethanol or DMSO did not exceed 0.2% in experiments: concentrations of 1% and above were found to increase albumin transfer across monolayers.

Statistical analysis

Since the resting transfer of trypan blue-labelled albumin across endothelial monolayers varied from cell batch to batch, each experiment was performed with its own internal controls. Results are presented as the mean ± s.e.mean, and comparisons were made either with Student's *t* test or the Mann-Whitney test when there was unequal variance in samples. A probability of 0.05 or less was considered significant.

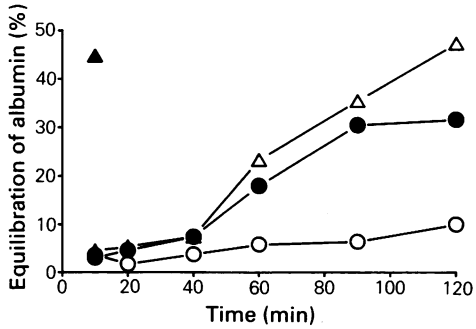

Figure 1 Time course showing the transfer of trypan blue-labelled albumin across confluent monolayers of pig aortic endothelial cells grown on polycarbonate membranes in the absence of drugs (○), and following treatment with ionophore A23187 (20 μM, △) or phorbol myristate acetate (6 μM, ●). The rapid transfer across membranes without cells is also shown (▲). Each point is the mean of 2–4 observations.

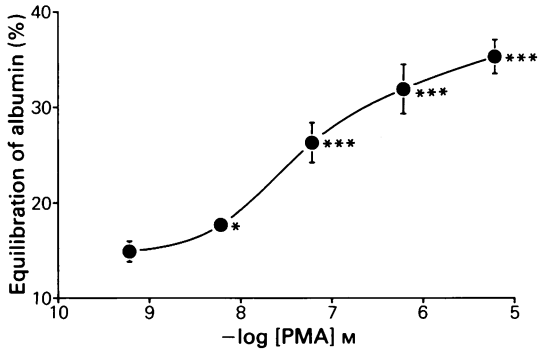

Figure 2 Concentration-response curve showing the ability of phorbol myristate acetate (PMA) to increase the transfer of trypan blue-labelled albumin across confluent monolayers of pig aortic endothelial cells during a 90 min incubation period. Each point is the mean of 5–6 observations; vertical bars show s.e.mean. * $P < 0.05$; *** $P < 0.001$, denotes significant difference from albumin transfer across untreated monolayers.

Results

Phorbol myristate acetate and A23187

Transfer of trypan blue-labelled albumin (4%) across polycarbonate membranes into the lower chamber was rapid: roughly 50% equilibration was achieved within 10 min and complete equilibrium within 60 min (Figure 1). When a confluent layer of pig aortic endothelial cells was present on membranes the transfer of labelled albumin was markedly restricted. Addition of PMA (6 μM) or ionophore A23187 (20 μM) increased the transfer of labelled albumin across endothelial monolayers in a time-dependent manner (Figure 1). The concentration-dependence of the increased transfer of labelled albumin induced by PMA and by ionophore A23187 during a 90 min incubation is shown in Figure 2 and Table 1, respectively. Combined exposure to sub-

Table 1 Effects of ionophore A23187, phorbol myristate acetate (PMA), forskolin and 8 bromo cyclic GMP (8 Br cGMP) on transfer of trypan blue-labelled albumin across monolayers of pig aortic endothelial cells

Stimulus	*Pretreatment*	*% equilibration of albumin*	n
None (control)‡	None	4.2 ± 0.2	4
A23187, 2 μM	None	9.8 ± 1.1***	4
A23187, 20 μM	None	24.2 ± 0.5***	4
None (control)†	None	11.6 ± 0.9	6
None	PMA, 60 nM	19.1 ± 2.1*	6
A23187, 2 μM	None	18.7 ± 2.6*	6
A23187, 2 μM	PMA, 60 nM	31.0 ± 3.6*	6
None (control)‡	None	14.8 ± 1.1	
None	Forskolin, 20 μM	8.7 ± 3.0	4
A23187, 20 μM	None	47.6 ± 2.0***	4
A23187, 20 μM	Forskolin, 20 μM	46.1 ± 2.2***	4
None (control)‡	None	8.0 ± 1.1	4
None	8 Br cGMP, 30 μM	7.7 ± 0.9	4
A23187, 20 μM	None	44.9 ± 2.8***	4
A23187, 20 μM	8 Br cGMP, 30 μM	49.6 ± 4.1***	4

Following exposure of endothelial monolayers to ionophore A23187 for 60 min (†) or 90 min (‡) the % equilibration of trypan blue-labelled albumin in the lower chamber was measured spectrophotometrically. The effects of pretreatment with PMA, forskolin, or 8 bromo cyclic GMP, for 2 min before addition of A23187 are also shown. Results are expressed as the mean ± s.e.mean. * $P < 0.05$; *** $P < 0.001$, denotes significant difference from control or a difference between two groups joined by a bar.

Table 2 Effects of phorbol myristate acetate (PMA), 4α-phorbol 12,13-didecanoate (4α-PDD), superoxide dismutase (SOD) and catalase (Cat), and flurbiprofen (FBP) on transfer of trypan blue-labelled albumin across monolayers of pig aortic endothelial cells

Stimulus	*Pretreatment*	*% equilibration of albumin*	n
None (control)	None	9.2 ± 1.3	6
PMA, 0.6 μM	None	25.1 ± 0.6***	6
4α-PDD, 0.6 μM	None	9.3 ± 0.9	6
None (control)	None	9.1 ± 1.0	6
None	SOD, 30 u ml^{-1} and Cat, 30 u ml^{-1}	8.6 ± 0.3	6
PMA, 0.6 μM	None	22.7 ± 1.0***	6
PMA, 0.6 μM	SOD, 30 u ml^{-1} and Cat, 30 u ml^{-1}	23.2 ± 0.8***	6
None (control)	None	7.0 ± 0.3	13
None	FBP, 10 μM	6.9 ± 0.5	12
PMA, 0.6 μM	None	17.2 ± 1.3***	6
PMA, 0.6 μM	FBP, 10 μM	17.5 ± 1.2***	6

Following exposure of endothelial monolayers to PMA or 4α-PDD for 90 min the % equilibration of trypan blue-labelled albumin in the lower chamber was measured spectrophotometrically. The effects of pretreatment with a combination of superoxide dismutase and catalase or with flurbiprofen for 2 min before addition of PMA are also shown. Results are expressed as the mean ± s.e.mean. ***$P < 0.001$, denotes significant difference from control.

maximal concentrations of PMA (60 nm) and ionophore A23187 (2 μM) for 60 min showed an additive effect (Table 1).

The inactive phorbol ester, 4α-PDD (0.6 μM, 90 min), lacked the ability of PMA (0.6 μM, 90 min) to increase transfer of labelled albumin across endothelial monolayers (Table 2). Parallel studies in which phase-contrast microscopy was used showed that endothelial cells underwent a shape change in response to PMA (0.6 μM) consistent with cell contraction, but remained adherent to the substrate. A23187 (20 μM) also caused cells to change shape but definite detachment of cells was evident.

Modulation of phorbol myristate acetate

A combination of superoxide dismutase (30 u ml^{-1}) and catalase (30 u ml^{-1}) had no effect on resting or PMA (0.6 μM)-induced transfer of labelled albumin across endothelial monolayers during a 90 min incubation period (Table 2). The cyclo-oxygenase inhibitor, flurbiprofen (10 μM), also was without effect on resting or PMA (0.6 μM)-induced transfer of labelled albumin (Table 2).

Treatment with each of 3 agents designed to elevate endothelial cyclic AMP content, i.e. dibutyryl cyclic AMP (30 μM), forskolin (20 μM), and (±)-isoprenaline (20 μM), inhibited the PMA (0.6 μM)-induced increase in transfer of labelled albumin without affecting resting transfer (Table 3, Figure 3). Propranolol (20 μM) blocked the ability of (±)-isoprenaline (20 μM) to inhibit PMA (0.6 μM)-induced transfer of labelled albumin (Figure 3).

Treatment with 2 agents designed to elevate endothelial cyclic GMP content, i.e. 8-bromo cyclic GMP (30 μM) or atriopeptin II (20 nM), had no effect on resting or PMA (0.6 μM)-induced transfer of labelled albumin (Table 3).

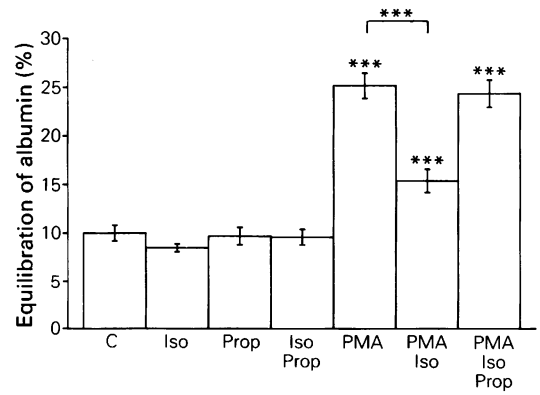

Figure 3 (±)-Isoprenaline (Iso, 20 μM) inhibits phorbol myristate acetate (PMA, 0.6 μM)-stimulated transfer of trypan blue-labelled albumin across monolayers of pig aortic endothelial cells and this is blocked by propranolol (Prop, 20 μM). Columns are the mean of 8–23 observations; vertical bars show s.e.mean. ***$P < 0.001$, denotes significant difference from control (C) monolayers, or a difference between groups joined by a bar.

Table 3 Effects of phorbol myristate acetate (PMA), forskolin, dibutyryl cyclic AMP (db cAMP), atriopeptin II (APII) and 8 bromo cyclic GMP (8 Br cGMP) on transfer of trypan blue-labelled albumin across monolayers of pig aortic endothelial cells

Stimulus	*Pretreatment*	*% equilibration of albumin*	n	
None (control)	None	2.1 ± 0.3	6	
None	Forskolin, 20 μM	1.8 ± 0.3	6	
PMA, 0.6 μM	None	6.8 ± 0.4***	6	**
PMA, 0.6 μM	Forskolin, 20 μM	4.5 ± 0.4***	6	**
None (control)	None	9.2 ± 1.3	6	
None	db cAMP, 30 μM	8.5 ± 0.5	6	
PMA, 0.6 μM	None	25.1 ± 0.6***	6	**
PMA, 0.6 μM	db cAMP, 30 μM	19.6 ± 1.4***	6	**
None (control)	None	13.3 ± 1.7	6	
None	APII, 20 nM	11.2 ± 2.0	6	
PMA, 0.6 μM	None	49.1 ± 2.2***	6	
PMA, 0.6 μM	APII, 20 nM	49.8 ± 1.8***	6	
None (control)	None	8.0 ± 0.6	6	
None	8 Br cGMP, 30 μM	7.2 ± 0.6	6	
PMA, 0.6 μM	None	34.0 ± 1.3***	6	
PMA, 0.6 μM	8Br cGMP, 30 μM	31.0 ± 2.9***	6	

Following exposure of endothelial monolayers to PMA the % equilibration of trypan blue-labelled albumin in the lower chamber was measured spectrophotometrically. The effects of pretreatment with forskolin, dibutyryl cyclic AMP atriopeptin II or 8 bromo cyclic GMP, for 2 min before addition of PMA are also shown. Results are expressed as the mean ± s.e.mean. ** $P < 0.01$; *** $P < 0.001$, denotes significant difference from control or a difference between groups joined by a bar.

Modulation of A23187

Treatment with forskolin (20 μM) or 8-bromo cyclic GMP (30 μM) had no effect on resting or ionophore A23187 (20 μM)-induced transfer of labelled albumin across endothelial monolayers during a 90 min incubation period (Table 1).

Bradykinin, histamine, platelet activating factor and thrombin

Four endothelial cell stimulants, i.e. bradykinin (0.1 μM), histamine (100 μM), platelet activating factor (0.2 μM) and thrombin (1 u ml^{-1}), had no effect on transfer of labelled albumin across endothelial monolayers during a 90 min incubation period (Table 4).

Discussion

The use of endothelial cell monolayers grown on polycarbonate membranes as an *in vitro* model of the vascular permeability barrier is now well established (Shasby *et al.*, 1985; Rotrosen & Gallin, 1986). An important characteristic of the permeability barrier i.e. the high permeability to small but not large molecular weight substances is conserved using this technique (Del-Vecchio *et al.*, 1987). This model has been applied mainly to venous and pulmonary artery endothelial cells in attempts to elucidate the mechanisms underlying inflammation and pulmonary oedema (Shasby *et al.*, 1982; Rotrosen & Gallin, 1986). The inflammatory mediator, histamine,

Table 4 Effects of histamine, bradykinin, thrombin and platelet activating factor (PAF) on transfer of trypan blue-labelled albumin across monolayers of pig aortic endothelial cells

Stimulus	*% equilibration of albumin*	n
None (control)	6.1 ± 0.7	5
Histamine, 100 μM	6.4 ± 0.6	6
Bradykinin, 0.1 μM	5.5 ± 1.0	6
Thrombin, 1 u ml^{-1}	6.6 ± 1.0	6
None (control)	11.7 ± 0.6	7
PAF, 0.2 μM	10.7 ± 0.4	7

Following exposure of endothelial monolayers to histamine, bradykinin, thrombin or platelet activating factor for 90 min the % equilibration of trypan blue-labelled albumin in the lower chamber was measured spectrophotometrically. Results are expressed as the mean ± s.e.mean.

has been observed to increase the transfer of high molecular weight markers across endothelial monolayers (Rotrosen & Gallin, 1986; Killackey *et al.*, 1986), consistent with its ability to increase vascular permeability *in vivo*. Inter-endothelial gap formation resulting from endothelial contraction mainly in post-capillary venules appears to be the basis of the increased vascular permeability *in vivo* (Majno & Palade, 1961). Recently, platelet activating factor, which promotes marked pulmonary oedema has been shown to induce a shape change, consistent with contraction, in cultures of pulmonary artery endothelial cells (Grigorian & Ryan, 1987). Changes in endothelial cell shape are triggered by calcium (Shasby *et al.*, 1985) or by activation of protein kinase C (Grigorian & Ryan, 1987).

In this study cultures of pig aortic endothelial cells restricted the passage of trypan blue-labelled albumin. In keeping with the *in vivo* finding that histamine and bradykinin promote plasma leakage only at venular endothelium (Svensjo *et al.*, 1979) we found that these agents did not increase the transfer of labelled albumin across aortic endothelial monolayers. Platelet activating factor and thrombin were also unable to promote increased albumin transfer despite having been shown to induce a shape change in and promote increased permeability of bovine pulmonary artery endothelial cells (Grigorian & Ryan, 1987; Minnear *et al.*, 1988; De-Michele *et al.*, 1988). Of these two agents only thrombin is known to have other effects on pig aortic endothelial cells e.g., stimulation of prostacyclin production (Pearson *et al.*, 1983).

Shasby *et al.* (1985) reported that calcium ionophore A23187 increased the transfer of albumin across monolayers of pig pulmonary artery endothelial cells and proposed that endothelial contraction was calcium-dependent. We found that ionophore A23187 induced a shape change, consistent with contraction, in pig aortic endothelial cells, and increased the permeability of monolayers to labelled albumin. This increased permeability was not solely due to inter-endothelial gap formation, but was also the result of detachment of cells. Elevation of endothelial cyclic AMP or cyclic GMP content using forskolin or 8-bromo cyclic GMP, respectively, had no effect on the ability of ionophore A23187 to induce increased transfer of labelled albumin. Bovine pulmonary artery endothelial cells have previously been reported to change from their normal polygonal morphology to an elongated spindle-like shape when exposed to the stimulant of protein kinase C (Grigorian & Ryan, 1987). We found that PMA induced a shape change in pig aortic endothelial cells. This occurred without detachment of cells and was associated with increased transfer of labelled albumin across endothelial monolayers. The ability to promote transfer of labelled albumin was concentration-dependent and was not shared by the inactive analogue, 4α-PDD and is likely to result from activation of protein kinase C. Phorbol esters stimulate production of superoxide radicals by endothelial cells (Matsubara & Ziff, 1986), and these can have adverse effects on endothelial cells (Shasby *et al.*, 1985) or their basement membrane (McCord, 1974; Greenwald & Moy, 1979), thus compromising the arterial endothelial barrier (Hennig & Chow, 1988). The lack of effect of superoxide dismutase and catalase on the PMA-induced increase in albumin transfer across aortic endothelial monolayers makes it unlikely that oxidant-induced injury was involved. It is more likely that activation of protein kinase C leads to phosphorylation of proteins involved in controlling cell shape. Two possible candidates are talin and vinculin, cytoskeletal proteins involved in regulation of cell shape and adhesion, since these are known substrates for protein kinase C (Litchfield & Ball, 1986; Werth *et al.*, 1983).

Elevation of cyclic AMP but not cyclic GMP content is known to relax epithelial cells and improve the integrity of tight junctions (Duffey *et al.*, 1981). Furthermore, *in vivo* studies have shown that stimulation of β_2-adrenoceptors inhibits the increase in vascular permeability induced by inflammatory agents, an effect caused by blockade of inter-endothelial gap formation at post-capillary venules (Svensjo & Grega, 1986; Grega, 1986; Marciniak *et al.*, 1978). *In vitro* studies with cultured pulmonary artery endothelial cells also suggest that elevation of cyclic AMP inhibits contraction and permeability (Stelzner *et al.*, 1988; Minnear *et al.*, 1988). In accord with these observations, we found that treatment with each of three agents designed to elevate cyclic AMP content i.e., dibutyryl cyclic AMP, forskolin and (±)-isoprenaline, caused a significant inhibition of PMA-induced transfer of labelled albumin across aortic endothelial monolayers without affecting resting transfer. The effect of (±)-isoprenaline was abolished in the presence of the β-adrenoceptor blocking agent, propranolol. Elevation of cyclic AMP content appears therefore to be a common mechanism whereby endothelial contractility and permeability is inhibited.

In contrast to the findings with cyclic AMP, elevation of cyclic GMP levels with either the membrane permeant analogue, 8 bromo cyclic GMP, or atriopeptin II (Martin *et al.*, 1988), failed to alter resting or PMA-induced transfer of labelled albumin across aortic endothelial monolayers. Cyclic GMP might, however, be involved in enhancing permeability at the post-capillary venule during inflammation (Chander *et al.*, 1988).

Vasodilator prostaglandins are known to potentiate vascular leakage during inflammatory states by

increasing blood flow (Williams, 1983). We found, however, that inhibition of cyclo-oxygenase using flurbiprofen had no effect on resting or PMA-induced transfer of labelled albumin across aortic endothelial monolayers.

In conclusion, our results using an *in vitro* model confirm well-established data obtained *in vivo* that inflammatory mediators have no effect on the permeability of aortic endothelium. Results with ionophore A23187 and PMA suggest that aortic endothelial contractility and permeability can be stimulated by elevation of calcium levels or activation of protein kinase C and that these two pathways are additive. Elevation of cyclic AMP but not cyclic GMP levels inhibits permeability increases induced by PMA. Pharmacological agents which elevate endothelial cyclic AMP levels and thereby inhibit transport of high molecular weight substances into the arterial wall might therefore warrant evaluation as anti-atherogenic drugs.

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Interactions of iloprost and sodium nitroprusside on vascular smooth muscle and platelet aggregation

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1 The aim of the study was to assess and quantify any synergism occuring between the stable analogues of prostacyclin (iloprost) and nitric oxide (sodium nitroprusside) with respect to both relaxation of vascular smooth muscle and inhibition of platelet aggregation in the rabbit.

2 Iloprost (0.3–3 ng ml^{-1}) and sodium nitroprusside (0.3–3 ng ml^{-1}) caused dose-dependent relaxation of the rabbit mesenteric and coeliac arteries.

3 Iloprost (0.3–30 ng ml^{-1}) and sodium nitroprusside (0.03–30 μg ml^{-1}) caused dose-dependent inhibition of rabbit platelet aggregation induced by adenosine diphosphate or collagen.

4 In combination, iloprost and sodium nitroprusside caused an inhibition of platelet aggregation that was 2–3 fold greater than would be expected by summation, while no such potentiation was observed on vascular smooth muscle.

5 Thus, our results indicate that under physiological conditions the mediators prostacyclin and endothelium-derived relaxing factor (NO) can exert a synergistic action on platelets, but have only an additive effect on vascular smooth muscle.

Introduction

Prostacyclin (PGI_2; Moncada *et al.*, 1976) activates adenylate cyclase (Tateson *et al.*, 1977; Gorman *et al.*, 1977) and the adenosine 3':5'-cyclic monophosphate (cyclic AMP) formed results in vascular smooth muscle relaxation and inhibition of platelet aggregation. Endothelium-derived relaxing factor (EDRF; Furchgott & Zawadzki, 1980) has been identified as nitric oxide (NO; Palmer *et al.*, 1987; Ignarro *et al.*, 1987) and activates guanylate cyclase (Mellion *et al.*, 1980). The guanosine 3':5'-cyclic monophosphates (cyclic GMP) formed also leads to relaxation of vascular smooth muscle (Rapoport *et al.*, 1983) and inhibition of platelet aggregation (Radomski *et al.*, 1987a). Activators of these two secondary messenger systems act synergistically to inhibit platelet aggregation (Levin *et al.*, 1982; Radomski *et al.*, 1987a; MacDonald *et al.*, 1988); an important finding since the receptor-mediated release of PGI_2 and NO from endothelial cells is coupled (de Nucci *et al.*, 1988). However, synergism of these mediators on the relaxation of vascular smooth muscle has not been demonstrated. Here we show that the stable analogues of PGI_2 and NO, iloprost and sodium nitroprusside (NP), do not act synergistically as relaxants of vascular smooth muscle. In addition, we have determined the degree of synergism between these drugs on rabbit platelets. Some of these results were presented to the British Pharmacological Society (Antunes *et al.*, 1988).

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Methods

Vascular smooth muscle

Spiral strips of rabbit mesenteric artery (RbMesA) and rabbit coeliac artery (RbCA) were denuded of endothelium and set up in a cascade (Vane, 1964). The tissues were superfused with oxygenated (95% O_2/5% CO_2) and warmed (37°C) Krebs solution containing indomethacin (5.6 μM) at a flow rate of 5 ml min^{-1}. In some experiments, U46619 (30 nM) was infused (0.05 ml min^{-1}) to contract the tissues. Iloprost and NP were infused (0.05 ml min^{-1}) for 5 min.

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Platelet aggregation

Male New Zealand White rabbits (2–2.5 kg) were treated with sodium pentobarbitone (Sagatal, 30 mg kg^{-1}, i.v.) for general anaesthesia and lignocaine (xylocaine, 2%) for local anaesthesia. The carotid artery was cannulated and blood collected into tri-sodium citrate (3.15% w/v) in a ratio of 9:1. The blood was centrifuged in a Petalfuge at low speed (200 *g*) for 8 min to produce platelet-rich plasma (PRP). Platelet-poor plasma (PPP) was obtained by further centrifugation of the PRP (Hermle centrifuge Z 230 M, FRG) at high speed (12,000 *g*) for 1 min.

Platelet aggregation was studied by use of a dual channel Payton aggregometer calibrated with PRP (0%) and PPP (100%) with respect to the degree of light transmission. Aliquots (0.5 ml) of PRP were added to siliconised cuvettes (37°C) and stirred (1000 rev min^{-1}). Platelets were incubated for approximately 1 min to establish a baseline and incubated with iloprost, NP or both for 1 or 5 min before the addition of a dose of adenosine diphosphate (ADP) or collagen which caused near maximal aggregation (approximately 90%).

The % inhibition of platelet aggregation was calculated from the maximum increase in light transmission observed over a 4 min period after addition of the aggregating agent, as compared to that of a control.

Preparation of washed platelets

Prostacyclin (300 ng ml^{-1}) was added to PRP and centrifuged (900 *g* for 18 min) in a Hereus Minifuge T centrifuge to sediment the platelets. The resulting supernatant was removed and replaced with an equal volume of Krebs solution (Ca^{2+}-free), warmed to 37°C. The pellet was gently resuspended and a further dose of prostacyclin (300 ng ml^{-1}) was added. The platelets were sedimented again by centrifugation (900 *g*, 18 min) and the platelet pellet resuspended in fresh Krebs solution (Ca^{2+}-free) at 37°C. $CaCl_2$ (1 mM) was added and the washed platelet suspension (WPS) kept at room temperature (Radomski & Moncada, 1983).

Materials

The composition of the Krebs solution was (mM): NaCl 118, $NaHCO_3$ 25, glucose 5.6, KCl 4.7, KH_2PO_4 1.2, $MgSO_4 \cdot 7H_2O$ 1.17 and $CaCl_2$ 2.5. Collagen (fibril suspension) was obtained from Hormon-Chemie (Munchen GMBH). ADP, indomethacin and NP were bought from Sigma Chemical Co (Poole, UK). Iloprost was a gift from Dr L. Sprazagala, Schering Co, (FRG) and PGI_2 was a gift from Dr B.J.R. Whittle, Wellcome Research Laboratories, U.K. U46619 (9,11-dideoxy-9α,11α-methanoepoxyprostaglandin $F_{2\alpha}$) was a gift from Dr J. Pike, Upjohn, Kalamazoo, MI.

Statistical comparison

All dose-response curves were analysed by use of a two-way analysis of variance followed by a least significant difference procedure (LSD) to determine the nature of the response (SPSS Inc., Chicago, Il., USA). A P value of <0.05 was considered statistically significant. ED_{50} values (dose responsible for 50% maximum relaxation or 50% inhibition of platelet aggregation) were calculated for comparison.

Results

Interactions of threshold doses of iloprost and sodium nitroprusside

Vascular smooth muscle Iloprost (0.3–30 ng ml^{-1}) or NP (0.3–30 ng ml^{-1}) caused dose-dependent relaxations of the RbMesA and RbCA precontracted with U46619. Co-infusion of a threshold dose of NP (0.3 ng ml^{-1}) did not significantly alter the iloprost dose-response curve on the RbMesA or the RbCA (Figure 1). Similarly, co-infusion of a threshold dose of iloprost (0.3 ng ml^{-1}) did not alter the NP dose-response curve on either vascular tissue. These data are included in Table 1, expressed as ED_{50} values.

Table 1 The effect of threshold concentrations of sodium nitroprusside (NP) and iloprost (Ilo) on iloprost and NP-induced relaxation of vascular smooth muscle

Tissue	*Drug* (ng ml^{-1})	ED_{50} (ng ml^{-1})
RbMesA	Ilo	7.4 ± 2.2
	Ilo + NP (0.3)	9.9 ± 2.2
	NP	13.9 ± 2.8
	NP + Ilo (0.3)	11.7 ± 2.7
RbCA	Ilo	8.4 ± 1.7
	Ilo + NP (0.3)	7.0 ± 1.5
	NP	16.1 ± 4.5
	NP + Ilo (0.3)	10.3 ± 2.3

Threshold concentrations of NP (0.3 ng ml^{-1}) or iloprost (0.3 ng ml^{-1}) did not significantly alter the ED_{50} values for iloprost or NP-induced relaxation of rabbit mesenteric artery (RbMesA) and rabbit coeliac artery (RbCA) precontracted with U-46619 (30 nM). Data are expressed as mean ± s.e.mean of at least four observations. No significant difference was detected as calculated by a two-way analysis of variance.

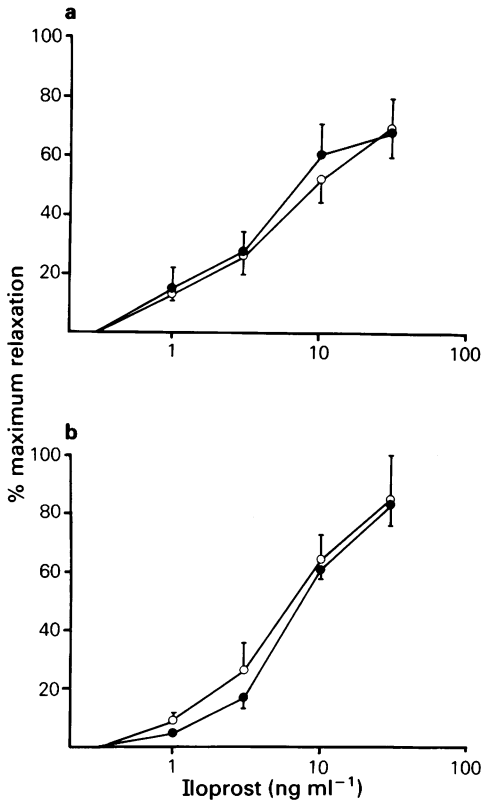

Figure 1 A threshold concentration of sodium nitroprusside (NP) did not act synergistically with iloprost on rabbit mesenteric (a) or rabbit coeliac (b) arteries precontracted with U-46619 (30 nM). The dose-response curve to iloprost alone (●) was not signficantly different from iloprost in the presence of 0.3 ng ml^{-1} NP (○). Data are expressed as mean of four observations. Vertical lines show s.e.mean.

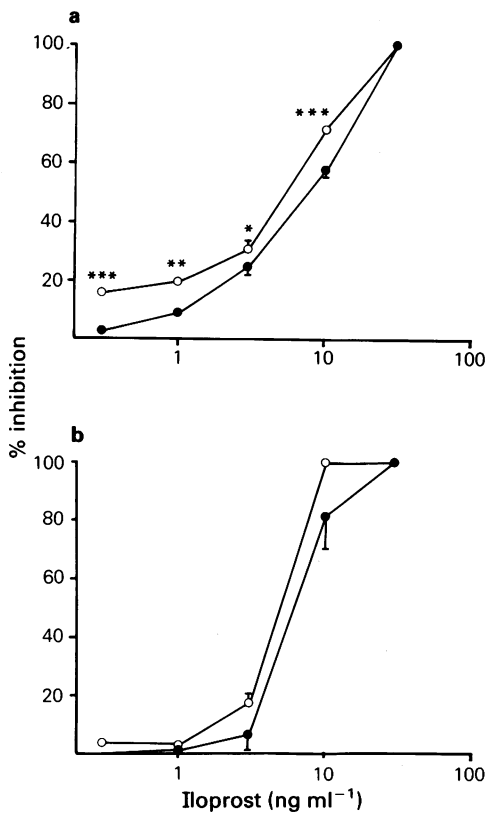

Figure 2 A threshold concentration of sodium nitroprusside (NP) acts synergistically with iloprost on inhibition of platelet aggregation induced by adenosine diphosphate (a) but not by collagen (b). The dose-response curve to iloprost alone (●) was significantly different from iloprost in the presence of 30 ng ml^{-1} NP (○) when using adenosine diphosphate, but not significantly different in the presence of 100 ng ml^{-1} NP when using collagen as the aggregating agent. Data are expressed as mean of four observations; vertical lines show s.e.mean. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$.

Platelet aggregation

In PRP iloprost (0.3–30 ng ml^{-1}) or NP (0.03–30 μg ml^{-1}) caused dose-dependent inhibition of platelet aggregation induced by ADP (4 μM) or collagen (4 μg ml^{-1}). A threshold dose of NP enhanced slightly the iloprost-induced inhibition of platelet aggregation (Figure 2), although only the dose-response curve obtained with ADP reached a statistically significant difference. Similar results were obtained with a threshold dose of iloprost on the inhibition of platelet aggregation induced by NP. These data are included in Table 2, expressed as ED_{50} values.

An increase in the incubation time from 1 to 5 min before challenge with collagen did not enhance the activity of NP. When collagen-induced aggregation of PRP was studied the potency-ratio of iloprost ($ED_{50} = 6.3 \pm 0.9$ ng ml^{-1}) and NP ($ED_{50} = 6000 \pm 2300$ ng ml^{-1}) was 1:952, while in WPS the potency ratio of iloprost ($ED_{50} = 2.7 \pm 0.9$ ng ml^{-1}) and NP ($ED_{50} = 1410 \pm 710$ ng ml^{-1}) was 1:517. The activity of NP as compared to iloprost was only slightly increased following the platelet washing procedure.

Interaction of effective doses of iloprost and sodium nitroprusside

Vascular smooth muscle Co-infusion of effective doses of iloprost (3 ng ml^{-1}) and NP (3 ng ml^{-1})

Table 2 The effect of threshold concentrations of sodium nitroprusside (NP) and iloprost (Ilo) on iloprost and NP-induced inhibition of platelet aggregation

Agonist	*Drug* (ng ml^{-1})	ED_{50} (ng ml^{-1})
ADP	Ilo	7.5 ± 0.4
	Ilo + NP (30)	5.2 ± 0.3*
	NP	1600 ± 100
	NP + Ilo (0.3)	1100 ± 200
Collagen	Ilo	6.3 ± 0.9
	Ilo + NP (100)	4.9 ± 0.1
	NP	6000 ± 2300
	NP + Ilo (0.3)	2500 ± 1000

Threshold concentrations of NP (30 ng ml^{-1} for adenosine diphosphate, 100 ng ml^{-1} for collagen) or iloprost (0.3 ng ml^{-1}) caused a reduction in the ED_{50} values for iloprost or NP-induced inhibition of platelet aggregation. Data are expressed as mean ± s.e.mean of four observations. Statistical significance was calculated by a two-way analysis of variance (* $P < 0.05$).

caused smooth muscle relaxation which was equivalent to the expected 'additive' result. A co-infusion of higher concentrations of iloprost and NP (10 ng ml^{-1}) similarly indicated a lack of synergism (Table 3).

Platelet aggregation Incubation of 2 or 4 ng ml^{-1} iloprost with PRP induced a small inhibition of collagen-induced platelet aggregation, similar to that induced by 1 or 2 μg ml^{-1} NP. However, co-incubation of 2 ng ml^{-1} iloprost and 1 μg ml^{-1} NP with PRP caused an inhibition of platelet aggregation equivalent to either 8 ng ml^{-1} iloprost or 4 μg ml^{-1} NP, exhibiting synergism (Figure 3).

Table 3 The effect of co-infusion of iloprost (Ilo) and sodium nitroprusside (NP) on vascular smooth muscle

Drug (ng ml^{-1})	% *maximum relaxation*	
	RbMesA	*RbCA*
Ilo (3)	22.8 ± 5.0	23.2 ± 6.8
NP (3)	17.8 ± 2.1	22.2 ± 5.4
Ilo (3) + NP (3)	41.2 ± 2.8	49.6 ± 11.7
Ilo (10)	56.8 ± 6.3	52.7 ± 11.3
NP (10)	42.2 ± 3.5	42.2 ± 8.2
Ilo (10) + NP (10)	91.4 ± 2.7	78.1 ± 10.0

This illustrates the summation of the relaxant action of iloprost and NP on rabbit mesenteric arteries (RbMesA) and rabbit coeliac arteries (RbCA) precontracted with U-46619 (30 nM). Data are expressed as mean ± s.e.mean of at least five experiments.

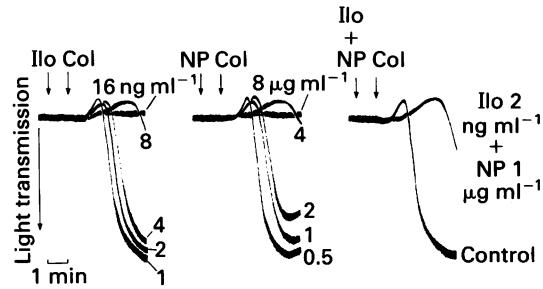

Figure 3 Synergism between effective doses of iloprost (Ilo) and sodium nitroprusside (NP) in inhibiting platelet aggregation induced by collagen (Col). The superimposed traces illustrate the dose-dependent effect of iloprost (1–16 ng ml^{-1}) and NP (0.5–8 μg ml^{-1}) on platelet aggregation. Incubation of 2 ng ml^{-1} iloprost in combination with 1 μg ml^{-1} NP had a greater than additive effect.

Similar results were obtained with ADP-induced platelet aggregation (Figure 4). The degree of inhibition obtained on incubation of PRP with iloprost and NP clearly had a greater than additive effect (Table 4).

Discussion

The demonstration of synergism between PGI_2 and EDRF in platelets has relied upon representation of dramatic inhibition of aggregation with combinations of low concentrations of these substances (Radomski *et al.*, 1987a; McDonald *et al.*, 1988) without presenting a full dose-response curve for quantitative analysis. Such data are essential in order to establish whether these agonists have a real

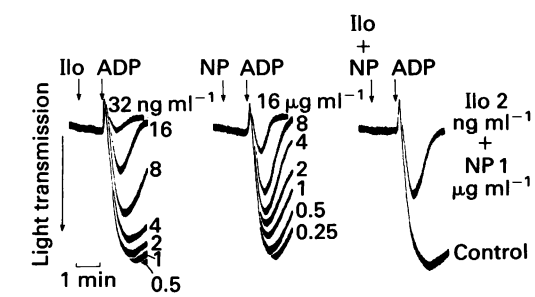

Figure 4 Synergism between effective doses of iloprost (Ilo) and sodium nitroprusside (NP) in inhibiting platelet aggregation induced by adenosine diphosphate (ADP). The superimposed traces illustrate the dose-dependent effect of iloprost (0.5–32 ng ml^{-1}) and NP (0.25–16 μg ml^{-1}) on platelet aggregation. Incubation of 2 ng ml^{-1} iloprost in combination with 1 μg ml^{-1} NP had a greater than additive effect.

Table 4 The effect of co-incubation of iloprost (Ilo) and sodium nitroprusside (NP) on inhibition of platelet aggregation

Drug ($ng\,ml^{-1}$)	*% inhibition* ADP	Collagen
Ilo (1)	3 ± 0	4 ± 2
NP (500)	14 ± 2	4 ± 2
Ilo (1) + NP (500)	35 ± 4	28 ± 9
Ilo (2)	11 ± 2	6 ± 2
NP (1000)	22 ± 0	12 ± 2
Ilo (2) ± NP (1000)	61 ± 5	74 ± 6

This illustrates the synergism between the inhibitory actions of iloprost and NP on adenosine diphosphate (ADP)- and collagen-induced platelet aggregation. Data are expressed as mean ± s.e.mean of three experiments.

synergistic action or merely an additive effect. In this work, we used NP and iloprost as stable analogues of nitric oxide and prostacyclin, in order to compare their interaction in platelets with that in smooth muscle.

With respect to inhibition of platelet aggregation, co-incubation of iloprost and NP showed a 2–3 fold increase in activity, above the expected additive effect. Interestingly, the synergism was most clearly observed when active concentrations were combined, presumably because increases in both cyclic AMP and cyclic GMP were needed. However, no synergism was observed on the relaxation of vascular smooth muscle, when using either threshold or active concentrations of iloprost or NP.

The finding that iloprost and NP act synergistically in platelets but not in smooth muscle may have therapeutic implications. In theory, the ability to potentiate the inhibition of platelets, while not potentiating the concomitant vasodilatation and fall in blood pressure would be beneficial in treatments directed at preventing aggregate formation without causing hypotension. For such a synergism to be exploited, a nitrovasodilator which has a similar potency on vascular smooth muscle and on platelets would be needed.

Although identical concentrations of iloprost and NP were required to relax vascular smooth muscle, 1000 times more NP than iloprost was needed to inhibit platelet aggregation. Since this difference was also observed with washed platelets, the difference in potency cannot be attributed to binding of NP to plasma proteins. Furthermore, the inhibitory activity of NP on platelets was not enhanced by increasing the incubation time, suggesting that a balance exists between release of NO and its breakdown to nitrite and nitrate. NP is thought to liberate NO spontaneously (see Waldman & Murad 1987), but authentic NO, when used exogenously, is approximately equipotent on vascular smooth muscle and on platelets (Hutchinson *et al.*, 1987; Radomski *et al.*, 1987b). We must assume, therefore, that the liberation of NO from NP is greatly accelerated by a mechanism present in vascular smooth muscle but absent from platelets. This mechanism may be similar to that proposed for the action of organic nitrates, such as glyceryl trinitrate, which are converted to NO enzymically (see Waldman & Murad 1987) or stimulated to release NO by thiol-containing compounds, such as cysteine (Feelisch & Noack, 1987). Indeed, like NP, glyceryl trinitrate has a weak inhibitory effect on platelet aggregation compared to its action on vascular smooth muscle (Schafer, 1980); a strong indication that platelets lack an efficient mechanism to generate NO from either organic nitrates or NP.

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Quinoxalines interact with the glycine recognition site of NMDA receptors: studies in guinea-pig myenteric plexus and in rat cortical membranes

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1 The effects of several quinoxalines, including 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and 6,7,dinitroquinoxaline-2,3-dione (DNQX), and of two kynurenates, kynurenate (KYNA) and 7-Cl-kynurenate (7-Cl-KYNA), have been evaluated on the N-methyl-D-aspartate (NMDA) receptors present in the guinea-pig ileum myenteric plexus preparation and on the strychnine-insensitive [^{3}H]-glycine binding sites of cortical membranes.

2 Quinoxalines and kynurenates antagonized in a non-competitive manner L-glutamate-induced contraction. Their IC_{50}s were (in μM): 5 for 7-Cl-KYNA, 7.5 for 6,7-Cl-3-hydroxy-2-quinoxaline carboxylate (6,7-Cl-HQCA), 20 for DNQX, 50 for CNQX, 76 for KYNA and 125 for 3-hydroxy-2-quinoxaline carboxylate (HQCA).

3 Glycine (5–50 μM) completely reversed the antagonism displayed by both quinoxalines and kynurenates. The interaction between glycine and the tested compounds appeared to be competitive in nature.

4 Quinoxalines and kynurenates displaced, in a concentration-dependent manner, [^{3}H]-glycine from its strychnine-insensitive binding sites present in rat cortical membranes. Their IC_{50}s for this action were (in μM): 0.45 for 7-Cl-KYNA, 0.6 for 6,7-Cl-HQCA, 2.4 for DNQX, 3.5 for CNQX, 20 for KYNA and 40 for HQCA.

5 When the IC_{50}s for the displacement effect of [^{3}H]-glycine binding were plotted against the IC_{50}s obtained in the myenteric plexus, a significant correlation was found.

6 These data show that quinoxalines and kynurenates may antagonize the responses to L-glutamate by interacting with the glycine recognition sites of the NMDA receptor ion channel complex.

Introduction

The quinoxalinediones, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, also known as FG9065) and 6,7, dinitroquinoxaline-2,3-dione (DNQX, FG9041), have been reported to be selective, potent and competitive antagonists for the quisqualate and the kainate subtypes of excitatory amino acid receptors (Honore' *et al.*, 1988; Fletcher *et al.*, 1988), and have therefore been proposed and utilized as powerful tools for detailed investigations on the physiological role of non N-methyl-D-aspartate (NMDA) receptors (Blake *et al.*, 1988; McBain *et al.*, 1988; Andreasen *et al.*, 1989; Davies *et al.*, 1989; Harris, 1989). However, quantitative pharmacological studies have revealed that both CNQX and DNQX also block NMDA responses in a non-competitive manner, probably interacting with the strychnine-insensitive glycine modulatory site of NMDA receptors (Birch *et al.*, 1988b; Foster, 1988; Verdoorn & Dingledine, 1988).

The myenteric plexus of the guinea-pig ileum contains excitatory amino acid receptors of the NMDA type, but not of the quisqualate or kainate type: the stimulation of these receptors causes release of acetylcholine and induces ileal contraction (Moroni *et al.*, 1986; Wiley *et al.*, 1987). As originally observed by Johnson & Ascher (1987) in cultured neuronal cells, glycine positively modulates NMDA

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receptors. This occurs also in the guinea-pig myenteric plexus (Luzzi *et al.*, 1988), while kynurenate (KNYA) competitively antagonizes glycine actions (Moroni *et al.*, 1989). These observations make the guinea-pig ileum myenteric plexus a useful tool for studying agonists, antagonists and modulators of the NMDA receptor ion channel complex.

In order to clarify whether or not quinoxalinediones interact with NMDA receptors we tested CNQX, DNQX and other quinoxaline derivatives such as 3-hydroxy-2-quinoxaline carboxylic acid (HQCA) and 6,7,dichloro-3-hydroxy-2-quinoxaline carboxylic acid (6,7-Cl-HQCA) in the guinea-pig myenteric plexus. Since we found an inhibitory activity for these compounds, we further characterized their pharmacological action by comparing, in this system, their effects with those of KYNA and 7-chlorokynurenate (7-Cl-KYNA). Both are thought to act at the glycine modulatory site of the NMDA receptor-ion channel complex, with 7-Cl-KYNA being considered more selective and more potent (Kessler *et al.*, 1987; Birch *et al.*, 1988a; Kemp *et al.*, 1988; Watson *et al.*, 1988). Parallel experiments were performed to evaluate the effects of quinoxalines on the strychnine-insensitive glycine binding sites present in rat cortical membranes.

Methods

Functional studies

Male guinea-pigs weighing 300–350 g were used and the longitudinal muscle of the ileum with the myenteric plexus attached was prepared, mounted and pre-incubated for 3 h in a Mg^{2+}-free Krebs solution as previously described in detail (Moroni *et al.*, 1986; Luzzi *et al.*, 1988). The tested antagonists, dissolved in small volumes, were added to the bath immediately after the closure of the superfusion system and 15–20 s before the challenge dose of L-Glu. When the ileal contraction reached its maximum they were washed out together with L-Glu by opening the superfusion system. There was a 15–20 min interval before testing another dose. To study the interaction between glycine and the antagonists, the different compounds were added to the closed system in this order: antagonist, glycine and challenge dose of L-Glu.

Calculation of functional IC_{50}s of the antagonists

In order to calculate the IC_{50}s of the antagonists, at least four doses of each molecule were tested in a single preparation. The response to 100 μM Glu was considered the maximal response (100%) and this was evoked before and after each dose of the supposed antagonist. The percentage inhibition of the ileal contraction induced by every dose of antagonist was evaluated and a regression curve was constructed. IC_{50}s were calculated as the mean dose that inhibited the response to 100 μM Glu by 50%. For each molecule at least 5 different preparations were used.

Binding studies

In order to study the interactions of these molecules with the strychnine-insensitive glycine recognition sites of central nervous system membranes, binding experiments in rat (male, Wistar strain) cortices were performed according to Kishimoto *et al.* (1981), using 20 nM 2-[^{3}H]-glycine (16.8 Ci mmol^{-1}) as ligand and 50 mM Tris-citrate buffer (pH 7.2) as standard medium. In order to reduce the concentrations of endogenous Glu and glycine, membranes, before being used in the binding assay, were resuspended (5 mg protein ml^{-1}) and dialyzed at 0–4°C for 24 h against the buffer (3 × 500 ml).

Statistical evaluation of the data

Analysis of the regression lines (slope, intercept and correlation coefficient), tests for parallelism and Schild plot were performed utilizing a computer programme as described by Tallarida & Murray (1984).

Chemicals

L-Glutamate (L-Glu), glycine and KYNA were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). CNQX, DNQX, HQCA, 6,7-Cl-HQCA and 7-Cl-KYNA were from Tocris Neuramin Ltd. (Essex, U.K.). DL-2-Amino-5-phosphonovaleric acid (DL-AP5) was obtained from Cambridge Research Biochemicals (Harston, U.K.) and 2-[^{3}H]-glycine was from Amersham (Amity-PG, Milano, Italy).

Results

Effects of quinoxalines on the guinea-pig myenteric plexus

Figure 1 shows that DNQX (10 μM), 6,7-Cl-HQCA (10 μM), HQCA (50 μM) and CNQX (10 μM) antagonized in an apparent non-competitive manner the effects of L-Glu on the guinea-pig myenteric plexus.

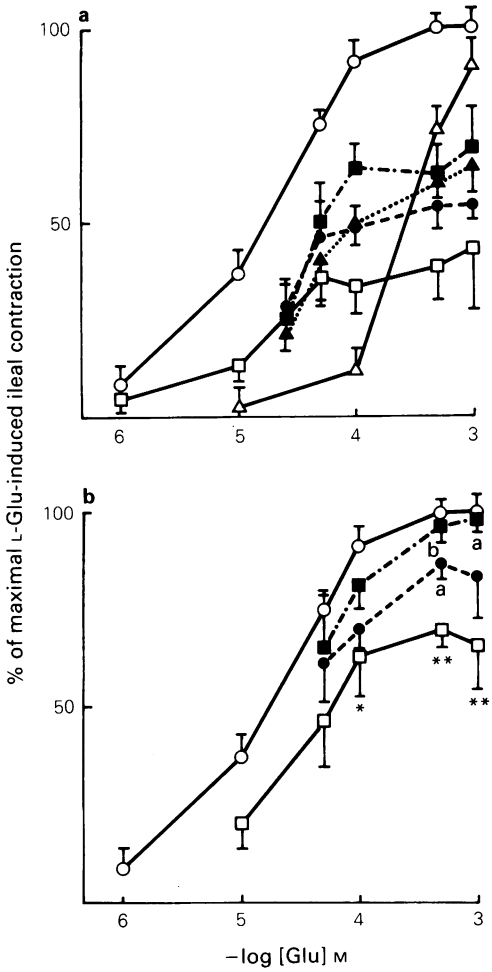

Figure 1 (a) Effects of 7-Cl-kynurenate (7-Cl-KYNA, □, 10 μM); 6,7-dinitroquinoxaline-2,3-dione (DNQX, ●, 10 μM); 6,7-Cl-3-hydroxy-2-quinoxaline carboxylate (6,7-Cl-HQCA, ▲, 10 μM); 3-hydroxy-2-quinoxaline carboxylate (HQCA, ■, 50 μM) and D,L-2-amino-5-phosphonovaleric acid (DL-AP5, △, 100 μM) on the dose-response curve (○) for L-glutamate (L-Glu)-induced guinea-pig myenteric plexus longitudinal muscle contraction. The maximal response (100% value) to 1 mM L-Glu was obtained at the end of each experiment. (b) The effects of different concentrations of glycine (●: 5 μM and ■: 50 μM) on 10 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) antagonism (□) of L-Glu-induced ileal contraction (○). CNQX and glycine were added to the bath 15–20 s before L-Glu. Vertical bars show s.e.mean of at least four experiments for each point in (a) and in (b). In (b) statistical significance was assessed by means of Student's *t* test.
* $P < 0.05$ vs Glu alone; ** $P < 0.01$ vs. Glu alone; [a] $P < 0.05$ vs. Glu + CNQX alone; [b] $P < 0.01$ vs. Glu + CNQX alone.

Their action was comparable to that of 7-Cl-KYNA (10 μM) and KYNA (300 μM) while DL-AP5 (100 μM) behaved as a competitive antagonist (see also Moroni *et al.*, 1989). The relative potencies of these compounds, calculated as their IC_{50}s (i.e. the concentration of antagonist which inhibited the response to 100 μM L-Glu by 50%), were (± s.e.mean): 5 ± 4 μM for 7-Cl-KYNA, 7.5 ± 0.9 μM for 6,7-Cl-HQCA, 20 ± 1.7 μM for DNQX, 50 ± 3.4 μM for CNQX, 76 ± 8.9 μM for KYNA and 125 ± 13 μM for HQCA.

Glycine dose-dependently reversed the L-Glu antagonistic effect of 10 μM CNQX (Figure 1b) and similar curves were obtained when the antagonists were 7-Cl KYNA, DNQX, 6,7-Cl HQCA, HQCA (data not presented) or KYNA (see Moroni *et al.*, 1989). The inhibitory action of each of these substances was completely reversed by increasing the concentration of glycine, thus suggesting they act as competitive antagonists of glycine.

In our experimental model, glycine dose-dependently potentiated the response to 50 μM L-Glu, and this curve was shifted to the right in the presence of 5, 10 and 30 μM DNQX (Figure 2a). Dose-ratios were 2.9 ± 0.9, 10.2 ± 2.2 and 22.7 ± 4.2 respectively, and the test for parallelism indicated that the slopes of these curves did not differ significantly. Schild analysis (Figure 2b) gave a pA_2 value of 5.6 ± 0.2 and slope not significantly different from −1 (−1.32 ± 0.39).

Effects of quinoxalines on the binding of [^{3}H]-glycine to rat cortical membranes

Rat cortical membranes had saturable, high affinity, strychnine-insensitive [^{3}H]-glycine binding sites, having a K_D of 0.2 ± 0.06 μM and a B_{max} of 3.9 ± 0.8 pmol mg^{-1} protein. Figure 3 shows that [^{3}H]-glycine was dose-dependently displaced by quinoxalines and by kynurenates, while glutamate (1 mM) and DL-AP5 (0.1 mM) were inactive. The IC_{50}s were (± s.e.mean): 0.45 ± 0.07 μM for 7-Cl-KYNA, 0.6 ± 0.06 μM for 6,7-Cl-HQCA, 2.4 ± 0.6 μM for DNQX, 3.5 ± 0.5 μM for CNQX, 20.2 ± 4.4 μM for KYNA and 43 ± 4.7 μM for HQCA. In Figure 4 the IC_{50}s of these compounds for inhibition of [^{3}H]-glycine binding were plotted against their IC_{50}s for inhibition of L-Glu-induced ileal contraction. Their displacing potencies correlated significantly with their relative pharmacological potencies (correlation coefficient $r = 0.96$, $P < 0.01$).

Discussion

It has been previously suggested that the quinoxalinediones CNQX and DNQX, at concentrations sig-

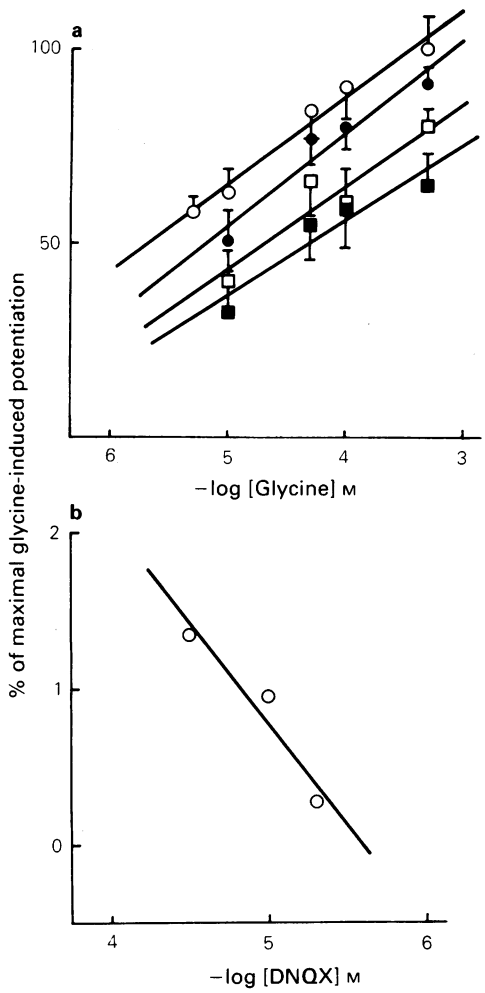

Figure 2 (a) Effects of (●) 5 μM, (□) 10 μM and (■) 30 μM 6,7-dinitroquinoxoline-2,3-dione (DNQX) on the (○) glycine dose-response curve for the potentiation of 50 mM L-glutamate (L-Glu)-induced ileal contraction. Each preparation was used for the construction of a dose-response curve to glycine in the absence and presence of one dose of DNQX. DNQX was added to the closed bath after glycine, 15–20 s before the challenge dose of L-Glu. The slopes of the curves were not significantly different. Dose-ratios are given in the text. Vertical bars are s.e.mean of at least 4 experiments for each point. (b) Schild plot of DNQX versus glycine potentiating effect. The pA_2 value was 5.6 ± 0.2 and the slope was not significantly different from −1 (−1.32 ± 0.39).

nificantly higher than those able to block competitively kainate and quisqualate receptors, may also affect receptors of the NMDA type (Birch *et al.*, 1988b; Blake *et al.*, 1988; Fletcher *et al.*, 1988; Honoré *et al.*, 1988). Our experiments, performed in

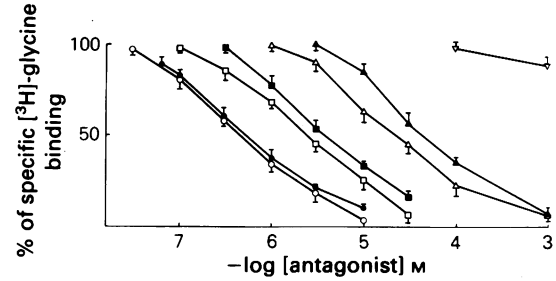

Figure 3 The dose-dependent displacing effect of (○) 7-Cl-KYNA, (●) 6,7-Cl-HQCA, (□) DNQX, (■) CNQX, (△) KYNA, (▲) HQCA and (▽) DL-AP5 on specific [^{3}H]-glycine binding to rat cortical membranes. Each point is the mean of at least four experiments run in triplicate. See text for IC_{50} values. The presence or the absence of L-Glu (up to 1 mM) did not affect the displacing effect of the antagonists. Vertical bars are s.e.mean. See Figure 1 legend for abbreviations.

the guinea-pig ileum myenteric plexus, which is a pharmacological preparation suitable for the study of molecules interacting both with the glutamate and the glycine recognition sites of the NMDA receptor ion channel complex (Moroni *et al.*, 1986; Luzzi *et al.*, 1988; Moroni *et al.*, 1989; Reggiani *et al.*, 1989), confirmed that CNQX and DNQX non-competitively inhibited NMDA responses and demonstrated that their actions may be competitively antagonized by glycine. This concept is sup-

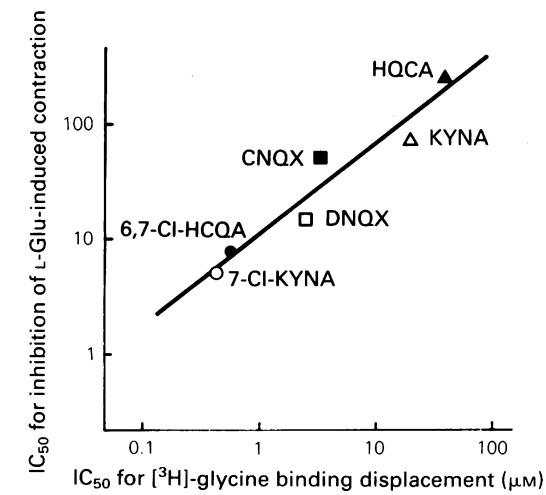

Figure 4 Correlation between the displacing potencies of quinoxalines and kynurenates on specific [^{3}H]-glycine binding and their antagonistic activities on L-glutamate (L-Glu)-induced ileal contraction. The line was constructed by linear regression. Correlation coefficient $r = 0.96$, $P < 0.01$. See Figure 1 legend for abbreviations.

ported (1) by the possibility of completely surmounting the antagonism of quinoxalinediones by increasing glycine concentrations in the bath, (2) by the observation that the glycine dose-response curve for the potentiation of L-Glu-evoked contraction shifts in a parallel manner when different concentrations of DNQX are used, and (3) by the finding that the slope of the Schild plot performed after these curves is not significantly different from −1. Further confirmation of these functional observations is derived from studies performed in cortical membranes, showing that [^{3}H]-glycine binding was displaced by quinoxalinediones and other quinoxalines in a manner resembling that of kynurenates.

Thus, quinoxalinediones appeared to have a pharmacological profile very similar to that of KYNA, 7-Cl-KYNA, and other quinoxalines, such as HQCA and 6,7-Cl-HQCA, which are also known to possess antagonist actions at the NMDA receptor level (Erez *et al.*, 1985; Frey *et al.*, 1988). The micromolar concentrations of CNQX and DNQX used in some electrophysiological studies to block selectively non-NMDA receptors (Blake *et al.*, 1988; Fletcher *et al.*, 1988; McBain *et al.*, 1988; Andreasen *et al.*, 1989; Davies *et al.*, 1989) were sufficient in the guinea-pig myenteric plexus to reduce the L-Glu-induced (NMDA receptor-mediated) ileal contraction. Furthermore, the concentrations of quinoxalinediones which displaced [^{3}H]-kainate and [^{3}H]-α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate (AMPA) binding (Honoré *et al.*, 1988) were in the same range as those able to displace [^{3}H]-glycine in our experiments.

The similarity between the pharmacological properties of quinoxalines and those of kynurenates may also be indicated by the observation that similar molecular transformations, namely the chloride substitution in KYNA and HQCA, strongly increased both the potency in displacing [^{3}H]-glycine and in inhibiting L-Glu-mediated ileum contraction. A significant correlation was indeed observed between the IC_{50} for displacement of [^{3}H]-glycine binding and the IC_{50} for the antagonism of L-Glu-induced ileal contraction. The lower doses of antagonists required for the inhibition of glycine binding may be due to the presence of endogenous glycine in the bathing solution or to diffusion problems of the molecules within the myenteric plexus preparation.

The association of [^{3}H]-glycine binding measurements in rat cortical membranes with functional studies on the guinea-pig ileum myenteric plexus has therefore proved to be a simple and reliable pharmacological approach for exploring the real capability of different drugs to interact with the glycine recognition site of the NMDA receptor ion channel complex.

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Glucocorticoids inhibit prostaglandin synthesis not only at the level of phospholipase A_2 but also at the level of cyclo-oxygenase/PGE isomerase

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1 Prostanoid synthesis was induced in bone marrow-derived macrophages by addition of exogenous arachidonic acid to the cell cultures. When the cells were preincubated with dexamethasone (10^{-7} and 10^{-6} M) overnight, prostaglandin synthesis was inhibited by $66.5 \pm 2.8\%$ and $56.7 \pm 2.9\%$ (mean $\pm$ s.d.; $n = 3$) respectively.

2 Endogenous membrane bound phospholipase A_2 was measured with labelled phospholipids used as substrates. The enzyme activity with phosphatidylcholine and phosphatidylethanolamine as substrates was inhibited by $27.0 \pm 8.3\%$ and $23.3 \pm 11.1\%$ ($n = 4$) respectively, in dexamethasone-treated macrophages compared to control cells. Neither the distribution of radiolabelled arachidonic acid among the different phospholipid species nor the release of arachidonic acid from prelabelled cells were significantly impaired by pretreatment of the macrophages with dexamethasone (1 μM).

3 The enzyme activity of the cyclo-oxygenase/prostaglandin E (PGE) isomerase was measured in cell membranes from control cells and dexamethasone-treated cells. It was inhibited by $40.0 \pm 8.4\%$ ($n = 4$) in dexamethasone-treated cells as compared to control cells. Thus, glucocorticoids inhibit not only phospholipase A_2 in these cells, but predominantly inhibit arachidonic acid metabolism subsequent to its release from phospholipids.

Introduction

Glucocorticoids exert their multiple metabolic functions by binding to cytoplasmic receptors and subsequent induction of catalytic or regulatory proteins (Rousseau, 1984). Their anti-inflammatory properties have been attributed in part to the liberation and enhanced synthesis of proteins, collectively called lipocortins, which inhibit phospholipase A_2, leading to a decreased eicosanoid synthesis (Flower, 1984). Such proteins have been isolated from various cells (Sato *et al.*, 1988, and references therein) and cloned from the human histiocytic lymphoma line U937 (Wallner *et al.*, 1986). Lipocortins were shown to be identical with another group of proteins, membrane cytoskeletal proteins which bind calcium and phospholipids including calpactins I and II, as well as other proteins (Huang *et al.*, 1986; Brugge, 1986). These proteins are very abundant and may account for up to 2.5% of total cell protein as shown in endothelial cells (Hullin *et al.*, 1989). Their exact physiological function has not yet been defined but it has been supposed that they may play a role in membrane-to-cytoskeleton linkage (Glenney *et al.*, 1987) or regulation of cell functions such as secretion (Hutton, 1986).

The effect of glucocorticoids on phospholipase A_2 is most often measured by the decreased release of fatty acids from prelabelled cellular lipids (e.g. Hong & Levine, 1976; Flower & Blackwell, 1979; Hirata *et al.*, 1980). An attempt to measure reduced endogenous phospholipase activity with exogenous substrate was not successful (Russo-Marie & Duval, 1982). The phospholipase A_2 inhibitory capacity of lipocortins is usually measured with pancreatic phospholipase and various labelled phospholipids, most often labelled *E. coli*, as substrate. It was shown recently at least in the case of some substrates, that lipocortins do not interact directly with the phospholipase A_2 but inhibit the enzyme by interaction with the substrate phospholipids (Davidson *et al.*, 1987; Aarsman *et al.*, 1987).

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By comparing arachidonic acid release and PGE_2 synthesis in unstimulated mouse peritoneal macrophages, Wood *et al.* (1984) found an inhibition of PGE_2 synthesis but not arachidonic acid release in hydrocortisone-treated cells. The molecular mechanism underlying these effects of hydrocortisone, however, was not further investigated. Therefore, in the present study we investigated the molecular mechanism of the inhibition of prostaglandin synthesis in macrophages by dexamethasone. For the first time we were able to measure a decreased enzyme activity of membrane-bound endogenous phospholipase A_2 after treatment of cells with dexamethasone. In addition, a marked decrease of cyclo-oxygenase/PGE isomerase activity was demonstrated which was even more pronounced than the inhibition of phospholipase A_2 activity. These findings indicate a more complex interference of glucocorticoids with arachidonic acid metabolism than has often been suggested.

Methods

Bone marrow cultures

Bone marrow-derived macrophages were cultivated in a similar way to that described by Fischer *et al.* (1984). Cells were flushed from the femora of six to eight week-old C57/B16 mice and cultivated in DMEM (4.5 g ml^{-1} glucose, Gibco, Karlsruhe, F.R.G.), supplemented with 1 mM sodium pyruvate, 2 mM glutamine, 1% non essential amino acids, 5×10^{-5} M 2-mercaptoethanol, 10% new born calf serum (Gibco), 5% horse serum (Boehringer Mannheim, F.R.G.) and 30% L-cell conditioned medium. Bone marrow cells were seeded in petri dishes (Greiner Nuertingen, F.R.G.) (10 ml, 1×10^5 cells ml^{-1}) and cultured at 37°C in a humidified atmosphere containing 10% CO_2. At day 5, 5 ml of the medium, containing non adherent cells, were removed and supplemented by 10 ml fresh medium. Medium (10 ml) was also exchanged on day 7. At day 8, the medium was removed and the adherent cells were washed twice with warm phosphate buffered saline. To remove adherent cells, the monolayer was incubated for 10 min on ice with 10 ml HEPES (5 mM) buffered RPMI 1640 (Gibco). Then the cells were flushed with a pipette, centrifuged and washed once with HEPES buffered RPMI. The cells were subcultured with 5 to 6×10^6 cells per petri dish in culture medium as described above. At day 11 the cells were flushed from the dishes and used for the experiments. Cell viability always exceeded 90% as determined by trypan blue exclusion. Homogeneity of the macrophage preparation was assessed by staining according to Giemsa and non specific esterase staining (Ichikawa *et al.*, 1966; Gould *et al.*, 1975).

L-cell conditioned medium

L 929 S cells were kindly provided by E. Ferber, Freiburg, F.R.G. They were kept in DMEM containing 10% foetal calf serum (Flesch & Ferber, 1986). Conditioned medium was prepared in 160 cm^2 culture flasks (Costar, Technomara, Fernwald, F.R.G.) with 5×10^6 cells per 50 ml medium. At day 8, the medium was collected, centrifuged and kept batchwise at −20°C.

Release of prostaglandins

Macrophages were preincubated over night in DMEM supplemented with 5 mg ml^{-1} bovine serum albumin (essentially fatty acid-free) in flat bottom micro titer plates (Nunc, Wiesbaden, F.R.G.) at a cell density of 5×10^5 cells ml^{-1}. If not indicated otherwise, dexamethasone was added during this preincubation period. Then the medium was removed and the cells were further incubated with DMEM and the respective concentration of dexamethasone in the presence or absence of arachidonic acid to induce prostaglandin synthesis. After the times indicated, the supernatant was removed, centrifuged and an aliquot used for the determination of the prostaglandin concentration.

The concentration of PGE_2 and thromboxane B_2 (TXB_2) was determined by a double antibody radioimmunoassay as described recently (Kaever *et al.*, 1988). The antibodies were kindly provided by V. Kaever, Hannover, F.R.G.

Release of arachidonic acid

Release of arachidonic acid was determined according to Wood *et al.* (1984). Macrophages were incubated over night as described above with 0.04 μCi [^{14}C]-arachidonic acid (Amersham Buchler, Braunschweig, F.R.G., specific activity 50 mCi $mmol^{-1}$). The cells were washed once with medium containing 5 mg ml^{-1} albumin and further incubated in medium supplemented with albumin in the presence or absence of thimerosal, an inhibitor of fatty acid reacylation. At the end of the incubation time, an aliquot of the supernatant was removed and the radioactivity therein determined by liquid scintillation counting. In control experiments, the liberated activity was shown to be more than 95% arachid-

onic acid when analyzed by thin layer chromatography (t.l.c.). However, all of the activity was once arachidonic acid esterified to complex lipids. Thus the determination of the total release activity could be taken as a measure of liberated arachidonic acid.

To analyze the distribution of labelled arachidonic acid among the different phospholipid classes, macrophages were incubated in multi well plates (24 wells, Costar) with 0.05 μCi [^{14}C]-arachidonic acid in the presence or absence of various concentrations of dexamethasone for 20 h. Then the medium was removed, and the cells lysed with 500 μl cold H_2O. The suspension was briefly sonicated and the lipids were extracted by chloroform/methanol and separated by t.l.c. (Goppelt & Resch, 1984). The amount of radioactivity of the individual lipid fractions was determined by liquid scintillation counting.

Determination of enzyme activities

Enzyme activities were determined in a crude membrane fraction from macrophages. For this purpose, the macrophages were incubated over night with or without dexamethasone in petri dishes in medium supplemented with albumin. At the end of the incubation period, the supernatant was replaced by cold buffer (20 mM Tris/HCl, pH 8.0, 5% saccharose). The plates were either kept on ice for 15 min or frozen to −20°C before use. The cells were scraped off by a rubber policeman and briefly sonicated in the cold. To prepare crude membranes, the homogenate was centrifuged at 600 *g* and the pellet resuspended in buffer and again sonicated. The supernatants were combined, supplemented with 0.1 mM $CaCl_2$ and centrifuged at 100,000 *g* for 1 h. The pellet was resuspended in buffer (Tris/HCl, saccharose) and kept on ice or frozen to −80°C. Protein was determined by measuring the native fluorescence at 280/340 nm (Resch *et al.*, 1972) with bovine serum albumin used as standard.

Determination of phospholipase A_2 activity

Phospholipase A_2 activity was determined essentially as described by Flesch & Ferber (1986) using phosphatidylcholine L-a-1-palmitoyl-2-arachidonyl (arachidonyl-1-[^{14}C]) (NEN, Boston, MA, U.S.A.) as substrate. The phospholipid was suspended in twice distilled water containing bovine serum albumin (essentially fatty acid-free) by brief sonication in the cold (final concentration in the assay: 1 μM lipid corresponding to about 25,000 c.p.m., 5 mg ml^{-1} albumin). The assay mixture contained 50 mM Tris/HCl, pH 8.5, 10 mM $CaCl_2$, 10 to 20 μg protein and the labelled lipid as substrate in a total volume of 250 μl in glass tubes. Control assays were performed with 10 mM EDTA instead of $CaCl_2$. After incubation for 60 min at 37°C the reaction was terminated by the addition of 375 μl cold isopropanol/1 N HCl (1/0.086, v/v). The glass tubes were kept in an ice bath and 700 μl heptane was added. The solution was shaken vigorously in order to obtain a homogeneous one phase system and the tubes were warmed up to room temperature to develop a two-phase system. After 30 min, 500 μl of the heptane phase were transferred to an Eppendorf tube containing 200 μl heptane and about 200 mg of silica to adsorb extracted phospholipid. The well mixed tubes were centrifuged at 13,000 *g* for 5 min and the radioactivity of 500 μl of the supernatant determined by liquid scintillation counting. The enzyme activity was calculated from the percentage of liberated fatty acid. In control experiments the heptane phase was analyzed by t.l.c. and contained exclusively free arachidonic acid. No diacylglycerol was detectable, which might have been generated by a phospholipase C activity.

Determination of cyclo-oxygenase/PGE isomerase activity

The synthesis of PGE_2 from arachidonic acid was determined in a crude membrane fraction similar as described by Kosaka *et al.* (1987). The assay mixture contained 50 mM HEPES, pH 7.4, 4 mM glutathione, 1 μM haemoglobin, 1 mM tryptophan, 20 to 40 μg protein and 1 μM arachidonic acid (sodium salt) as substrate in a total volume of 200 μl. As control, 5×10^{-5} M diclophenac was added. After incubation for 10 min at 37°C the reaction was terminated by the addition of 20 μl acetylsalicylate (5×10^{-4} M) and quick freezing in liquid nitrogen. The PGE_2 concentration was determined by radioimmunoassay. There were no crossreactivities with any of the constituents of the assay.

Materials

Dexamethasone phosphate (9α-fluoro-16α-methylprednisolone phosphate, Decadron) and acetylsalicylate were obtained from MSD Sharp and Dohme, München, F.R.G. In the dilutions used, the solvent of dexamethasone did not influence the parameters measured. Thimerosal (ethylmercurythiosalicylate), arachidonic acid (sodium salt) and actinomycin D were from Sigma, München, F.R.G. Arachidonic acid (free acid) was obtained from NuChek Prep, Elysian, U.S.A. The fatty acid was kept in ethanol/toluol (4/1, v/v). Before use, the organic solvent was evaporated and the arachidonic acid diluted in the

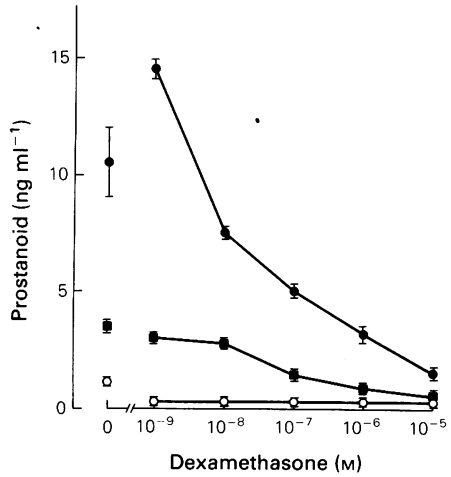

Figure 1 Concentration-dependent inhibition of prostanoid synthesis from exogenous arachidonic acid by dexamethasone. Macrophages were preincubated for 20 h in the presence or absence of dexamethasone in the concentrations indicated. Then the medium was exchanged and the cells were further incubated in the presence (● PGE_2; ■ thromboxane) or absence (○ PGE_2) of arachidonic acid (3×10^{-6} M) for 1 h. The concentration of thromboxane in the absence of arachidonic acid was below the detection limit. Data are results of a representative experiment out of a series of 3 similar ones. Vertical bars are the range of duplicate incubations.

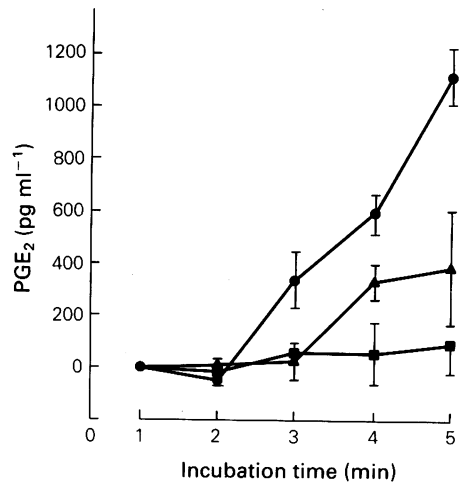

Figure 2 Short time kinetics of the induction of prostaglandin synthesis in bone marrow-derived macrophages. After preincubation for 20 h without (●) or with dexamethasone (▲ 10^{-7} M; ■ 10^{-6} M), the cells were kept in fresh medium under the same conditions for about 30 min. Then arachidonic acid was added (3×10^{-6} M) and the prostaglandin release determined after the times indicated. Vertical bars are the range about the means of two experiments each with duplicate incubations.

appropriate medium by brief sonication. Diclofenac (*o*-(2,3 dichloranilino)-phenylacetic acid) was kindly provided by Ciba-Geigy, Werth, F.R.G.

Statistical tests

When arachidonic acid or prostaglandin release was measured, means of duplicate incubations were determined under each experimental condition. When enzyme activities were measured, duplicate assays were performed for each cell preparation. To compare the values obtained from different primary cell cultures, the mean of each set of control cells was taken as 100% and the mean of the respective cells incubated with hexamethasone calculated as a percentage of the control. Percent data are given as means ± s.d. of n different cells cultures. The statistical significance of the inhibition values so obtained was calculated using the non-paired two-tailed Student's t test. A P value <0.05 was considered to indicate a statistically significant difference between two sets of data.

Results

Inhibition of prostanoid synthesis from exogenous arachidonic acid in dexamethasone-treated macrophages

Bone marrow-derived macrophages were obtained in high yield (about 20×10^6 cells from 1×10^6 stem cells) as a homogeneous preparation of mature macrophages. After cultivation in the presence of serum and L-cell conditioned medium as a source of colony stimulating factor, the cells were further incubated in medium supplemented with bovine serum albumin (5 mg ml^{-1}). The cells were induced to increase prostaglandin synthesis by exogenously added arachidonic acid. Prostanoid synthesis was inhibited by dexamethasone-pretreatment in a concentration-dependent manner. A representative experiment of the inhibition of PGE_2 and thromboxane synthesis after 1 h incubation with arachidonic acid (3×10^{-6} M) is shown in Figure 1. The synthesis of PGE_2 was inhibited by 66.5 ± 2.8% and 56.7 ± 2.9% by 10^{-6} M and 10^{-7} M dexamethasone respectively ($n = 3$, $P < 0.0001$ versus control). Thromboxane synthesis, which was about one third

of PGE_2 synthesis, was inhibited to the same extent. The amount of prostanoids released was not significantly different after 1, 3 and 5 h of incubation with arachidonic acid, indicating a fast synthesis during the first hour of incubation. Thus prostaglandin synthesis was measured during the first 5 min of arachidonic acid incubation (Figure 2). The value determined after 1 min was considered as background and thus set to zero. In control cells, an increase in PGE_2 synthesis was evident already after 3 min of exposure to arachidonic acid. The synthesis was almost completely inhibited by pretreatment with 10^{-6} M dexamethasone and to a lesser extent by pretreatment with 10^{-7} M dexamethasone.

When macrophages were incubated with dexamethasone and arachidonic acid simultaneously, no inhibition of prostanoid synthesis was observed (Figure 3). They had to be preincubated with dexamethasone for at least 3–5 h before an inhibitory effect became apparent. To show the necessity of RNA synthesis for the dexamethasone-induced inhibition of prostaglandin synthesis, macrophages were treated with actinomycin D prior to dexamethasone incubation (Table 1). At the end of the incubation time, cell viability was 60 ± 6% compared to control cells under the different conditions used. RNA synthesis was inhibited completely (>97%) as determined by [^{3}H]-uridine incorporation. The inhibitory effect of dexamethasone on PGE_2 synthesis was reversed by pretreatment of the cells with actinomycin D.

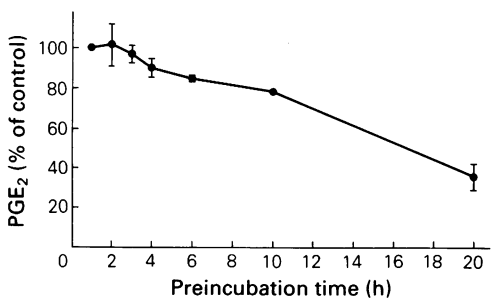

Figure 3 Importance of the preincubation for dexamethasone-induced inhibition of prostaglandin synthesis. Macrophages were preincubated with or without dexamethasone (10^{-6} M) for the times indicated. Then the medium was removed and medium containing arachidonic acid (3×10^{-6} M) with or without dexamethasone was added to the cultures for 1 h. PGE_2 was determined in the supernatants by radioimmunoassay. Data are percentage PGE_2 compared to non-treated control cells (means ± s.d. of 3 experiments).

Table 1 Effect of actinomycin D on the dexamethasone-induced inhibition of prostanoid synthesis

Macrophage incubation mixture	*Pretreatment conditions*	
	Control	*Actinomycin D*
Medium	1033 ± 113	1436 ± 123
Dexamethasone	559 ± 75	1549 ± 175
Medium + arachidonic acid	4716 ± 760	5668 ± 761
Dexamethasone + arachidonic acid	1251 ± 27	6181 ± 347

Bone marrow-derived macrophages were preincubated for 3 h with actinomycin D ($10\,\mu g\,ml^{-1}$). They were further incubated with dexamethasone (10^{-6} M) for 14 h. Part of the cells were stimulated during the last hour of the incubation period with exogenous arachidonic acid (10^{-5} M). PGE_2 ($pg\,ml^{-1}$) was determined in the supernatants by radioimmunoassay. Data are means ± s.d. of triplicate incubations.

Effect of conditioned media on PGE_2 synthesis

Macrophages were preincubated overnight without dexamethasone. Then the medium was removed and conditioned medium added from cells that had been preincubated in the presence or absence of dexamethasone (10^{-6} M). To induce prostaglandin synthesis, arachidonic acid or thimerosal were added as stimuli. Thimerosal is a mercury compound, which inhibits the reacylation of liberated endogenous arachidonic acid and thus increases the pool of free arachidonic acid available for prostanoid synthesis (Goppelt-Struebe *et al.*, 1986). The calculation of PGE_2 synthesis was corrected for the different amount of PGE_2 introduced by the conditioned medium. Compared to medium from control cells, the conditioned medium from dexamethasone-treated cells was able to inhibit prostaglandin release: after 60 min stimulation with thimerosal (10^{-4} M) or arachidonic acid (10^{-5} M) PGE_2 synthesis was inhibited by 18 ± 5% and 23 ± 9% ($n = 3$, $P < 0.05$ versus control), respectively. Inhibition of arachidonic acid release from prelabelled cells was not detectable under the same experimental conditions.

Effect of dexamethasone on arachidonic acid distribution among phospholipids

When bone marrow-derived macrophages were incubated with [^{14}C]-arachidonic acid, the fatty acid was rapidly incorporated into phospholipids, primarily

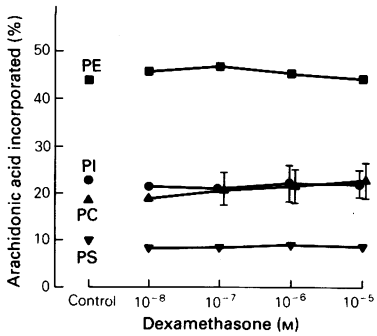

Figure 4 Distribution of arachidonic acid among different phospholipid species. Macrophages were incubated for 20 h with [^{14}C]-arachidonic acid in the presence of dexamethasone in the concentrations indicated. Lipids were extracted and separated as described in Methods. Vertical bars are the range of 2 experiments. If not indicated otherwise, the range was smaller than the symbols used. PE, phosphatidylethanolamine, PI phosphatidylinositol, PS, phosphatidylserine, PC phosphatidylcholine.

phosphatidylcholine, and neutral lipids. With prolonged incubation, arachidonic acid was rearranged among the different lipid species; after 20 h of incubation the fatty acid was esterified almost exclusively to phospholipids with most of the activity associated with phosphatidylethanolamine. After incubation of the macrophages for 20 h the distribution of arachidonic acid was not significantly altered, when the cells were incubated in the presence of various concentrations of dexamethasone (Figure 4).

Release of arachidonic acid from prelabelled cells

Macrophages were prelabelled for 20 h with arachidonic acid in the presence or absence of dexamethasone (10^{-6} M). Then the washed cells were further incubated in medium supplemented with albumin to trap liberated fatty acid. To enhance the arachidonic acid release the reincorporation was inhibited by thimerosal, which inhibits the lysophosphatide acyltransferase (Goppelt-Struebe *et al.*, 1986). The ratio of fatty acid released from dexamethasone-treated cells (10^{-6} M) to that released from control cells, was 0.94 ± 0.05 ($n = 6$) in the absence and 0.91 ± 0.25 ($n = 4$) in the presence of thimerosal (10^{-4} M), measured after 1 h. As can be seen from the large standard deviation, in some experiments there was even an increase in arachidonic acid release from dexamethasone-treated macrophages, but no significant decrease as expected from the prostanoid synthesis, which was inhibited by over 60% under the same conditions.

Table 2 Inhibition of phospholipase A_2 and cyclo-oxygenase/PGE isomerase activity in membranes of dexamethasone-treated macrophages

	% *inhibition*
Phospholipase A_2	
substrate: phosphatidylcholine	27.0 ± 8.3*
substrate: phosphatidylethanolamine	23.3 ± 11.1*
Cyclo-oxygenase/PGE isomerase	40.0 ± 8.4**

Membranes were prepared from macrophages which had been incubated for 20 h with or without dexamethasone (10^{-6} M). Enzyme activities were determined as described in Methods. Data are percentage inhibition compared to non-treated control cells ($n = 4$, $*P < 0.02$, $**P < 0.01$)

Inhibition of phospholipase A_2 by dexamethasone

The activity of phospholipase A_2 was measured in membranes obtained from macrophages that had been incubated with dexamethasone for 20 h. This incubation did not influence the adherence or the optical appearance of the cells. The amount of protein recovered in the cell homogenates and membranes from dexamethasone-treated cells and control cells was not significantly different. Phospholipase A_2 activity was determined with radiolabelled phospholipids as substrates. Cleavage of arachidonic acid from phosphatidylcholine and phosphatidylethanolamine, labelled with arachidonic acid in position 2, was inhibited in the membranes from dexamethasone-treated cells (Table 2).

Inhibition of cyclo-oxygenase/PGE isomerase activity

The conversion of exogenously added arachidonic acid to PGE_2 was measured in a crude membrane fraction. Membranes, prepared from dexamethasone-treated cells, showed a significantly reduced cyclo-oxygenase/PGE_2 isomerase activity (Table 2).

Discussion

The inhibitory effect of glucocorticoids on prostaglandin synthesis has been demonstrated in various cell types and tissues, especially in inflammatory tissues (Flower, 1988). It was attributed to the induction of lipocortins, which are characterized by their ability to inhibit phospholipase A_2. This mechanism, however, was not consistent with all the experimental evidence obtained in different types of inflamma-

tion models (Calignano *et al.*, 1985; Foster & McCormick, 1985). When healthy men or rabbits were treated with glucorticoids, the whole body production of prostanoids measured as urinary excretion was not diminished (Naray-Fejes-Toth *et al.*, 1984; Rosenkrantz *et al.*, 1985). The increased PGE_2 synthesis observed in some cells such as fibroblasts (Chandrabose *et al.*, 1978), mast cells (Robin *et al.*, 1985) or renal medullary interstitial cells (Erman *et al.*, 1986 and references therein), upon glucocorticoid administration may add to this effect. Thus, the effect of glucocorticoids on prostanoid synthesis varies depending on the cell system, adding to the complexity of glucocorticoid action. At variance with many other groups, Moore & Hoult (1980) could show a decrease in the activity of prostaglandin synthase and an increase in the activities of prostaglandin degrading enzymes in different tissues of rats by glucocorticoids and an action at a later stage than phospholipase A_2 was also suggested by Wood *et al.* (1984), investigating hydrocortisone effects in macrophages. Thus there was evidence that the action of glucocorticoids on arachidonic acid metabolism is more complex than a generalized phospholipase A_2 inhibition.

One of the arguments against a regulation distal to phospholipase A_2 was the reversibility of steroid-induced inhibition of prostanoid synthesis by exogenously added arachidonic acid (Russo-Marie & Duval, 1982; Parente *et al.*, 1984; Fan & Lewis, 1985).

In our experiments prostanoid synthesis was inhibited by dexamethasone when arachidonic acid was added to the cells as inducing agent. The discrepancy with the reports cited may be due to different experimental conditions: in those experiments arachidonic acid and dexamethasone were added simultaneously and PGE_2 secretion measured after a few hours. In our kinetic experiments we could show that arachidonic acid is very rapidly metabolized to prostaglandins: an increased PGE_2 synthesis was measurable after 3 min of incubation with arachidonic acid and came to an end within 1 h. As the action of glucocorticoids is dependent on protein synthesis, preincubation with dexamethasone was an absolute prerequisite to detect any effects. Inhibition of PGE_2 synthesis by dexamethasone was abolished by pretreatment of the cells with actinomycin D, excluding any direct glucocorticoid effects. When exogenous arachidonic acid is added to macrophages, the arachidonic acid is rapidly incorporated into complex lipids, phospholipids and neutral lipids. Dexamethasone did not significantly influence the distribution of [^{14}C]-arachidonic acid among the various phospholipid species, as was also reported for cultured rat kidney cells (Russo-Marie & Duval, 1982). Release of arachidonic acid from prelabelled macrophages (peritoneal macrophages from mouse and RAW_{264} cells) had been shown not to be influenced by hydrocortisone (Wood *et al.*, 1984). These results were confirmed by our experiments with bone marrow-derived macrophages and dexamethasone. Also the conditioned medium did not interfere with the arachidonic acid release, but inhibited prostaglandin synthesis. These findings correlate with the rather modest inhibition of phospholipase A_2 activity by about 25%.

Inhibition of the membrane-bound phospholipase A_2 was measured with two types of substrates, phosphatidylcholine and phosphatidylethanolamine. Lipocortin was shown recently to inhibit pancreatic phospholipase A_2 with phosphatidylethanolamine as substrate but not with phosphatidylcholine (Rothut *et al.*, 1987). At the moment, however, we do not have any evidence for the mechanism of the glucocorticoid-induced intracellular inhibition of phospholipase A_2, bound to the particular fraction of macrophages. As we could show recently, phospholipase A_2 activity is also inhibited in dexamethasone-treated phorbolester-differentiated U937 cells (Koehler *et al.*, 1989). However, the expression of lipocortins is not inducible by glucocorticoids in these cells (Isacke *et al.*, 1989). Thus other mechanisms of inhibition of phospholipase A_2 may also be operative in the glucocorticoid-treated cells.

The modest inhibition of phospholipase A_2 activity and the failure to detect effects of dexamethasone on phospholipid metabolism suggested additional regulatory mechanisms. Endogenous arachidonic acid is metabolized to cyclo-oxygenase or lipoxygenase products. It is not quite clear whether exogenous arachidonic acid is a direct substrate for the cyclo-oxygenase or whether it is first esterified to phospholipids and becomes a substrate after cleavage by a phospholipase A_2. Esterification seems to be necessary for leukotriene formation but not for prostanoid synthesis in resident macrophages (Scott *et al.*, 1982). The very fast PGE_2 synthesis observed in bone marrow-derived macrophages suggested a direct conversion, which was inhibited by dexamethasone. Thus the data indicated an effect of dexamethasone on the cyclo-oxygenase/PGE isomerase system. This could be confirmed by the direct measurement of the cyclo-oxygenase/PGE isomerase system. The conversion of arachidonic acid was inhibited in membranes from dexamethasone-treated macrophages compared to membranes from control cells. A similar inhibition of prostaglandin synthase in rats was also shown by Moore & Hoult (1980). After this manuscript was submitted, Bailey *et al.* (1988) showed decreased mRNA levels of cyclo-oxygenase in vascular smooth muscle cells and Raz *et al.* (1989) showed decreased levels of cyclo-

oxygenase in fibroblasts. This indicates that the effects of glucocorticoids on cyclo-oxygenase are not restricted to a certain cell type but seem to be of more general nature.

Thus we could show a dexamethasone-induced inhibition of an endogenous membrane-bound phospholipase A_2 activity. However, our results also indicate that there are additional regulatory mechanisms that do not lead to a general inhibition of arachidonic acid metabolism but to a more selective inhibition of prostanoid synthesis.

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Selective anti-platelet aggregation synergism between a prostacyclin-mimetic, RS93427 and the nitrodilators sodium nitroprusside and glyceryl trinitrate

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1 Citrated platelet-rich plasma from human donors was used to examine turbidometrically the platelet aggregation response to collagen (2.5 $\mu g\,ml^{-1}$) and ADP (1.6 $\mu g\,ml^{-1}$).

2 With collagen as an aggregating agent, the limited (35% maximal inhibition) inhibitory effects of glyceryl trinitrate (GTN, 0.78–50 $\mu g\,ml^{-1}$) were markedly potentiated by threshold (3.3–10 $ng\,ml^{-1}$) concentrations of RS93427, an orally active prostacyclin-mimetic. Almost complete inhibition of aggregation could then be produced.

3 A threshold concentration of RS93427 (3.3 $ng\,ml^{-1}$) similarly potentiated the ability of sodium nitroprusside (NaNp, 0.78–10 $\mu g\,ml^{-1}$) to inhibit collagen-induced platelet aggregation. There was an 8 fold reduction in the IC_{25} concentration of NaNp.

4 Threshold concentrations of the nitrodilators were also able to potentiate the anti-aggregatory effects of RS93427 (0.03–30 $ng\,ml^{-1}$) on collagen-induced platelet aggregation. With threshold concentrations of either GTN (6.3–25 $\mu g\,ml^{-1}$) or NaNp (0.3–1.3 $\mu g\,ml^{-1}$), the mean IC_{50} concentration of RS93427 was reduced 4 or 6 fold, respectively, while the IC_{25} concentration was reduced 6 or 10 fold, respectively.

5 No similar synergistic interactions were seen between RS93427 and the nitrodilators when ADP was used as an aggregating agent.

6 In spontaneously hypertensive rats, the dose-response for the hypotensive response to bolus doses of RS93427 was not altered by concomitant steady state infusion of a threshold dose (1 $\mu g\,kg^{-1}\,min^{-1}$) of GTN.

7 Possible therapeutic implications of these findings are discussed.

Introduction

The stable, orally active prostacyclin-mimetic, RS93427-017, (hereafter called RS93427) is calcium *bis*-[Z-4-{(3'S,1S,2S,3R,6S)-2-(3'-cyclohexyl-3'-hydroxyprop-1-ynyl)-3-hydroxybicyclo[4.2.0]oct-7-ylidene}]butyrate. It is a potent inhibitor of aggregation of human platelets but is much less active on the platelets of most other species (Willis *et al.*, 1987). It shares with prostacyclin an ability to reduce systemic blood pressure via a marked fall in peripheral resistance (Willis *et al.*, 1987).

The nitrodilators, sodium nitroprusside and glyceryl trinitrate, are thought to act via the intracellular generation of nitric oxide (NO) which then elevates intracellular guanosine 3' : 5'-cyclic monophosphate (cyclic GMP) (see Waldman & Murad, 1987; Vane *et al.*, 1987; Sneddon *et al.*, 1988a). The activity of 'endothelium-derived relaxing factor' (EDRF), a previously uncharacterized unstable vasodilator derived from the vascular endothelium (Furchgott & Zawadski, 1980) has recently been attributed to NO (Ignarro *et al.*, 1987; Kahn & Furchgott, 1987; Palmer *et al.*, 1987).

The present studies were stimulated by a report by Radomski *et al.* (1987a) in which both natural EDRF and NO were found to work synergistically with prostacyclin in inhibiting aggregation of washed human platelets in response to collagen, thrombin, and adenosine diphosphate (ADP). This same group (Radomski *et al.*, 1987b) also found that NO inhibited adhesion of platelets to surfaces,

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including collagen fibrils, an effect mediated by increased platelet cyclic GMP and not shared by physiological concentrations of prostacyclin, which only elevated cyclic AMP. Similar findings have been reported by Sneddon *et al.* (1988b) for platelet adhesion to vascular endothelium. Together, these data suggest existence of a homeostatic process by which vascular prostacyclin (PGI_2) and NO, released together by a coupled mechanism, may attenuate localized platelet reactivity (de Nucci *et al.*, 1988).

We now describe a study of mutual anti-aggregatory synergism between RS93427 (an orally active prostacyclin-mimetic) and two nitrodilators, sodium nitroprusside and glyceryl trinitrate. A preliminary account of these findings has been communicated to the British Pharmacological Society (Loveday *et al.*, 1988).

Methods

Platelet aggregation

Human whole blood was collected from drug-free volunteers (21–50 years old) of either sex via a 19 gauge butterfly infusion set into 0.1 vol of 3.8% (w/v) sodium citrate. Platelet-rich plasma (PRP) was then prepared by centrifugation at 750 *g* for 4 min. The remaining blood was centrifuged at 1470 *g* for 6 min to prepare platelet-poor plasma (PPP). Siliconised aggregation cuvettes (7.92 mm in diameter) containing 200 μl PRP (platelet count = 485,000–600,000 μl^{-1}) and 200 μl PPP were prepared; and four microlitres of a stock solution of RS93427 (final concentration: 0.033–30 ng ml^{-1}; 0.095–87 nM) in 0.01 M Tris buffer, pH 8.0, or Tris vehicle alone, was then added. Then 4 μl of GTN stock solution in 70% ethanol, or ethanol vehicle alone, was added to the appropriate cuvettes at a final minimal inhibitory concentration of GTN (see below). Alternatively, 4 μl of a stock solution of NaNp in water, or water vehicle alone, was added at a final minimal inhibitory concentration of NaNp (see below).

In other experiments, minimal inhibitory concentrations (see below) of RS93427 were added to similar cuvettes in which 4 μl of GTN or NaNp stock solution or corresponding vehicle had been added to the plasma-diluted PRP. Final concentrations of GTN were 0.78–50 μg ml^{-1} (7.5–480 μM), and final concentrations of NaNp were 0.08–10 μg ml^{-1} (0.3–38 μM).

The platelets were immediately stirred for 1.5 min (1,000 r.p.m., 37°C) in the light path of a Payton dual-channel aggregation module. Aggregation of the platelets was then induced by adding either collagen (1 μl; final concentration of 2.5 μg ml^{-1}) or ADP (4 μl; final concentration of 1.6 μg ml^{-1}, 3.75 μM). Aggregation of the platelets was recorded as the increase in light transmission for 5 min (with collagen as the aggregation stimulus) or for 3 min (with ADP as the stimulus), during which times full but submaximal aggregation responses were attained. Aggregation measurements were calculated as the percentage inhibition of maximum amplitude of aggregation obtained with control cuvettes to which only the appropriate vehicle solutions had been added.

Determination of threshold concentrations of glyceryl trinitrate and sodium nitroprusside For each aggregation inducer used (collagen or ADP), the minimal inhibitory concentrations of GTN or NaNp were chosen that gave <14% inhibition when added alone (pH 8 Tris buffered vehicle only). These concentrations, determined for each donor prior to their use in conjunction with RS93427, were 6.3–25 μg ml^{-1} (60–240 μM) and 0.78–1.6 μg ml^{-1} (7.5–15.4 μM) for GTN with collagen or ADP as the stimulus, respectively, and 0.3–1.3 μg ml^{-1} (1.1–5.0 μM) and 0.08 μg ml^{-1} (0.3 μM) for NaNp with collagen or ADP as the stimulus, respectively.

Determination of threshold concentrations of RS93427 Similarly, minimal inhibitory concentrations of RS93427 were chosen that gave <14% inhibition when added alone (vehicle only). These concentrations, determined for each donor prior to use in conjunction with nitrodilators, were 3.3–10 ng ml^{-1} (9.5–29 nM) with collagen as inducer and 0.33 ng ml^{-1} (0.95 nM) in all cases with ADP as inducer.

Blood pressure studies

Male spontaneously hypertensive rats (SHR) of 250–350 g were used throughout. The animals were initially anaesthetized with pentobarbitone sodium (70 mg kg^{-1}, i.p.) before surgical preparation. The left femoral artery was cannulated with PE50 polyethlyene tubing for blood pressure measurement, and both femoral veins were similarly cannulated for drug administration. Blood pressure and heart rate were monitored on a Grass polygraph via Statham 23Gb transducers.

Following surgery, anaesthesia was maintained by intravenous infusion of pentobarbitone (0.018 ml min^{-1}) at a rate of 20 mg kg^{-1} h^{-1} and the animal preparation allowed to stabilize for at least 20 min before administration of RS93427 or GTN was started.

Mean blood pressure was calculated as the diastolic blood pressure plus one-third of the pulse pressure. The ED_{25} was defined as the dose that resulted in a lowering of mean blood pressure of

25 mmHg or an increase in heart rate of 25 beats min^{-1}. The values were obtained graphically from the dose-response curves.

Dose-response curves were first obtained for injections of GTN and RS93427 administered as bolus doses in 1 ml kg^{-1} dose volumes, washed in through the catheter with 0.2 ml of 0.9% w/v NaCl solution. Eight rats were treated with cumulative i.v. doses of 0.3, 1, 3, 10 and 30 $\mu g\,kg^{-1}$ of RS93427 at intervals of 15 min (allowing baseline blood pressure to return to normal). Similarly, another 8 rats were injected with GTN at 0.1, 0.3, 1, 3, 10, 100 and 300 $\mu g\,kg^{-1}$. Repeated administration (16 sequential doses) of GTN (3 $\mu g\,kg^{-1}$, i.v.) at intervals of 15 min did not result in a diminished response ($P < 0.05$, $n = 4$).

Using this dose-response information, a threshold dose (1 $\mu g\,kg^{-1}\,min^{-1}$) of GTN was continuously infused in another group and the dose-response curve to ascending bolus doses of RS93427 was again examined; parallel studies were performed in which only the vehicle (0.9% saline) for GTN was infused at 0.018 ml min^{-1}.

Materials

SHR were obtained from Charles River Breeding Laboratories, Kingston, N.Y., U.S.A. Platelet aggregation studies were performed in a dual channel aggregation module (Payton Associates, Buffalo, N.Y., U.S.A.) in which PPP anti-coagulated with sodium citrate (Sigma Chemical Co., St. Louis, MO, U.S.A.) was used. Aggregation was induced by adenosine diphosphate (ADP, Sigma) or collagen (Chrono-par, Chronolog, Havertown, PA, U.S.A.). Tris buffer used for dissolving drug was also obtained from Sigma. NaNp was obtained from Aldrich Chemical Co., Milwaukee, WI, U.S.A. and GTN used in platelet aggregation studies was manufactured by Taylor Pharmaceutical Co. (Decatur, IL, U.S.A.) and supplied by Dey Laboratories (Napa, CA, U.S.A.). GTN used in blood pressure studies was obtained from Parke-Davis (Morris Plains, NJ, U.S.A.).

Statistical analysis

All data are presented graphically as means ± standard errors of the mean (s.e.mean). IC_{50} and IC_{25} are defined as the concentration of aggregation inhibitory agent required for 50% inhibition or 25% inhibition, respectively, of aggregation. Two approaches were used to analyse the data: comparison of IC_{50} and IC_{25} values for each pair of aggregation inhibition curves, and concentration-to-concentration comparisons between each pair of aggregation inhibition curves.

Regression curves were fitted from the data of each donor by least squares techniques, plotting percentage inhibition of aggregation versus ln(dose), as the logarithm provided a more consistently linear fit. These individual regression curves were then used to calculate an IC_{25} and IC_{50} value for each donor. Mean $\ln(IC_{25})$ and mean $\ln(IC_{50})$ values were then analyzed by one-way analysis of variance, accounting for subjects (paired t test).

Inhibition of aggregation due to each combination of agents also was compared with the effect of the single agent on a concentration-by-concentration basis by one-way analysis of variance (ANOVA) at each dose, accounting for subjects (paired t test).

For blood pressure studies, a two-way repeated measures analysis of variance was used, followed by pairwise comparison between time points or between treatment groups. The comparisons were adjusted by Fisher's LSD strategy and Dunn's procedure.

In all cases, statistical significance was assumed at $P < 0.05$.

Results

Figure 1a shows that RS93427, at a threshold anti-aggregatory concentration (3.3 $\mu g\,ml^{-1}$, confirmed for each donor) potentiated the ability of NaNp to inhibit aggregation induced by collagen (2.5 $\mu g\,ml^{-1}$). A clear leftward shift in the NaNp dose-response curve was produced (Figure 1a). A statistically significant 8 fold reduction in IC_{25} concentration (from 2.3 ± 1.3 $\mu g\,ml^{-1}$ to 0.3 ± 0.1 $\mu g\,ml^{-1}$) was seen. Significant enhancement of the activity of NaNp by RS93427 was seen at 0.6 and 1.25 $\mu g\,ml^{-1}$ of NaNp (Figure 1a).

By contrast, when ADP (1.6 $\mu g\,ml^{-1}$) was used to induce aggregation (Figure 1b), no such potentiation of the activity of NaNp by the threshold concentration (0.33 ng ml^{-1}) of RS93427 was observed.

Figure 2 shows the effects of similar studies with glyceryl trinitrate (GTN), which had only a weak intrinsic inhibitory effect on platelet aggregation. (The ethanolic vehicle had no effect alone on collagen-induced platelet aggregation: −0.4 ± 2.6% inhibition with 0.7% ethanol.) Again, threshold concentrations of RS93427 (3.3–10 ng ml^{-1}, determined empirically for each donor) markedly potentiated the inhibitory effects of GTN on collagen-induced aggregation, as seen by a clear leftward shift in the GTN dose-response curve (Figure 2a). Indeed, GTN was now rendered capable of producing an 87% maximal inhibition, compared to only 35% in the absence of RS93427. Although it was not possible in all donors to estimate quantitatively the IC_{50} value for GTN in the absence of RS93427 or the IC_{25} value in the presence of RS93427, significant enhancement of the

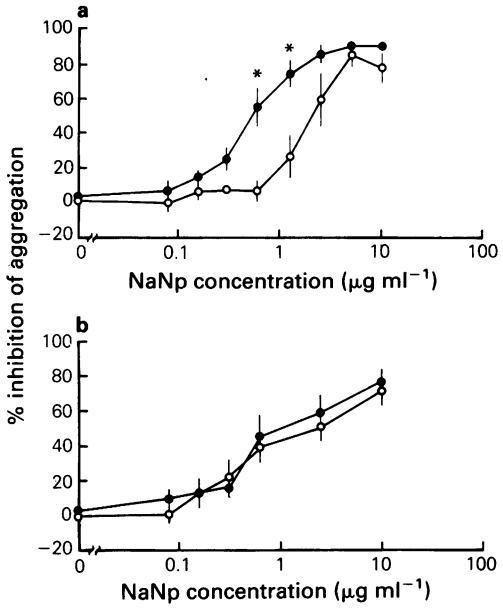

Figure 1 Effects of threshold concentrations of RS93427 on inhibition of human platelet aggregation by ascending concentrations of sodium nitroprusside (NaNp). (a) Effects on aggregation induced by collagen. Human citrated platelet-rich plasma (PRP) was induced to aggregate for 4 min by addition of 1 μl of a microfibrillar suspension of collagen (final concentration of 2.5 μg ml^{-1}). Ascending concentrations of NaNp together with a threshold concentration (3.3 ng ml^{-1}; confirmed empirically for each donor) of RS93427 were added to 400 μl aliquots of plasma-diluted PRP stirred (1,000 r.p.m., 37°C) in the light path of a Payton dual channel aggregation module. (○) NaNp + vehicle ($n = 4$); (●) NaNp + RS93427 ($n = 4$). * $P < 0.05$ vs vehicle; one-way ANOVA at each dose. (b) Effects on aggregation induced by ADP. Conditions were as described in (a), except that 4 μl of ADP (final concentration of 1.6 μg ml^{-1}) was used to induce aggregation and the threshold concentration of RS93427 was 0.33 ng ml^{-1}. (○) NaNp + vehicle ($n = 5$); (●) NaNp + RS93427 ($n = 5$). It can be seen that RS93427 markedly potentiates the effects of NaNp on collagen-induced aggregation, but not upon ADP aggregation.

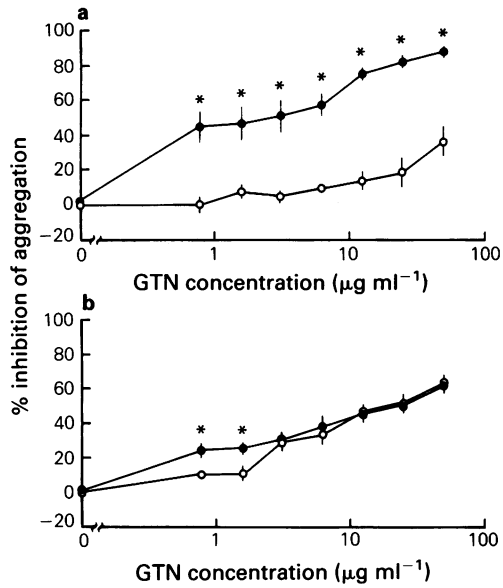

Figure 2 Effects of threshold concentrations of RS93427 on inhibition of human platelet aggregation by ascending concentrations of glyceryl trinitrate (GTN). Conditions are as described in the legend to Figure 1, except that ascending concentrations of GTN were added with a threshold concentration of RS93427. (a) Effects on aggregation induced by collagen suspension (2.5 μg ml^{-1}). Threshold concentrations of RS93427 were 3.3–10 ng ml^{-1}, chosen empirically for each donor. (○) GTN + vehicle ($n = 6$); (●) GTN + RS93427 ($n = 6$). * $P < 0.05$ vs vehicle; one-way ANOVA at each dose. (b) Effects on aggregation induced by ADP (1.6 μg ml^{-1}). The threshold concentration of RS93427, confirmed empirically for each donor, was 0.33 ng ml^{-1}. (○) GTN + vehicle ($n = 5$); (●) GTN + RS93427 ($n = 5$). * $P < 0.05$ vs vehicle; one-way ANOVA at each dose. GTN was able to inhibit only partially aggregation induced by ADP, with an even more limited effect on collagen-induced aggregation. However, in the presence of threshold concentrations of RS93427, a marked inhibition of collagen-induced aggregation was produced; the effects of GTN on ADP-induced aggregation were not similarly potentiated.

activity of GTN by RS93427 was seen at all GTN concentrations from 0.78 to 50 μg ml^{-1} (Figure 2a).

As previously described for NaNp, a threshold concentration (0.33 ng ml^{-1}) of RS93427 had no meaningful effect on inhibition of ADP-induced aggregation by GTN (Figure 2b).

With collagen as the aggregating agent, threshold concentrations of NaNp (0.3–1.3 μg ml^{-1}) and GTN (6.3–25 μg ml^{-1}) also potentiated the ability of RS93427 to inhibit platelet aggregation (Figure 3a and b). The IC_{25} of RS93427 was reduced 10.5 fold (from 6.7 ± 1.2 to 0.6 ± 0.1 ng ml^{-1}) by NaNp and 6 fold (from 4.8 ± 0.7 to 0.8 ± 0.2 ng ml^{-1}) by GTN. There was a similar, but less marked, reduction in IC_{50} by 6 fold (from 13.1 ± 2.8 to 2.1 ± 0.3 ng ml^{-1}) and 3.5 fold (from 9.0 ± 1.2 to 2.6 ± 0.7 ng ml^{-1}) by NaNp and GTN, respectively. NaNp significantly enhanced the activity of RS93427 at concentrations of 3.3 and 10 ng ml^{-1} (Figure 3a). Similarly, GTN significantly enhanced the activity of RS93427 at concentrations of 0.033, 0.33, 3.3 and 10 ng ml^{-1} (Figure 3b).

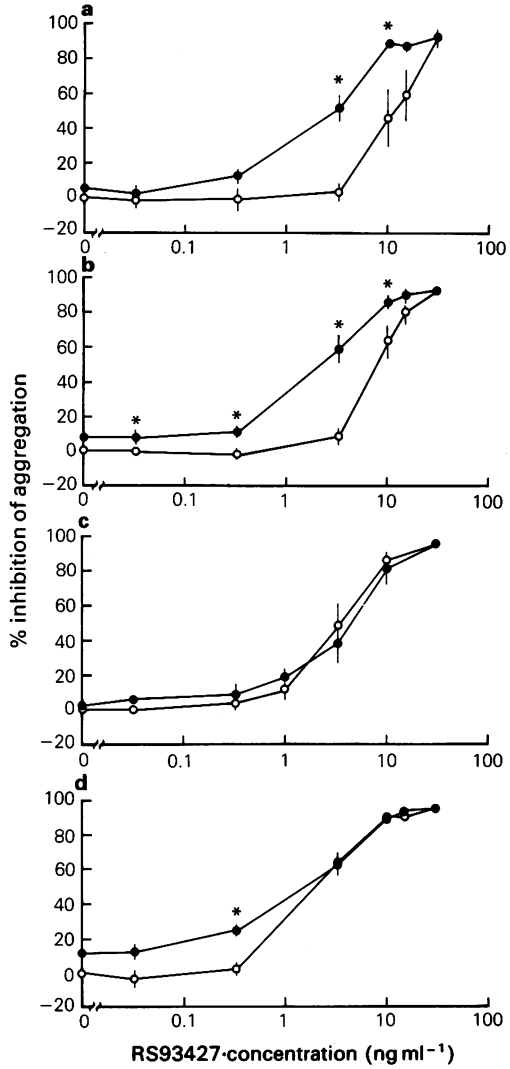

Figure 3 Effects of threshold concentrations of sodium nitroprusside (NaNp) and glycerol trinitrate (GTN) on aggregation response to ascending concentrations of RS93427. Conditions were as described in Figure 1, except that ascending concentrations of RS93427 were examined in the presence of threshold concentrations of NaNp or GTN. (a) Collagen-induced aggregation: marked potentiation by NaNp of the anti-aggregatory effects of RS93427. Threshold concentrations of NaNp were 0.3–1.3 $\mu g\,ml^{-1}$, chosen empirically for each donor. (○) RS93427 + vehicle ($n = 6$); (●) RS93427 + NaNp ($n = 6$). * $P < 0.05$ vs vehicle; one-way ANOVA at each dose. (b) Collagen-induced aggregation: marked potentiation by GTN of the anti-aggregatory effects of RS93427. Threshold concentrations of GTN were 6.3–25 $\mu g\,ml^{-1}$, chosen empirically for each donor. (○) RS93427 + vehicle ($n = 12$); (●) RS93427 + GTN ($n = 12$). * $P < 0.05$ vs vehicle; one-way ANOVA at each dose. (c) ADP-induced aggregation: lack of potentiation by NaNp of the anti-aggregatory effects of RS93427. The threshold concentration of NaNp, confirmed empirically for each donor, was 0.08 $\mu g\,ml^{-1}$. (○) RS93427 + vehicle ($n = 5$); (● RS93427 + NaNp ($n = 5$). (d) ADP-induced aggregation: lack of potentiation by GTN of the anti-aggregatory effects of RS93427. Threshold concentrations of GTN were 0.78–1.56 $\mu g\,ml^{-1}$, chosen empirically for each donor. (○) RS93427 + vehicle ($n = 5$); (●) RS93427 + GTN ($n = 5$). * $P < 0.05$ vs vehicle; one-way ANOVA at each dose.

By contrast, when ADP was used as the aggregating agent, threshold concentrations of neither NaNp (0.08 $\mu g\,ml^{-1}$; Figure 3c) nor GTN (0.78–1.56 $\mu g\,ml^{-1}$; Figure 3d) meaningfully altered the aggregation-inhibitory activity of RS93427.

Results for blood pressure and heart rate are shown in Figure 4. Effects on blood pressure of ascending doses of GTN are shown in Figure 4a ($ED_{25} = 2\,\mu g\,ml^{-1}$, i.v.). It can also be seen that the hypotensive dose-response for RS93427 ($ED_{25} = 4\,\mu g\,ml^{-1}$, i.v.) and the tachycardic dose-response ($ED_{25} = 6\,\mu g\,ml^{-1}$, i.v.) were not altered by infusion of a threshold concentration of GTN ($1\,\mu g\,kg^{-1}\,min^{-1}$) (Figure 4b and c).

Discussion

Our results complement those of Radomski *et al.* (1987a) and of MacDonald *et al.* (1988) who reported upon the synergistic interactions between NO (or EDRF) and PGI_2 in saline suspensions of washed human platelets. These effects were seen with collagen, ADP, platelet activating factor (PAF) and thrombin as aggregating agents. By contrast, we have examined platelets suspended in citrated platelet-rich plasma and have used the stable PGI_2-mimetic RS93427 together with two nitrodilators (NaNp and GTN) that may act via release of endogenous NO. We observed strong synergistic interactions for aggregation induced by collagen but not by ADP.

We have also examined blood pressure interactions in one species (the rat) and failed to show synergistic effects between RS93427 and GTN in the vasculature *in vivo*.

A similar lack of synergistic effects on vascular smooth muscle has been seen *in vitro* with another PGI_2-mimetic (iloprost) examined in combination with NaNp (Antunes *et al.*, 1988; P.S. Lidbury, personal communication). However, the same authors found that the synergistic anti-aggregatory effects of iloprost with NaNp were only weak (about two fold above the expected additive effect), although not spe-

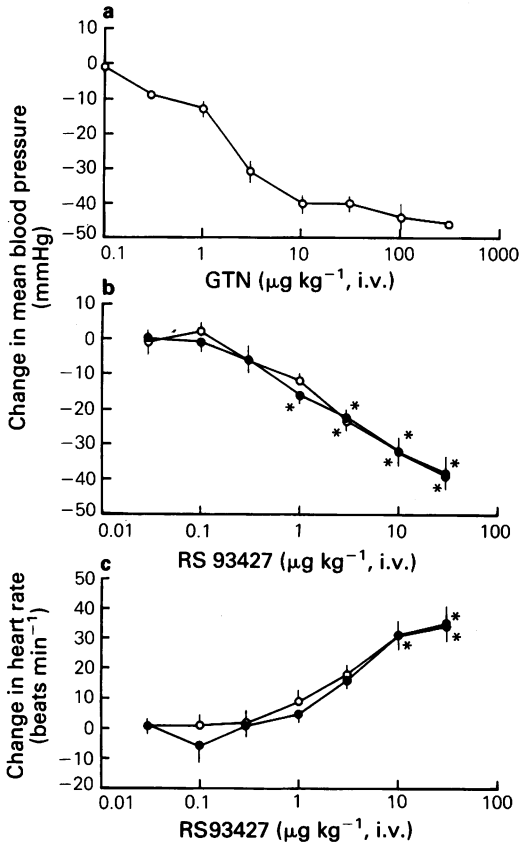

Figure 4 Effects on blood pressure and heart rate of glyceryl trinitrate (GTN) and RS93427. (a) Effect of GTN on blood pressure in anaesthetized spontaneously hypertensive rats. The predose mean blood pressure of the rats was 96 ± 4 mmHg ($n = 3$). (b) Inability of threshold concentrations of GTN to potentiate the hypotensive response to RS93427 in anaesthetized spontaneously hypertensive rats. The baseline blood pressure responses of the groups receiving saline (○) or GTN (○) were 82 ± 3 and 78 ± 8 mmHg, respectively ($n = 8$ each). * $P < 0.05$, compared to pre-dose controls (c) Effect of RS93427 on heart rate in anaesthetized spontaneously hypertensive rats. The baseline heart rate responses of the groups receiving saline (○) or GTN (○) were 264 ± 12 and 274 ± 8 beats min^{-1} ($n = 8$ each). * $P < 0.05$ compared to pre-dose levels. RS93427 was injected in cumulative bolus doses of 0.3 to 140 μg kg^{-1} (1 ml kg^{-1}) during infusions of either saline (0.18 ml min^{-1}) or a threshold dose (1 μg kg^{-1}) of GTN. There were no significant pairwise differences between RS93427 and RS93427 + GTN for either the hypotensive or the tachycardic responses.

cific to the agent used to induce platelet aggregation. These results are reminiscent of earlier findings by Levin *et al.* (1982) who showed some synergism between the anti-aggregatory effects of PGI_2 and NaNp for collagen or adrenaline-induced aggregation examined using platelet-rich plasma.

Differences in prostanoid activity are possibly responsible for these differences between aggregating agents. It is known, for instance, that RS93427 differs from iloprost in having a marked specificity for inhibition of human rather than animal platelets, an activity partially reminiscent of prostaglandin D_2 (Willis *et al.*, 1987 and unpublished data).

Perhaps the interactive effects between RS93427 and the nitrodilators represent a functional synergism only. Thus, RS93427 (via elevation of platelet cyclic AMP; see Mills & MacFarlane, 1977; Moncada & Vane, 1979) prevents the platelet-platelet aggregation process that amplifies the initial stimulus of adhesion of platelets to collagen. This adhesion process would itself be inhibited by GTN and NaNp via prior conversion to NO (Radomski *et al.*, 1987b; Sneddon *et al.*, 1988b) which then elevates platelet cyclic GMP, also resulting in inhibition of platelet aggregation *per se*.

This dual action on adhesion and aggregation is then a likely explanation for the apparent synergistic interactions of RS93427 with the nitrodilators. If this hypothesis were true, then similar synergistic effects should not be seen when platelet aggregation is induced directly without prior adhesion of the platelets to a surface.

Indeed, no synergistic response between RS93427 and the nitrodilators was seen when ADP was used as the aggregating agent. ADP is the archetypal soluble aggregating agent (Born & Cross, 1962). In addition to directly inducing the platelets to stick to one another (i.e., aggregate), it also induces a secondary aggregation response due to release of aggregation-inducing arachidonate metabolites and platelet granular contents (Willis, 1978).

Similarly, in the spontaneously hypertensive rat, no potentiating effects of GTN were seen on the hypotensive response to RS93427 (and its compensatory increase in heart rate). The fall in blood pressure is due to the vasodilator response to relaxation of vascular smooth muscle that can be produced by increases in cyclic AMP or cyclic GMP (Vane *et al.*, 1987). Therefore, by producing the same effect, it is unlikely that RS93427 or the nitrodilators could act synergistically. Correspondingly, the effects of the PGI_2-mimetic iloprost and NaNp seem no more than additive in ability to relax precontracted strips of rabbit aorta (Antunes *et al.*, 1988; P.S. Lidbury, personal communication).

Upon rupture of an atherosclerotic plaque, platelets adhere to exposed collagen and other material and then clump, releasing materials that induce further clumping, coagulation and vasospasm. Such processes are thought to occur in sudden death produced by coronary thrombosis (Wissler, 1984).

Thus, co-administration of RS93427 with a nitrodilator may be an important new therapeutic approach. Vasodepressor effects of the compound might be minimised in comparison with the enhancement of its inhibitory effects on adhesion-stimulated platelet aggregation.

In addition, possible new usefulness of nitrodilator compounds as anti-thrombotic agents is suggested. Daily oral administration of RS93427 in doses insufficient to produce any noticeable effects on platelet aggregation or blood pressure may render a nitrodilator such as GTN able to produce significant anti-thrombotic effects *in vivo* in the coronary circulation, against a background of coronary artery vasodilatation.

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Effect of cromakalim on contractions in rabbit isolated renal artery in the presence and absence of extracellular Ca^{2+}

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1 The inhibitory effects of the K^+ channel activator, cromakalim, upon contractions to noradrenaline, histamine and caffeine were examined in rabbit isolated renal artery. For comparison, the effects of pinacidil, dazodipine and sodium nitroprusside were also studied in some experiments.

2 In normal Krebs solution, cromakalim (1 μM) produced a 39.1% reduction in area under the curve (AUC) of the noradrenaline concentration-response, and a 61.8% reduction in the histamine AUC. Ca^{2+} removal (with EGTA 0.1 mM) gave an 80.0% reduction in the noradrenaline AUC and a 74.5% reduction in the histamine AUC. The combination of Ca^{2+} removal and cromakalim (1 μM) had no further effect on the noradrenaline responses (a reduction of 78.4% in AUC), but produced a significantly greater reduction in the histamine AUC (86.2%).

3 $LaCl_3$ (1 mM) reduced the noradrenaline AUC by 74.8% and gave an 81.8% reduction in the response to a single (EC_{90}) histamine concentration. $LaCl_3$ (1 mM) plus cromakalim (1 μM) produced no further reduction in the noradrenaline AUC (71.9%) but gave a significant further reduction of the histamine response (94.6%).

4 Pinacidil (3 μM) reduced the noradrenaline AUC by 35.5%. Pinacidil (3 μM) plus $LaCl_3$ (1 mM) produced the same reduction in the noradrenaline AUC (80.9%) as $LaCl_3$ alone (80.9%).

5 In both normal and Ca^{2+}-free Krebs solution, cromakalim (0.1, 1.0 and 10 μM) produced concentration-related inhibition of the contraction to caffeine (10 mM). This inhibition was antagonised by the K^+ channel blocker, glibenclamide (3 μM). Similarly, pinacidil (0.3, 3.0 and 30 μM) produced a glibenclamide-sensitive inhibition of the caffeine contraction. At equi-vasorelaxant concentrations, dazodipine (0.01, 0.1 and 1.0 μM) and sodium nitroprusside (0.03, 0.3 and 3.0 μM) had no significant effect on caffeine contractions.

6 The data show that the K^+ channel activators, cromakalim and pinacidil, unlike the Ca^{2+} channel blocker, dazodipine, or the guanylate cyclase activator, sodium nitroprusside, can inhibit the contraction which results from caffeine-induced Ca^{2+} release. Cromakalim and pinacidil, however, inhibit only the component of the noradrenaline response resulting from Ca^{2+} influx (tonic component) and not that resulting from Ca^{2+} release (phasic component). Cromakalim may affect both components of the histamine contraction.

Introduction

The anti-hypertensive agent, cromakalim, is thought to relax vascular smooth muscle *in vitro* by activating K^+ channels in the cell membrane. The efflux of K^+ ions from the vascular smooth muscle cell hyperpolarises the membrane towards the K^+ equilibrium potential (Hamilton *et al.*, 1986; Weir & Weston, 1986). Hyperpolarisation opposes the ability of depolarising agents such as KCl to open voltage-operated Ca^{2+} channels (VOC). Thus cromakalim inhibits contractions induced by KCl, although high concentrations (>60 mM) of KCl, produce contractions which are resistant to cromakalim (Weir & Weston, 1986; Clapham & Wilson, 1987). This lack of effect is probably a consequence of overwhelming depolarisation produced by the high KCl concentrations.

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Cromakalim also inhibits contractions induced by receptor agonists such as noradrenaline, histamine, 5-hydroxytryptamine (5-HT) and angiotensin II (Clapham & Wilson, 1987; Cain & Nicholson, 1988; Cook *et al.*, 1988; Wilson, 1988), even though contractions to these agents are less dependent on depolarisation than the equivalent contractions to KCl (Mulvany *et al.*, 1982; Bolton *et al.*, 1984; Haeusler, 1985; Neild & Kotecha, 1987). Unlike contractions to KCl, contractions to all concentrations of the receptor agonists are inhibited.

Noradrenaline contracts vascular smooth muscle partly by opening receptor-operated Ca^{2+} channels (ROC) to admit Ca^{2+} into the cell, and partly by the release of Ca^{2+} from intracellular stores (Deth & van Breemen, 1974). Histamine also utilises both of these Ca^{2+} pools to produce contraction (Carrier & Howell, 1982; Daniel, 1984). Part of the present study was therefore designed to investigate whether cromakalim can inhibit the component of the contraction to noradrenaline and histamine which results from intracellular Ca^{2+} release.

Although caffeine has a number of actions on smooth muscle, its contractile activity in blood vessels is a consequence of Ca^{2+} release from intracellular stores, with little or no stimulation of Ca^{2+} influx (Itoh *et al.*, 1981). The effects of cromakalim on contractions to caffeine were therefore compared with its effects on noradrenaline and histamine contractions.

Rabbit isolated renal artery was selected for these experiments since this tissue has been shown to be highly dependent on the release of intracellular Ca^{2+} for contraction to noradrenaline (Bevan *et al.*, 1986). Furthermore, preliminary experiments showed that, of the various arteries of the rabbit which were studied (renal, mesenteric, pulmonary, carotid, femoral and aorta), the renal artery gave the largest and most reproducible contractions to caffeine. In some experiments the K^+ channel activator, pinacidil (Bray *et al.*, 1987), the dihydropyridine Ca^{2+} channel blocker, dazodipine (PY 108-068: Hof, 1985) and the guanylate cyclase activator, sodium nitroprusside (Kukovetz *et al.*, 1979) have been used for comparison with cromakalim.

Preliminary accounts of this work were presented to the British Pharmacological Society, July 1988 and December 1988.

Methods

Tissue preparation

The left renal artery was removed from male New Zealand White rabbits (1.5–2.5 kg), and cut transversely into four equal rings. The tissues were suspended between fine stainless steel hooks in a 10 ml bath and isometric tension recorded via Dynamometer UF1 transducers. Most experiments used a bicarbonate-buffered Krebs solution (composition in mM: NaCl 118.4, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, D-glucose 10.1). Where Ca^{2+}-free conditions were used, $CaCl_2$ was omitted from the Krebs solution and EGTA 0.1 mM included. Since $LaCl_3$ forms a $La_2(CO_3)_3$ precipitate in Krebs solution, experiments involving $LaCl_3$ were carried out in a HEPES buffered salt solution (composition in mM: NaCl 120, KCl 6.0, $CaCl_2$ 2.5, $MgCl_2$ 1.2, HEPES 5.0, D-glucose 11.4). Both solutions were gassed with 95% O_2 plus 5% CO_2 and maintained at 37°C.

Contractions to noradrenaline and histamine

Preliminary experiments involved the construction of concentration-response curves to noradrenaline and histamine. The agonist was added cumulatively in half log units until the maximum response was attained. From the mean ($n = 5$) curves, noradrenaline and histamine EC_{30}, EC_{60} and EC_{90} values were estimated. These concentrations were used in subsequent experiments. In these, each of the four rings of artery were contracted with the EC_{30} of either noradrenaline or histamine. After washout over 15 min, the tissues were incubated for 15 min with either (a) normal Krebs (control), (b) a single submaximal concentration of cromakalim (1 μM), (c) Ca^{2+}-free Krebs or (d) Ca^{2+}-free Krebs plus cromakalin (1 μM). Tissues were then re-challenged with the noradrenaline or histamine EC_{30}. After a 30 min washout, the cycle was repeated for the noradrenaline or histamine EC_{60} then for the EC_{90}.

Other experiments were performed with noradrenaline and histamine in HEPES buffer, where the four treatment groups were (a) HEPES buffer (control), (b) cromakalim (1 μM), (c) $LaCl_3$ (1 mM) and (d) $LaCl_3$ (1 mM) plus cromakalim (1 μM). These experiments differed in that only one concentration of noradrenaline or histamine was used per tissue since tissues were no longer viable after $LaCl_3$ washout. Pinacidil (3 μM) was also examined in place of cromakalim in some experiments where noradrenaline was the contractile agent. Preliminary experiments determined that 1 mM was the minimum $LaCl_3$ concentration necessary to abolish KCl-induced contractions in this tissue.

Contractions to caffeine

Each of the four rings of artery were contracted four times with caffeine (10 mM) in Krebs solution. Fol-

lowing the initial contraction in each tissue, the preparations were washed out for 15 min. Thereafter, the three remaining contractions in each tissue were obtained after incubation with either: (a) cromakalim (0.1, 1.0 and 10 μM); (b) cromakalim (0.1, 1.0 and 10 μM) all in the presence of glibenclamide (3 μM); (c) pinacidil (0.3, 3.0 and 30 μM); (d) pinacidil (0.3, 3.0 and 30 μM) all in the presence of glibenclamide (3 μM); (e) dazodipine (0.01, 0.1 and 1.0 μM); (f) sodium nitroprusside (0.03, 0.3 and 3.0 μM); (g) glibenclamide (3 μM) in each case (glibenclamide controls); (h) untreated (time-matched controls). The tissues were incubated with cromakalim, pinacidil, dazodipine and sodium nitroprusside for 15 min before exposure to caffeine. Where cromakalim or pinacidil was combined with glibenclamide, the tissues were pre-incubated for 10 min with glibenclamide. In preliminary experiments where the tissues were contracted with (30 mM) KCl, it was established that: (i) the potency-ratio for relaxation was approximately 1:3:10:30 for dazodipine:sodium nitroprusside:cromakalim:pinacidil; (ii) relaxant responses to these drugs occurred within 15 min; (iii) a 10 min pre-incubation with glibenclamide was sufficient to antagonise the relaxant effects of cromakalim and pinacidil.

A further experiment was performed to study the effect of cromakalim on caffeine contractions under Ca^{2+}-free conditions. The design was similar to that above except that five contractions to caffeine (10 mM) were obtained per tissue. A contraction was first obtained in Ca^{2+}-containing Krebs solution. Subsequent contractions, treatments as in groups (a), (b), (g) and (h) above, were obtained in Ca^{2+}-free Krebs solution. Washouts between responses were made in normal Krebs solution.

Treatment of results

Inhibition of contractions to noradrenaline and histamine were quantified in terms of area under the noradrenaline or histamine 3-point concentration-response curve (AUC). The % reduction in AUC was calculated for individual experiments and the mean $\pm$ s.e.mean % reduction shown. Significant differences in AUC between different treatment groups were assessed by use of Dunnett's test for multiple comparisons. In the caffeine experiments, significant differences between contractions in the presence of test drug and the pretreatment contraction were also assessed by use of Dunnett's test.

Drugs

Cromakalim was synthesized in these laboratories. Pinacidil monohydrate and dazodipine were gifts from Leo and Sandoz respectively. Sodium nitroprusside, histamine dihydrochloride, noradrenaline bitartrate, $LaCl_3$, EGTA and all reagents constituting the physiological salt solutions were purchased from Sigma.

Results

Effect of cromakalim on noradrenaline and histamine contractions in normal and Ca^{2+}-free Krebs solution

In normal Krebs solution, contractions to noradrenaline EC_{30}, EC_{60} and EC_{90} (0.27, 2.3 and 21 μM, respectively) were similar in the pretreatment and time-matched control curves (Figure 1a). Cromakalim (1 μM) inhibited the noradrenaline contractions (Figure 1b) resulting in a 39.1 $\pm$ 5.2% reduction in AUC. In Ca^{2+}-free Krebs (with EGTA), there was a large reduction in the noradrenaline contractions (Figure 1c) amounting to an 80.0 $\pm$ 2.4% reduction in AUC. Cromakalim (1 μM) in Ca^{2+}-free Krebs produced a similar reduction (78.4 $\pm$ 2.6%) in AUC (Figure 1d). In the presence of cromakalim, the response to noradrenaline was frequently biphasic in nature (Figure 2b). The latter (tonic) component of the noradrenaline response was preferentially inhibited by cromakalim, although the inhibition was partially overcome at the higher noradrenaline concentrations (Figure 2a, b, c). Ca^{2+} removal abolished the tonic component, even at the highest noradrenaline concentration (Figure 2d).

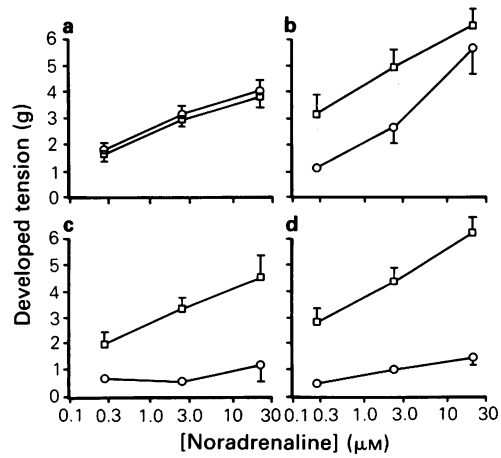

Figure 1 Mean (n = 6) contractions to noradrenaline in rabbit isolated renal artery. (□) Pretreatment responses. (○) Responses (a) in time-matched control; (b) with cromakalim (1 μM); (c) in Ca^{2+}-free/EGTA Krebs; (d) in Ca^{2+}-free/EGTA Krebs with cromakalim (1 μM). Vertical lines show s.e.mean.

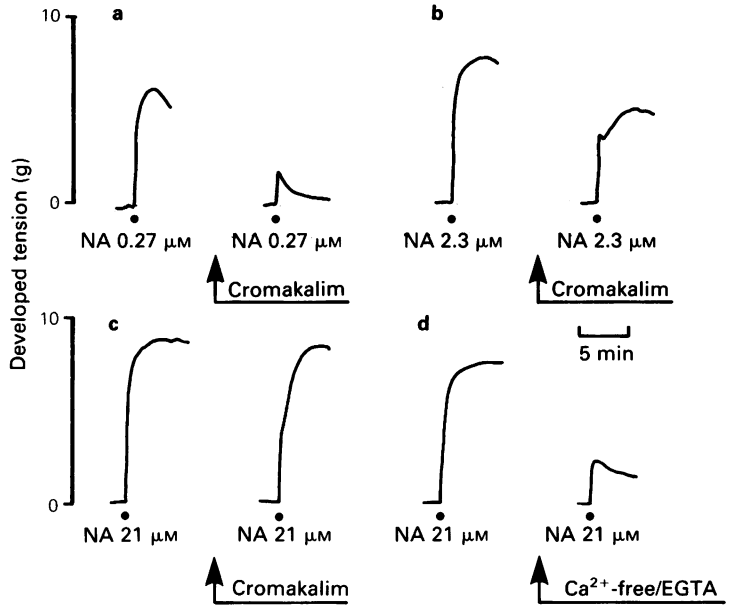

Figure 2 Tracing of responses to noradrenaline in rabbit isolated renal artery. Traces (a), (b) and (c) illustrate the effect of a 15 min incubation with cromakalim (1 μM) on the noradrenaline (NA) EC_{30}, EC_{60} and EC_{90} responses, respectively. Trace (d) shows the effect of a 15 min incubation in Ca^{2+}-free + EGTA (0.1 mM) Krebs solution on the noradrenaline EC_{90} response.

Contractions to histamine EC_{30} EC_{60} and EC_{90} (1.0, 3.5 and 23 μM respectively) were also similar in pretreatment and time-matched controls (Figure 3a). Cromakalim (1 μM) produced marked inhibition of histamine contractions (Figure 3b) corresponding to a 61.8 ± 6.2% reduction in AUC, a significantly ($P < 0.01$) greater effect than on noradrenaline contractions.

Ca^{2+} removal gave a 74.5 ± 5.0% reduction in AUC (Figure 3c), but cromakalim (1 μM) in Ca^{2+} Krebs (Figure 3d) gave a significantly ($P < 0.05$) greater reduction, of 86.2 ± 2.6%, than Ca^{2+} removal alone. As with noradrenaline, the tonic component of the histamine response was antagonised by cromakalim in a reversible manner, and abolished by Ca^{2+} removal.

Effect of cromakalim on noradrenaline and histamine contractions in the absence and presence of $LaCl_3$

Contractions to noradrenaline in HEPES-buffered solution were, as in Krebs solution, similar in pretreatment and time-matched controls (Figure 4a). Cromakalim (1 μM) inhibited contractions to noradrenaline (Figure 4b) to give a 31.8 ± 4.7% reduction in AUC. $LaCl_3$ (1 mM) produced a marked inhibition of the noradrenaline contractions (Figure 4c) corresponding to a 74.8 ± 3.2% reduction in AUC. As with Ca^{2+} removal, only the phasic part of the noradrenaline contraction remained in the presence of $LaCl_3$. The combination of cromakalim (1 μM) and $LaCl_3$ (1 mM), however, gave no further inhibition (Figure 4d), a reduction in AUC of 71.9 ± 2.9%.

$LaCl_3$ completely abolished the histamine EC_{30} and EC_{60} contractions. Thus only the data for the histamine EC_{90} response is presented ($n = 5$). Cromakalim (1 μM) reduced this single contraction by 46.0 ± 12.3%. $LaCl_3$ (1 mM) inhibited the contraction by 81.8 ± 7.5%. The combination of cromakalim and $LaCl_3$ produced an inhibition of 94.6 ± 3.5%, significantly ($P < 0.05$) greater than the inhibition by $LaCl_3$ alone.

Effect of pinacidil on noradrenaline contractions in the absence and presence of $LaCl_3$

As in the cromakalim experiments, noradrenaline control contractions were reproducible (Figure 5a). Pinacidil (3 μM) inhibited the noradrenaline responses (Figure 5b) resulting in a 35.5 ± 6.6% reduction in AUC.

$LaCl_3$ (1 mM) inhibited the contractions (Figure 5c), to give an 80.9 ± 5.1% reduction in AUC. The combination of pinacidil (3 μM) with $LaCl_3$ (1 mM)

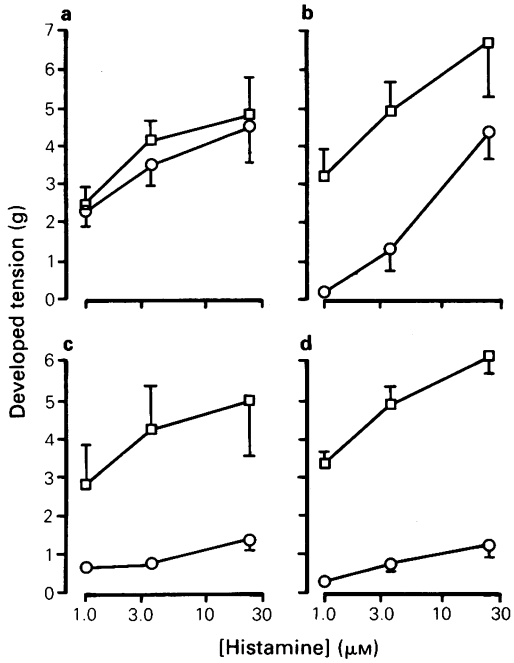

Figure 3 Mean ($n = 5$) contractions to histamine in rabbit isolated renal artery. (□) Pretreatment responses. (○) Responses: (a) in time-matched control; (b) with cromakalim (1 μM); (c) in Ca^{2+}-free/EGTA Krebs; (d) in Ca^{2+} free/EGTA Krebs with cromakalim (1 μM). Vertical lines show s.e.mean.

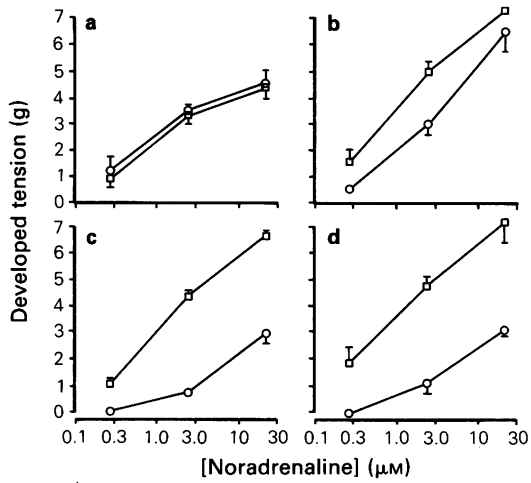

Figure 4 Mean ($n = 6$) contractions to noradrenaline in rabbit isolated renal artery. (□) Pretreatment responses. (○) Responses: (a) in time-matched control; (b) with cromakalim (1 μM); (c) with $LaCl_3$ (1 mM); (d) with $LaCl_3$ (1 mM) plus cromakalim (1 μM). Vertical lines show s.e.mean.

(Figure 5d) had no further effect, a reduction in AUC of 80.9 ± 2.0%.

Effect of vasorelaxant drugs on contractions to caffeine

Caffeine produced a rapid contraction which returned to baseline within 2 min. Cromakalim (0.1, 1.0 and 10 μM) produced significant, concentration-related inhibition of the caffeine (10 mM) contraction (Table 1). In the presence of glibenclamide (3 μM), the inhibitory effect of cromakalim 0.1 and 1.0 μM was abolished and that of 10 μM reduced. Similarly, pinacidil (0.3, 3.0 and 30 μM) inhibited the caffeine contractions. Glibenclamide again antagonised the effect. Dazodipine and sodium nitroprusside had no significant effect on caffeine contractions. Glibenclamide (3 μM) itself did not affect the contractions, and the time-matched control responses were consistent throughout. Contractions to caffeine were not significantly ($P > 0.05$) reduced in changing from normal to Ca^{2+}-free Krebs solution (Table 2). As in normal Krebs solution, cromakalim produced a concentration-related inhibition of the caffeine (10 mM) contraction which was sensitive to antagonism by glibenclamide. The time-matched controls were maintained throughout, as were all but the last of the glibenclamide control caffeine responses.

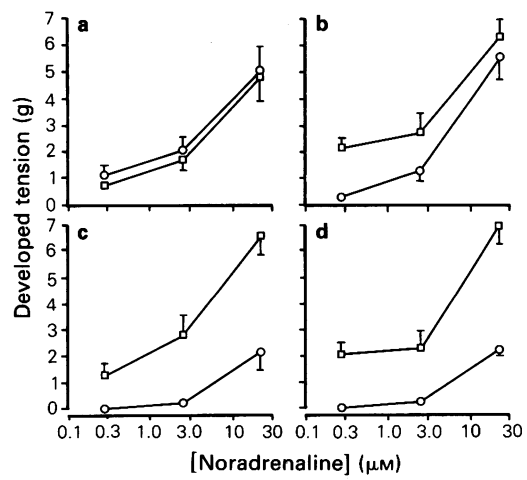

Figure 5 Mean ($n = 5$) contractions to noradrenaline in rabbit isolated renal artery. (□) Pretreatment responses. (○) Responses: (a) in time-matched control; (b) with pinacidil (3 μM); (c) with $LaCl_3$ (1 μM); (d) with $LaCl_3$ (1 mM) plus pinacidil (3 μM). Vertical lines show s.e.mean.

Table 1 Effect of vasorelaxant drugs on the contraction (mg) to caffeine (10 mM) in normal Krebs solution

	Initial contraction	*2nd contraction*	*3rd contraction*	*4th contraction*
Time matched control	898 ± 124	902 ± 113	901 ± 136	862 ± 120
Cromakalim	847 ± 122	[0.1] 613 ± 61**	[1.0] 410 ± 68**	[10] 273 ± 33**
Cromakalim + glibenclamide	887 ± 164	[0.1] 900 ± 127	[1.0] 887 ± 141	[10] 533 ± 83*
Pinacidil	1167 ± 126	[0.3] 933 ± 126*	[3.0] 700 ± 84**	[30] 480 ± 101**
Pinacidil + glibenclamide	1260 ± 169	[0.3] 1240 ± 166	[3.0] 1273 ± 178	[30] 877 ± 150*
Dazodipine	1040 ± 116	[0.01] 940 ± 191	[0.1] 1000 ± 206	[1.0] 1250 ± 248
Sodium nitro-prusside	760 ± 141	[0.03] 880 ± 201	[0.3] 760 ± 198	[3.0] 597 ± 147
Glibenclamide control	1057 ± 140	1093 ± 131	1100 ± 133	1103 ± 134

Data shown are means ± s.e.mean (n = 6–18).
Concentration of drug used shown [in μM]. Significant difference from pretreatment: * $P < 0.05$, ** $P < 0.01$; Dunnett's test. The glibenclamide concentration was 3 μM.

Table 2 Effect of vasorelaxant drugs on the mean contraction (mg) to caffeine (10 mM) in Ca^{2+}-free Krebs solution

	Ca^{2+} containing Krebs	*Initial contraction*	*2nd contraction*	*3rd contraction*	*4th contraction*
Time matched control	503 ± 59	447 ± 42	490 ± 74	493 ± 84	483 ± 73
Cromakalim	1057 ± 131	917 ± 138	[0.1] 800 ± 107	[1.0] 460 ± 40**	[10] 407 ± 40**
Cromakalim + glibenclamide	833 ± 108	727 ± 127	[0.1] 827 ± 102	[1.0] 697 ± 126	[10] 363 ± 46**
Glibenclamide control	613 ± 62	513 ± 70	527 ± 61	517 ± 56	367 ± 70*

Data shown are means ± s.e.mean (n = 6).
Concentration of drug used shown [in μM]. Significant difference from pretreatment: * $P < 0.05$, ** $P < 0.01$; Dunnett's test. The glibenclamide concentration was 3 μM.

Discussion

The contractile response of vascular smooth muscle to noradrenaline and histamine involves both the release of intracellular Ca^{2+}, the phasic component, and the influx of extracellular Ca^{2+}, the tonic component (Deth & van Breemen, 1974; Carrier & Howell, 1982). The extent of cell membrane depolarisation associated with the contractile activity of these receptor agonists in vascular smooth muscle remains a matter of controversy (see Johansson & Somylo, 1980; Hermsmeyer, 1983).

It is evident, however, that in isolated arteries such as rat small mesenteric (Mulvany *et al.*, 1982), guinea-pig anterior mesenteric (Bolton *et al.*, 1984) and rabbit pulmonary (Haeusler, 1985), the degree of depolarisation associated with a given contraction is considerably smaller for noradrenaline than for KCl. This applies to other receptor agonists such as histamine, 5-HT and angiotensin II (see Johansson & Somlyo, 1980). Nevertheless, cromakalim is an effective inhibitor of contractions to all of these agents (Cain & Nicholson, 1988; Cook *et al.*, 1988; Wilson, 1988). Thus it would appear that this inhibitory activity of cromakalim is not a result of hyperpolarisation opposing agonist-induced opening of VOC. The remaining possibilities are that cromakalim opposes the ability of agonists to open ROC or that cromakalim inhibits the contractions resulting from agonist-induced intracellular Ca^{2+} release. The present results suggest that the former is the more likely mechanism and the latter does not occur, at least with noradrenaline responses.

Cromakalim inhibited contractions to histamine and noradrenaline in normal Krebs and was more effective against histamine. Pinacidil, at a 3 fold

higher concentration than cromakalim, produced a similar inhibition to cromakalim on the noradrenaline response. Removal of extracellular Ca^{2+} gave a large reduction in noradrenaline and histamine contractions. This finding was unexpected, at least for noradrenaline, since rabbit renal artery has been shown to have a low dependence on extracellular Ca^{2+} for its contraction to noradrenaline (Bevan *et al.*, 1986). Chelating agents such as EGTA and EDTA can, if given in sufficient concentration for a long enough period of time, remove intracellularly bound Ca^{2+} from vascular smooth muscle (Wheeler & Weiss, 1979). Thus prolonged incubation with a high concentration of EGTA in a tissue which is highly dependent on intracellular Ca^{2+} for its contraction to noradrenaline could reduce the size of the noradrenaline response. This, however, appears extremely unlikely in this situation as only a 15 min incubation with the lowest effective concentration of EGTA (0.1 mM) was used. A reduction in the noradrenaline or histamine response could also be caused by membrane instability as a result of Ca^{2+} removal: this can be prevented by the inclusion of $MgCl_2$ (10 mM) (Aaronson & Jones, 1985). In preliminary experiments of the present series (unpublished), the inclusion of $MgCl_2$ (10 mM) did not prevent the large reduction in response to noradrenaline or histamine after Ca^{2+} removal. It must therefore be concluded that rabbit renal artery is largely dependent upon extracellular Ca^{2+} for contraction to noradrenaline and histamine. This is supported by the $LaCl_3$ experiments. When Ca^{2+} influx was presumably abolished by $LaCl_3$ (1 mM) (Deth & van Breemen, 1974), a concentration which certainly abolished KCl-induced contraction, a similar large reduction in the noradrenaline and histamine responses was again observed.

Removal of extracellular Ca^{2+} with EGTA, or preventing its influx with $LaCl_3$, abolished the tonic component of the noradrenaline response. Cromakalim also inhibited the tonic part of the contraction to the low noradrenaline concentration, although this component partially returned at the higher noradrenaline concentrations. These data are consistent with a reversible inhibitory action of cromakalim on noradrenaline-induced Ca^{2+} influx. Pinacidil had a similar profile indicating that this is a feature of K^+ channel activator drugs.

Cromakalim had no additional inhibitory effect on noradrenaline contractions following Ca^{2+} removal. Similarly, cromakalim and pinacidil had no further effect after inhibition of Ca^{2+} influx with $LaCl_3$. Thus K^+ channel activators do not appear to inhibit the component of the noradrenaline contraction, associated with the release of Ca^{2+} from intracellular stores, the phasic component.

With histamine as the contractile agent, cromakalim produced a small, but nonetheless, significant further reduction in contraction in combination with Ca^{2+} free/EGTA or $LaCl_3$, than either of these treatments alone. This could possibly explain the greater effect of cromakalim on histamine contractions than on noradrenaline contractions in normal Krebs. It is, however, difficult to reconcile this result with current theory regarding both the mechanism of action of cromakalim and the mechanism of histamine- and noradrenaline-induced Ca^{2+} release. Beyond the α-adrenoceptor and the histamine H_1-receptor, noradrenaline and histamine share a common pathway, via the second messenger inositol-1,4,5-trisphosphate (IP_3), culminating in Ca^{2+} release from intracellular stores (Berridge, 1986). Thus, the only site for a differential action of cromakalim on the noradrenaline and histamine responses is at the receptor site. However, cromakalim has no affinity for either the [^{3}H]-prazosin binding site (Coldwell & Howlett, 1987) or the [^{3}H]-mepyramine binding site (D. Nelson, personal communication). Confirmation of this differential effect in a tissue with a higher dependence of Ca^{2+} release for contraction would allow firmer conclusions to be drawn.

The finding that cromakalim antagonises contractions to caffeine is novel. All effects of cromakalim described previously could be explained by an indirect action (membrane hyperpolarisation) on Ca^{2+} influx through VOC (KCl contractions) or ROC (noradrenaline contractions). Although there may be some effect of cromakalim on histamine-induced Ca^{2+} release (see above), it is likely that the greater part of the effect of cromakalim is on histamine-induced Ca^{2+} influx. The lack of involvement of Ca^{2+} influx in the contractile response to caffeine (Itoh *et al.*, 1981) was confirmed both by the response to caffeine in Ca^{2+}-free Krebs and the absence of an inhibitory effect of the Ca^{2+} channel blocking agent, dazodipine.

The K^+ channel blocking agent, glibenclamide, has been shown to inhibit the vasorelaxant effect of cromakalim with a pA_2 value of 7.2 (Wilson, 1989). In the present study, glibenclamide, at a concentration (3 μM) which had no effect on caffeine contractions *per se*, antagonised the inhibitory effect of cromakalim on caffeine contractions. Pinacidil also inhibited caffeine contractions and this was also antagonised by glibenclamide. Sodium nitroprusside and dazodipine, however, at equivalent vasorelaxant concentrations had no significant effect on caffeine contractions. These data provide indirect evidence that the inhibitory effect upon caffeine contractions is a result of the K^+ channel activating property of cromakalim and pinacidil.

The results raise the question as to how K^+ channel activators can discriminate between contractions in response to Ca^{2+} release induced by nor-

adrenaline and caffeine. The answer may reside either in the pool of Ca^{2+} which noradrenaline and caffeine release, or the mechanism by which they release that Ca^{2+}. Whether or not noradrenaline and caffeine release Ca^{2+} from exactly the same store remains a matter of some debate. It has been shown that the store released by the two agents is identical (Itoh *et al.*, 1982); Leijten & van Breemen, 1984). However, others suggest that the overlap of stores is not complete (Deth & Casteels, 1977; Saida & van Breemen, 1984). It is, however, evident that noradrenaline and caffeine do not release Ca^{2+} by the same mechanism. As discussed earlier, noradrenaline-induced Ca^{2+} release is mediated by IP_3 (Berridge & Irvine, 1984; Hashimoto *et al.*, 1986). In contrast, caffeine is thought to stimulate or potentiate Ca^{2+}-induced Ca^{2+} release (Itoh *et al.*, 1981; Karaki *et al.*, 1987). It would therefore appear that K^+ channel activators can interfere with the latter mechanism but not the former. Whether the inhibition of caffeine contractions is a result of K^+ channel activation in the cell membrane and subsequent hyperpolarisation, or of K^+ channel activation at some other site remains to be determined.

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The effects of dihydropyridine compounds in behavioural tests of dopaminergic activity

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1 The effects of the dihydropyridine calcium channel blocker nifedipine and the activator Bay K 8644 were investigated in different behavioural tests involving dopaminergic systems. These were the discriminative stimulus induced by amphetamine, rotational behaviour in rats with unilateral 6-hydroxydopamine (6-OHDA) lesions and apomorphine-induced yawning in rats.

2 The yawning induced by apomorphine ($40\,\mu g\,kg^{-1}$ s.c.) was significantly potentiated by nifedipine (5–10 mg kg^{-1} i.p.). Bay K 8644 (0.05–0.5 mg kg^{-1} i.p.) dose-dependently inhibited yawning induced by apomorphine ($80\,\mu g\,kg^{-1}$ s.c.) and, at 0.4 mg kg^{-1}, inhibited the nifedipine potentiation of apomorphine-induced yawning. In contrast to their effects on apomorphine-induced yawning, nifedipine and Bay K 8644 had no effect on apomorphine-induced penile erection.

3 Bay K 8644 (0.06–0.5 mg kg^{-1} i.p.) and nifedipine (5–20 mg kg^{-1} i.p.) had no dose-related effect on the discrimination performance of rats trained to discriminate amphetamine from saline. However, nifedipine dose-dependently reduced the response rate of amphetamine-treated rats. Bay K 8644 had no effect on this measure except at high doses that also caused disruption.

4 Neither nifedipine (5–10 mg kg^{-1} i.p.) nor Bay K 8644 (0.06–0.5 mg kg^{-1} i.p.) affected the turning behaviour induced by amphetamine (1 mg kg^{-1} i.p.) in rats with unilateral 6-OHDA lesion of the medial forebrain bundle, and did not induce turning themselves.

5 As the dihydropyridine compounds affected apomorphine-induced yawning but not penile erection, and did not affect amphetamine-induced rotation or drug discrimination, it seems unlikely that they are affecting dopamine release *in vivo*.

Introduction

It has previously been shown that the dihydropyridine L-type calcium channel activator methyl-1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate (Bay K 8644) can increase dopamine turnover in the CNS of rats and mice (Pileblad & Carlsson, 1987; Bolger *et al.*, 1988; Bourson *et al.*, 1989). These authors have postulated that this is due to a direct effect on dopamine release as calcium is known to be important in neurotransmitter release mechanisms (Ashley *et al.*, 1984; Augustine *et al.*, 1987). Furthermore, *in vitro* studies using potassium-stimulated release from brain slices suggest that L-type calcium channels may play a role in this process (Middlemiss, 1985; Middlemiss & Spedding, 1985). In order to examine the functional significance of this interaction between the L-type calcium channel and CNS dopamine systems we have studied the effects of the dihydropyridine calcium channel blocker nifedipine and the activator Bay K 8644 in several behavioural tests of dopaminergic activity. Tests were chosen that may be expected to demonstrate an effect on release *in vivo*, such as rotational behaviour in rats with unilateral 6-hydroxydopamine (6-OHDA) lesions (Ungerstedt, 1971), apomorphine-induced yawning (Gower *et al.*, 1984) and the discriminative stimulus induced by amphetamine. Rats with unilateral 6-OHDA lesions of the nigrostriatal pathway will turn towards the lesioned side when treated with a dopamine releaser, such as amphetamine, because transmitter can only

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be released from the intact presynaptic sites on the non-lesioned side (Ungerstedt, 1971). In the case of apomorphine-induced yawning there is considerable evidence that the low doses of apomorphine required to induce yawning are acting on dopamine autoreceptors and thereby reducing dopamine release (Protais *et al.*, 1983; Dourish & Hutson, 1985). Finally, the amphetamine discriminative stimulus seems to rely on the ability of amphetamine to release dopamine (Schechter & Cook, 1975). The drug discrimination technique also has the advantage that animals can continue to show a discrimination despite a moderate degree of disruption. This feature was considered important as Bay K 8644 produces a spasticity-like syndrome at high doses (i.e. above 0.5 mg kg^{-1} i.p.) that may interfere with tests relying on a measure of locomotor activity (Bourson *et al.*, 1989).

Some of these results have been presented in abstract form to the British Pharmacological Society (Bourson *et al.*, 1986).

Methods

Animals

Male Sprague–Dawley rats (Charles River, France) weighing 250–350 g were used throughout. Before experimental use they were housed in wire cages in groups of 6 in temperature (22 ± 1°C) and humidity (55% R.H.) controlled animal quarters on a 12 h light–dark cycle with lights on between 6 h 00 min and 18 h 00 min. All animals had free access to food and tap water, except those used for the drug discrimination experiments which were maintained on a restricted diet as detailed below.

Apomorphine-induced yawning and penile erection

All experiments were carried out between 8 h 30 min and 12 h 30 min. The rats were observed in groups of 4, each rat having received a different pretreatment. Immediately following the s.c. injection of apomorphine, the rats were placed in individual clear glass beakers (18 cm high × 14.5 cm diameter) containing a layer of sawdust. A mirror was positioned behind the beakers to permit all round observation of the rats. The number of yawns was counted by direct observation for 30 min from the time the rats were placed in the beakers. In some experiments the incidence of penile erection was also recorded. Each rat was tested once only.

Amphetamine drug discrimination

Apparatus Testing was carried out using five standard operant cages (LI 830, Letica Scientific Instruments, Barcelona) with a food dispenser centred between two levers. The cages were housed separately in sound and light attenuating chambers and illuminated by a house light. The apparatus was programmed using solid state logic modules driven by an Apple IIe computer.

Training The training and testing procedures have been described in detail previously (Tricklebank *et al.*, 1987). Rats were deprived of food by 75–80% of their free-feeding weight and trained to press the right or left levers for food reinforcement (45 mg dustless Precision Pellets, Bioserv. Inc., New Jersey). Once the lever pressing response was established by use of continuous reinforcement, the conditions were changed to a fixed ratio (FR 10) schedule. Fifteen minutes before daily (Monday–Friday) test sessions lasting 14 min, the rats were injected with either amphetamine (0.8 mg kg^{-1} i.p.) or saline vehicle. Half the rats were trained to press the left lever and half the right lever for food reward following amphetamine treatment. In both cases pressing on the alternate lever was reinforced following saline administration. Drug (D) or saline (S) was given to each rat according to one of two repeating sequences (DSSDSSDDSD or SSDDSDDSSD). Each day the total number of responses made on both levers before the first reward and the total number of responses on each lever during the session were recorded. The criterion for correct discrimination was that the total number of responses before the first reward did not exceed 14.

Test of stimulus generalisation and antagonism Experiments were carried out once a week and started once the rats had achieved the discrimination criterion in at least 80% of the preceeding 15 training sessions. On experimental days the sessions lasted 3 min and no reinforcement was given. The total number of responses on each lever was recorded and also the total number of responses at the time when a score of 10 was obtained on one of the levers. Generalisation to amphetamine occurred when the rat pressed 10 times on the amphetamine-appropriate lever before pressing more than 4 times on the saline-appropriate lever. Experimental groups consisted of not less than six rats and the same rats were used to study different test drugs over a number of experimental sessions.

Unilateral 6-OHDA lesion of the medial forebrain bundle

Rats were pretreated with desmethylimipramine (25 mg kg^{-1} i.p.) approximately 30 min before the injection of 6-OHDA in order to block the uptake of the neurotoxin into the noradrenergic fibres and thus

produce a selective degeneration of the dopaminergic neurones. The rats were subsequently anaesthetized with pentobarbitone sodium (Clin-Midy, France; 30 mg kg^{-1} i.p.) and placed in a stereotaxic frame. 6-OHDA was dissolved in saline containing ascorbic acid (0.2 mg ml^{-1}) to a concentration of 4 μg μl^{-1}, and 2 μl of this solution were slowly injected into the right medial forebrain bundle (MFB) at the co-ordinates AP = −2.0 mm, L = +1.5 mm from the bregma and 8 mm below the dura according to the atlas of Pellegrino & Cushman (1967). The injection was made over a period of 5 min 30 s, the needle was left in place for a further 30 s to allow diffusion of compound away from the injection site.

Recording of rotational behaviour The rat was placed in a rotometer similar to that described by Ungerstedt & Arbuthnott (1970). The rotational behaviour was recorded automatically as the number of 360° turns towards or away from the lesioned side very 5 min during the 60 min following administration of amphetamine.

Drugs and solutions

The following compounds were used: nifedipine, Bay K 8644 (Bayer AG, FRG), 6-hydroxydopamine hydrobromide, desmethylimipramine hydrochloride, apomorphine hydrochloride (all from Sigma, U.S.A.), D-amphetamine sulphate (Fabriques de Laire, France).

All solutions were prepared immediately before use. Nifedipine and Bay K 8644 were dissolved in polyethyleneglycol 50% (PEG 50%) and were protected from light by wrapping the bottles in aluminium foil. All other drugs were dissolved in sterile 0.9% w/v NaCl solution. Apart from apomorphine, which was administered subcutaneously, and 6-OHDA which was administered into the medial forebrain bundle, all drugs were injected intraperitoneally.

Statistics

Data from yawning, penile erection and rotation experiments were analysed by the Kruskal–Wallis analysis of variance for non-parametric data. If this was significant, a Mann–Whitney U-test was used to compare control and test groups. Response rates from the drug discrimination experiments were analysed by Student's t test. The response rates were calculated as the mean of the number of lever presses min^{-1} of those rats in each treatment group that made more than 10 presses on any one lever, irrespective of their discrimination performance. Differences between groups were considered as significant if $P < 0.05$.

Results

Effect of nifedipine and Bay K 8644 on yawning and penile erection induced by apomorphine

Administration of apomorphine (20, 40 and 80 μg kg^{-1} s.c.) dose-dependently induced yawning (1.6 ± 0.7; 9.3 ± 2.9; 14.8 ± 2.6 yawns 30 min^{-1} respectively, mean ± s.e.mean, $n = 8$). A higher dose (120 μg kg^{-1} s.c.) was a less effective inducer of yawning (8.1 ± 2.0 yawns 30 min^{-1}; $n = 8$). Nifedipine (1.25 to 20 mg kg^{-1} i.p.) dose-dependently potentiated yawning induced by apomorphine (40 μg kg^{-1} s.c.), whereas given alone nifedipine induced only a low incidence of yawning (Figure 1). In contrast to the clear potentiation of yawning behaviour by nifedipine, no potentiation of penile erections induced by the same dose of apomorphine (1.7 ± 0.4 erections 30 min^{-1}) was observed (1.4 ± 0.3 and 1.5 ± 0.3 erections 30 min^{-1} for 2.5 and 10 mg kg^{-1} nifedipine, respectively; $n = 8$). Nifedipine (10 mg kg^{-1} i.p.) did not induce penile erections when given alone (PEG 50%: 0.1 ± 0.1; nifedipine 0.1 ± 0.1 erections 30 min^{-1}, $n = 8$).

Following i.p. administration, Bay K 8644 (0.05–0.5 mg kg^{-1} i.p.) dose-dependently inhibited apomorphine-induced yawning (Figure 2). Furthermore,

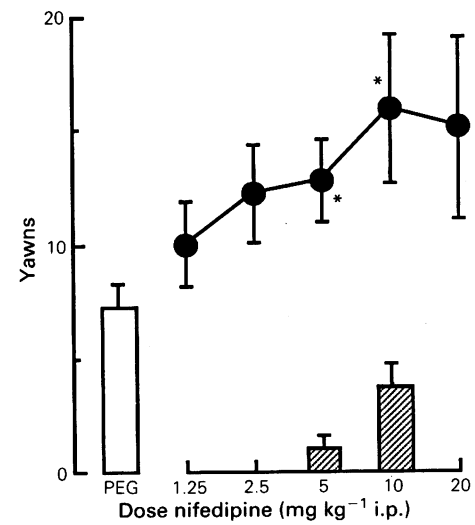

Figure 1 Effect of nifedipine on yawning induced by apomorphine (40 μg kg^{-1} s.c.). Nifedipine was administered intraperitoneally 30 min before apomorphine. Each point represents the mean number of yawns for 8–16 rats observed over a 30 min period immediately following apomorphine administration. Vertical lines show s.e.mean. The hatched columns represent the effects of nifedipine alone. *$P < 0.05$ compared to PEG 50%-treated controls by the Mann–Whitney U-test.

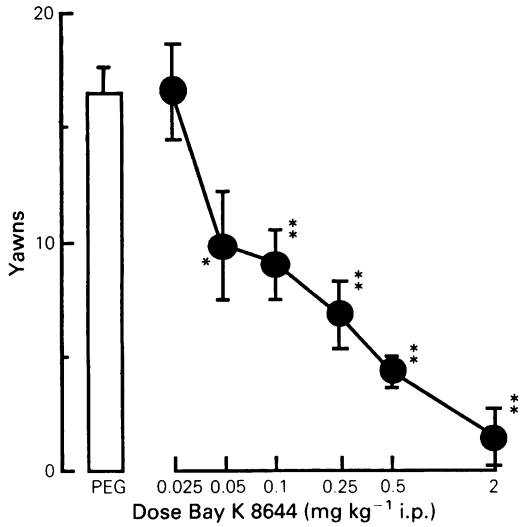

Figure 2 Effect of Bay K 8644 on yawning induced by apomorphine (80 μg kg^{-1} s.c.). Bay K 8644 was administered intraperitoneally 60 min before apomorphine. Each point represents the mean number of yawns for 8 rats observed over a 30 min period immediately following apomorphine administration. Vertical lines show s.e.mean. $^{*}P < 0.05$, $^{**}P < 0.01$ compared to PEG 50%-treated controls by the Mann–Whitney U-test.

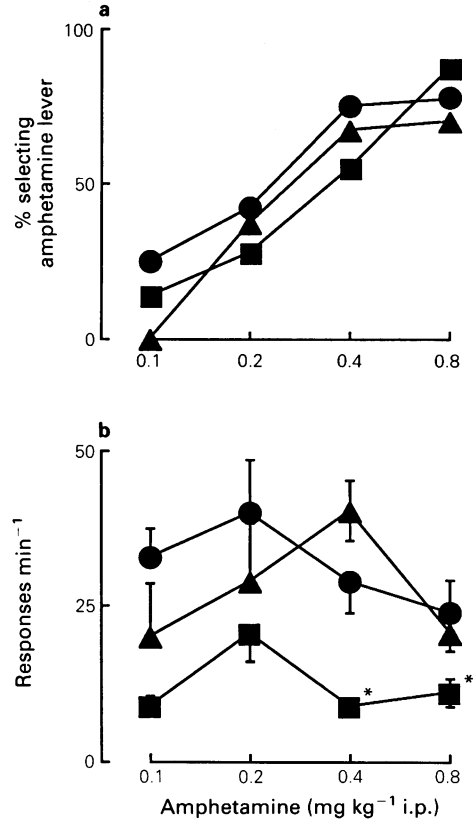

Figure 3 Effect of nifedipine on the dose-response relationship to amphetamine in rats trained to discriminate amphetamine (0.8 mg kg^{-1} i.p.) from saline. Nifedipine (20 mg kg^{-1}; ■) or PEG 50% (▲) was injected 15 min before amphetamine which was administered 15 min before the test. (●) Show the dose-response to amphetamine with no pretreatment. $^{*}P < 0.05$ compared to PEG 50%-treated controls by use of Student's t test.

the potentiation of apomorphine-induced yawning by nifedipine (10 mg kg^{-1}) was reversed by prior administration of Bay K 8644 (0.4 mg kg^{-1}). Thus, apomorphine (40 μg kg^{-1}) induced 14.8 ± 3.0 yawns 30 min^{-1} ($n = 8$) which was increased to 37.4 ± 7.3 following nifedipine pretreatment ($P < 0.01$ compared to vehicle pretreatment, Mann–Whitney U-test) and unaffected by administration of both nifedipine and Bay K 8644 (14.3 ± 3.6 yawns 30 min^{-1}; $P < 0.01$ compared to nifedipine pretreatment alone, Mann–Whitney U-test). Bay K 8644, at doses that significantly inhibited yawning, did not affect penile erections induced by apomorphine (PEG 50% pretreatment: 1.3 ± 0.3 erections 30 min^{-1}; Bay K 8644 0.05 mg kg^{-1}: 1.3 ± 0.4; 0.25 mg kg^{-1}: 1.0 ± 0.2; $n = 8$). Bay K 8644 (0.25 mg kg^{-1} i.p.) did not induce penile erections when given alone.

Effect of nifedipine and Bay K 8644 on the discriminative stimulus effects of amphetamine

Amphetamine (0.1–0.8 mg kg^{-1} i.p.) dose-dependently increased the percentage of rats selecting the amphetamine appropriate lever and pretreatment with either PEG 50% or nifedipine (20 mg kg^{-1} i.p.) had no significant effect on this dose-response relationship (Figure 3). When different doses of nifedipine were tested in combination with a submaximal dose of amphetamine (0.4 mg kg^{-1} i.p.), there was no dose-related effect on the ability of the animals to detect this dose of amphetamine, although a slight potentiation was obtained with 10 mg kg^{-1} of nifedipine (Table 1). Further studies with a lower dose of amphetamine (0.2 mg kg^{-1} i.p.) in combination with nifedipine (10 mg kg^{-1} i.p.) failed to confirm the potentiating effect of the dihydropyridine compound (treatment with either amphetamine alone or with amphetamine + nifedipine made 20% of rats select the amphetamine lever).

Unlike its effect on discrimination performance, nifedipine consistently and dose-dependently

Table 1 The effect of nifedipine or Bay K 8644 on the discriminative stimulus induced by amphetamine

Treatment	*Dose* (mg kg^{-1} i.p.)	*% amphetamine-lever responses*	*Responses min*$^{-1}$	*n/N*
PEG 50%		69	27.5 ± 5.9	13/13
Nifedipine	5	66	22.6 ± 4.4	9/13
	10	100	15.9 ± 2.9	12/14
	20	85	14.2 ± 2.7*	14/14
PEG 50%		40	28.9 ± 6.7	13/13
Bay K 8644	0.06	14	27.5 ± 9.9	7/7
	0.125	50	20.8 ± 6.5	10/12
	0.25	30	24.2 ± 6.9	10/12
	0.5	0	6.0	1/6

Rats were trained to discriminate amphetamine (0.8 mg kg^{-1} i.p.) from saline and the effects of nifedipine and Bay K 8644 were studied in combination with 0.4 and 0.2 mg kg^{-1} i.p. amphetamine respectively. Nifedipine was administered 15 min, and Bay K 8644 45 min, before amphetamine. n/N: number of animals responding (n) of the number tested (N). *$P < 0.05$ compared to PEG 50% treated control groups by means of Student's t test.

reduced the response rate of amphetamine-treated rats (Table 1 and Figure 3). However, Bay K 8644 (0.06–0.5 mg kg^{-1} i.p.) had no significant effect on either the discrimination performance, or on the response rates of rats receiving amphetamine (0.2 mg kg^{-1} i.p.; Table 1). At the highest dose used there was a clear disruption of lever pressing behaviour which corresponded to the appearance of the locomotor and postural effects of Bay K 8644.

Effects of nifedipine and Bay K 8644 on rotational behaviour

Amphetamine dose-dependently induced ipsilateral rotation in rats with unilateral lesions of the MFB (0.25 mg kg^{-1}: 48 ± 7; 0.5 mg kg^{-1}: 116 ± 20; 1 mg kg^{-1}: 152 ± 18; 2 mg kg^{-1}: 221 ± 31 turns 60 min^{-1}; n = 12–20). From these results a dose of 1 mg kg^{-1} was chosen for further study with the dihydropyridine compounds, as it produced a reliable rotational response which was well below the maximum that could be obtained. Following pretreatment with PEG 50% amphetamine induced 187 ± 33 ipsilateral turns 60 min^{-1} (mean ± s.e.mean; n = 7) and this response was not significantly altered by 45 min pretreatment with Bay K 8644 at either 0.06, 0.12, 0.25 or 0.5 mg kg^{-1} (202 ± 26, 153 ± 38, 161 ± 61 and 173 ± 18 turns 60 min^{-1} respectively; n = 5–7). Nifedipine (10 min pretreatment) also failed to affect the rotational behaviour induced by amphetamine (PEG 50% pretreatment: 122 ± 45 turns 60 min^{-1}; nifedipine 5 mg kg^{-1}: 129 ± 21; 10 mg kg^{-1}: 135 ± 50; n = 5). Neither compound significantly induced rotations themselves over the same dose-ranges. Higher doses of Bay K 8644 could not be tested due to its inhibitory effects on locomotor activity.

Discussion

Although the involvement of the L-type calcium channel in neurotransmitter release is controversial, experiments with the dihydropyridine calcium channel activator Bay K 8644 consistently produce results suggesting that it increases either neurotransmitter release or turnover, both *in vitro* (Middlemiss, 1985; Middlemiss & Spedding, 1985) and *in vivo* (Bolger *et al.*, 1988; Bourson *et al.*, 1989). Results with the dihydropyridine calcium channel blockers are less clearcut; Middlemiss (1985), Middlemiss & Spedding (1985) and Bourson *et al.* (1989) found no effect on either release or metabolite levels, whereas others have demonstrated inhibition of neurotransmitter release (Nordström *et al.*, 1986; Pileblad & Carlsson, 1986) or even, in the case of dopamine, an increase (Nordström *et al.*, 1986). The effects of Bay K 8644, referred to above, can be reversed by the dihydropyridine calcium channel blockers suggesting that when activated, L-type calcium channels can affect neurotransmitter release, but that normally they play a negligible role. Although the previous *in vivo* experiments do not differentiate between increased release and increased turnover, data from the *in vitro* studies suggest that it is neurotransmitter release that contributes to the raised metabolite levels. The experiments described in this paper were designed to investigate the functional interaction of the L-type calcium channel with dopamine release in behavioural models where dopamine release, or its modulation, is thought to play a major role. Although apomorphine-induced yawning was potentiated by nifedipine and inhibited by Bay K 8644, penile erection induced by the same compound, and both the stimulus effects of amphetamine and the rotational behaviour induced by amphetamine were unaffected by either compound.

Apomorphine-induced yawning is thought to be the result of reduced dopamine release following stimulation of presynaptic dopamine receptors (Mogilnicka & Klimek, 1977; Protais *et al.*, 1983; Gower *et al.*, 1984; Stähle & Ungerstedt, 1984; Stoessl *et al.*, 1987), and its potentiation by nifedipine and inhibition by Bay K 8644 are consistent with previous work suggesting an involvement of L-type calcium channels in dopamine release. The mutual antagonism between nifedipine and Bay K 8644 of their effects on yawning behaviour is also consistent with such an involvement, although the present data cannot distinguish between an inhibition by Bay K 8644 of the nifedipine potentiation or of the apomorphine response itself. However, apomorphine-induced penile erections are thought to be due to the same mechanism as that involved in the yawning response (Gower *et al.*, 1984) and the inability of the dihydropyridine compounds to affect this parameter argues against an interaction with either apomorphine itself or dopamine release mechanisms. This conclusion is further supported by the lack of effect of either nifedipine or Bay K 8644 on amphetamine drug discrimination and on rotation in unilaterally lesioned rats, either alone or in combination with amphetamine.

It is possible that the negative results we obtained with the dihydropyridine compounds in combination with amphetamine were due to the use of amphetamine itself, which releases dopamine from several storage pools (Chiueh & Moore, 1975) and whose effect is not receptor-mediated as is the case with apomorphine. Such mechanisms may be less susceptible to modulation via dihydropyridine-sensitive calcium channels than those involved in apomorphine-induced changes in dopamine release, although several studies have suggested a role for calcium in amphetamine-induced behavioural changes. Fung & Uretsky (1980) have shown that intrastriatal injection of EGTA could inhibit amphetamine-induced rotation in mice. Also, Grebb (1986) has demonstrated that nifedipine can antagonise amphetamine-induced hyperactivity. However, the specificity of these effects to the dopamine releasing ability of amphetamine is questionable; the effects seen by Grebb (1986) occurred at doses higher than those we have used and are possibly due to other effects of calcium antagonists that are seen at high doses (De Feudis, 1987).

Nencini & Woolverton (1988) have recently demonstrated that nimodipine weakly inhibited the discriminative stimulus effects of amphetamine, but considered this to be a non-specific effect as it was not dose-related, of small magnitude and associated with a significant reduction in response rate. Our results are more clearcut in that neither nifedipine nor Bay K 8644 had an effect on the discrimination performance by rats, although both reduced the response rate. As both the discriminative effects and rotational behaviour induced by amphetamine have been well characterised as being mediated primarily by increased dopamine release (Ungerstedt, 1971; Schechter & Cook, 1975), our results would suggest that at these doses the dihydropyridine compounds do not affect amphetamine-induced dopamine release. It should be noted that the effects on dopamine turnover that we previously demonstrated for Bay K 8644 were seen using higher doses than were possible in the present study (Bourson *et al.*, 1989), due to the appearance of interfering behaviours. This might also explain why the dihydropyridine compounds did not induce rotation themselves.

These data suggest that at the doses we have used dihydropyridine compounds are not able to modify dopamine release *in vivo* and that the effects on apomorphine-induced yawning are specific to that behaviour.

Clearly, further experiments are needed to determine the mechanisms responsible for the potentiation of apomorphine-induced yawning by nifedipine. Compounds that interact with several other transmitter systems, such as physostigmine (Holmgren & Urba-Holmgren, 1980; Gower, 1987), adrenocorticotropic hormone (Serra *et al.*, 1987) and oxytocin (Argiolas *et al.*, 1986), also induce yawning and it is possible that the dihydropyridine compounds affect one or other of these systems. An interaction with the cholinergic system in particular is suggested by the observation that physostigmine and pilocarpine induce yawning but not penile-erections (Gower, 1987). This is not the case for other compounds such as oxytocin (Argiolas *et al.*, 1986) and adrenocorticotropic hormone (Serra *et al.*, 1987). Further experiments are currently underway to examine this hypothesis.

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Effect of D,L-carnitine on the response of the isolated heart of the rat to ischaemia and reperfusion: relation to mitochondrial function

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1 The effect of 100 μM (20 μg ml^{-1}) of D,L-carnitine was studied on the isolated heart of the rat subjected to 30 min of low flow ischaemia followed by reperfusion.

2 In untreated hearts ($n = 30$) ischaemia produced an almost total loss of contractility ($P < 0.05$ compared with non-ischaemic time control) which was accompanied by an increase in resting tension of approximately 235% ($P < 0.05$). Ventricular arrhythmias developed during ischaemia in 100% ($P < 0.05$) of untreated hearts studied. Following reperfusion, untreated hearts recovered 16.3% of contractile function and demonstrated a 60% elevation in resting tension. The incidence of reperfusion-associated ventricular fibrillation was 60%.

3 Carnitine treatment produced no effect on either the contractile depression or the elevation in resting tension during ischaemia but did significantly decrease the incidence of arrythmias at the termination of ischaemia to 63.3% ($n = 30$, $P < 0.05$). In the presence of carnitine, contractile recovery at the end of reperfusion was significantly increased to 30.2% ($n = 10$, $P < 0.05$) and the elevation in resting tension was decreased to 30% ($n = 10$, $P > 0.05$). The incidence of ventricular arrhythmias during reperfusion was significantly reduced by carnitine.

4 Two populations of mitochondria, subsarcolemmal (SLM) and interfibrillar (IFM) isolated at the end of the ischaemic period exhibited an overall increase in oxidative phosphorylation rates as well as uncoupled oxygen consumption; both phenomena were more pronounced with IFM. Carnitine generally potentiated this response. A 29% and 38% inhibition in atractyloside-sensitive ADP uptake was observed in SLM and IFM, respectively, following ischaemia, which was partially prevented by carnitine.

5 After 10 min of reperfusion, adenosine diphosphate (ADP) uptake in SLM was further reduced to 55% of control whereas with IFM, uptake was not different from that seen at the end of ischaemia. Mitochondria isolated from hearts after 30 min of reperfusion revealed a significantly depressed oxidative phosphorylation as well as ADP/ATP translocase activity. These defects were partially reversed in hearts perfused with carnitine.

6 Our study demonstrates that D,L-carnitine protects the rat isolated heart against injury associated with ischaemia and reperfusion through a mechanism associated with improved mitochondrial function.

Introduction

Myocardial ischaemia is associated with the intracellular accumulation of various factors known to be associated with tissue injury, including long-chain acyl CoA (Opie, 1979; Piper & Das, 1987), inorganic phosphate (Pi) (van Belle *et al.*, 1986) and free radicals (Garlick *et al.*, 1987). These factors have been shown to exert deleterious effects on isolated mitochondria including inhibition of oxidative phosphorylation and adenine nucleotide (ADP/ATP) translocase activity (Opie, 1979; Shug, 1979; Ferrari *et al.*, 1988). Various investigators have shown that the deleterious effects of a number of these compounds could be prevented by carnitine or its analogues (Opie, 1979; Shug & Subramanian, 1987). These observations suggest the possibility that carnitine may protect the ischaemic reperfused heart through a mechanism possibly involving the preservation of mitochondrial function (Shug & Sub-

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ramanian, 1987). This concept has not been substantially documented and is complicated by a number of factors. First, although the possible protective effect of carnitine is attractive, a number of studies have shown a distinct lack of protection (Dipalma 1986; Bahl & Bressler, 1987), and indeed a toxic influence of this compound on the ischaemic and reperfused heart (Hearse *et al.*, 1980) has been reported. A second important point to consider is the fact that the relative contribution of mitochondrial dysfunction to the ischaemic reperfused myocardium still remains controversial, particularly with respect to acute ischaemic-reperfusion conditions (Reimer & Jennings, 1986). Thus, a quantitative description linking mitochondrial and cardiac function is lacking. This issue is complicated by numerous factors such as the existence of multifactorial contributing mediators to ischaemic-reperfusion injury (Reimer & Jennings, 1986), the heterogeneous responses of cardiac subsarcolemmal (SLM) and interfibrillar mitochondria (IFM) to ischaemia and reperfusion (van Jaarsveld & Lochner, 1982; Duan & Karmazyn, 1989) as well as the fact that severity of injury may determine the response of the heart to cardioprotective intervention by various factors including carnitine (Paulson *et al.*, 1984). The purpose of the present study, therefore, was to examine the potential effect of a relatively low concentration of D,L-carnitine on the ischaemic reperfused isolated heart. A major focus of the study was to examine this pharmacological intervention on mitochondrial function after subjecting the heart to various treatments and, in particular, to assess the relationship between mitochondrial function and cardiac recovery after reperfusion. Because of the identification of two populations of cardiac mitochondria (Palmer *et al.*, 1977) as well as the prior suggestion of differential responses of these populations to ischaemic and reperfusion insults, both SLM as well as IFM populations were evaluated.

Methods

Animals

Male Sprague-Dawley rats weighing 250–300 g were purchased from Canadian Hybrid Farms (Centreville, Nova Scotia, Canada). Animals were housed in Dalhousie University's Animal Care Facilities in accordance with guidelines of the Canadian Council on Animal Care (Ottawa, Ontario, Canada).

Heart perfusion

Rats were killed by decapitation and the hearts were immediately excised and immersed in a small crucible containing ice-cold Krebs-Henseleit buffer which produced an immediate cessation of contraction. The composition of the buffer (in mM) was as follows: NaCl 120, KCl 4.63, $NaHCO_3$ 20, KH_2PO_4 1.17, $MgCl_2$ 1.2, $CaCl_2$ 1.25, and glucose 8 as substrate; pH was 7.4. The heart was squeezed a few times with fingers to dislodge any clotted blood in the coronary vasculature. The heart was mounted via the aorta on a stainless steel cannula and perfusion with Krebs-Henseleit buffer was started immediately at a constant flow rate of $10\,ml\,min^{-1}$ regulated by a Watson-Marlow peristaltic pump. The buffer was continuously gassed with a 95% O_2: 5% CO_2 mixture and the entire system was regulated at 37°C. The atria were removed and the ventricles were electrically stimulated at 2 times threshold voltage at a frequency of 5 Hz. Contractile force and coronary perfusion pressure were recorded on a Grass Model 7 polygraph using standard techniques (Karmazyn, 1986a). Surface electrical activity was obtained by inserting one fine gauge platinum electrode into the apex while a second electrode was connected to the steel cannula. Hearts were considered arrhythmic when the incidence of extrasystoles was greater than 20 per min and was of at least one min duration.

Following an initial 30 min equilibration period at normal flow rates, low-flow global ischaemia was induced for 30 min by reducing the flow rate to $1\,ml\,min^{-1}$ with or without a further 30 min of reperfusion at $10\,ml\,min^{-1}$. Initial experiments were done in which carnitine was present throughout ischaemia and reperfusion, reperfusion only or ischaemia only. These preliminary studies revealed a complete absence of any effect of carnitine when it was present only during the latter 2 treatments; we have therefore described here results obtained with carnitine present during the complete perfusion sequence. Time control hearts were perfused for a similar duration as experimental hearts at normal flow rates.

Mitochondrial isolation

Two populations of cardiac mitochondria were isolated after the appropriate perfusion protocol according to the procedure of Palmer *et al.* (1977) except that identical isolation buffer containing (in mM) KCl 100, MOPS 50, EGTA 2 and 0.2% bovine serum albumin, pH 7.4 was used for both populations. The final suspension medium for both populations consisted of (in mM) KCl 125, $MgCl_2$ 5 and Tris 2, pH 7.4.

Oxidative phosphorylation

Mitochondrial oxidative phosphorylation was determined in a Gilson oxygraph equipped with a Clark electrode as described in detail previously (Karmazyn, 1986b). Oxidative phosphorylation was

assessed in terms of state 3 respiration rate (QO_2) following the addition of 600 nmol adenosine diphosphate (ADP), respiratory control index (RCI), ADP:oxygen ratio (ADP:O) and oxidative phosphorylation rate (OPR). Either pyruvate-malate (10 mM–2.5 mM) or glutamate-malate (10 mM–2.5 mM) was used as substrate. Uncoupled oxygen consumption was determined in the presence of 0.1 mM 2,4-dinitrophenol with glutamate-malate as substrate.

ADP/ATP translocase activity

ADP/ATP translocase activity was determined in a reaction medium containing (in mM) KCl 110, Tris 20 and EDTA 1 to which had been added 0.02 μCi of [^{14}C]-ADP, pH 7.4 using a modified procedure of Duee & Vignais (1969). The reaction was started by the addition of 0.5 mg mitochondrial protein and terminated at either 0 or 80 s later with 0.1 mM atractyloside. These durations were selected from initial studies showing saturation of the reaction after 40 s. The mixture was centrifuged at 25,000 *g* for 5 min and the supernatant discarded. The pellet was washed twice with medium, dissolved in 0.2 ml of 0.1 N NaOH and counted for radioactivity. ADP/ATP translocase activity was determined by the atractyloside-sensitive ADP uptake per mg mitochondrial protein.

Chemicals

Most drugs, chemicals and reagents were purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.) and were of the highest purity available. Nagarse was purchased from Enzyme Development Corporation (New York, N.Y., U.S.A.). [^{14}C]-ADP was obtained from DuPont Canada Inc. (Dorval, Quebec, Canada).

Statistical analysis

Data were analyzed by one-way analysis of variance with Student-Newman-Keuls (SNK) test for inter-treatment comparisons, except results for mechanical function which were analyzed by Student's *t* test for unpaired data. Frequency of arrhythmias was analyzed using a 2 × 2 contingency table (Zar, 1984). Differences were considered significant when $P < 0.05$.

Results

Cardiac mechanical function and incidence of arrhythmias

In time control experiments, hearts subjected to 90 min of normal perfusion exhibited an average loss in contractile force of approximately 25% as compared to initial values. Carnitine produced no effect on this loss of force. Resting tension remained at the initial 2 g setting over the entire 90 min perfusion period both in the presence and absence of carnitine (control data not shown).

The mechanical response to ischaemia and reperfusion is shown in Figure 1. The reduction in coro-

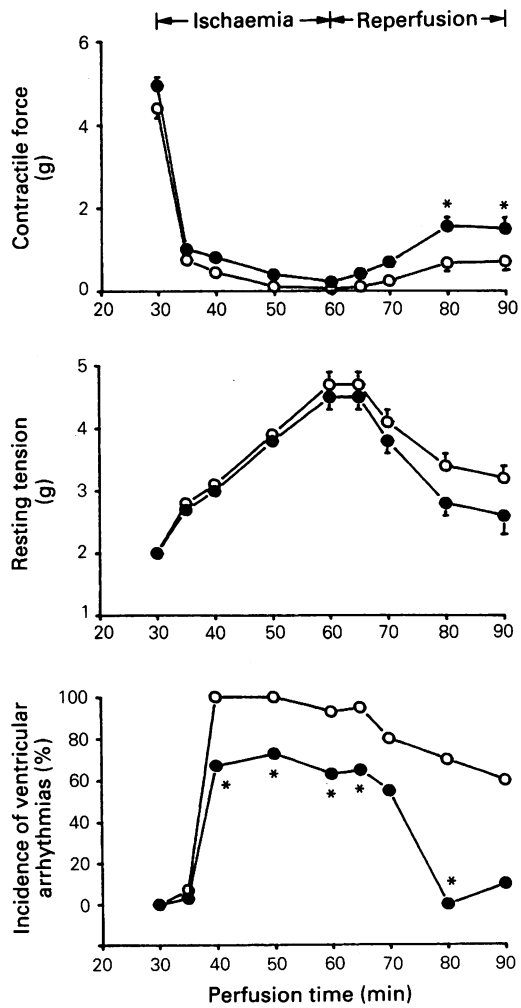

Figure 1 Response of the rat isolated heart to 30 min ischaemia and reperfusion in control hearts (○) or in hearts treated with carnitine (●). For mechanical data each point represents the mean with vertical bars indicating the s.e.mean. Data were pooled from all experiments including hearts not subjected to reperfusion; $n = 30$ for each treatment from 30 to 60 min; $n = 20$ at 70 min; $n = 10$ at 80 and 90 min. Where absent for contractile force and resting tension s.e.mean were smaller than the size of the symbol. * $P < 0.05$ compared with values in the absence of carnitine.

nary flow produced a rapid reduction in contractility and a concomitant progressive elevation in resting tension. The changes in both parameters were significant ($P < 0.05$) after 5 min of ischaemia and remained significant for the duration of the ischaemic, and indeed reperfusion periods. Carnitine produced no effect on either contractile force or resting tension changes associated with the initial ischaemic period. Reperfusion produced a gradual reduction in resting tension and recovery of contractile force; the response of both parameters was substantially influenced by the presence of carnitine. In untreated hearts recovery of force was 16.32% of pre-ischaemic values after 30 min of reperfusion. Carnitine significantly increased recovery to 30.2% of pre-ischaemic values. Furthermore, in the presence of carnitine resting tension was moderately reduced, particularly after 20 and 30 min of reperfusion; however differences were not statistically significant.

Figure 1 also shows the incidence of arrhythmias associated with ischaemia and reperfusion. In untreated hearts arrhythmias persisted throughout both ischaemic and reperfusion conditions. Most observable arrhythmias were in the form of extrasystoles, as described in Methods. With carnitine the incidence of all arrhythmias throughout ischaemia was significantly reduced and at the end of reperfusion arrhythmias were virtually totally abolished by carnitine (Figure 1). During ischaemia, ventricular fibrillation was observed in 29 of 30 untreated hearts whereas 6 of 10 hearts developed ventricular fibrillation during reperfusion; in 2 of these 6 hearts the fibrillation was persistent. In camitine-treated hearts the incidence of all arrhythmias at the end of ischaemia was 63.3%, which was significantly lower than control. The total cumulative incidence of fibrillation during ischaemia in the presence of carnitine was 23 of 30 hearts (77%) whereas 4 of 10 hearts fibrillated during reperfusion although none of these were persistent. However, these reductions in carnitine-treated hearts were not statistically significant. No arrhythmias were ever observed in time control perfused hearts either with or without carnitine throughout the 90 min perfusion period (not shown).

Mitochondrial function

Effect of ischaemia Mitochondria isolated after 30 min of whole heart ischaemia exhibited few changes when pyruvate-malate was used as the respiratory substrate (Figure 2). Small increases in respiratory rates (state 3 Qo_2 and OPR) were found, particularly with IFM and in mitochondria isolated from carnitine-treated hearts. With glutamate-malate, the stimulation in respiration rates was more pronounced and under these conditions, significant

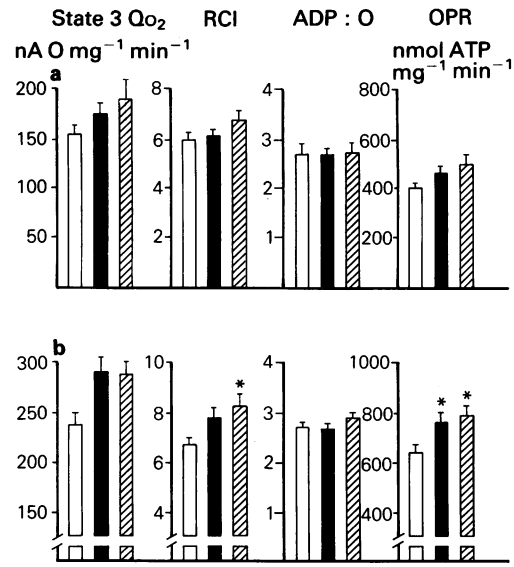

Figure 2 State 3 respiration (Qo_2), respiratory control index (RCI), ADP:oxygen ratio (ADP:O) and oxidative phosphorylation rate (OPR) with pyruvate-malate as substrates for subsarcolemmal (a) and interfibrillar (b) mitochondria isolated from non-ischaemic hearts (open columns), or from hearts subjected to 30 min ischaemia without reperfusion in the absence (solid columns) or presence (hatched columns) of carnitine. Each column represents the mean with vertical bars indicating the s.e.mean of $n = 10$. $^*P < 0.05$ compared with non-ischaemic values.

for both populations (Figure 3). Furthermore, a stimulation in RCI was also seen. Uncoupled oxygen consumption in mitochondria treated with 2,4-dinitrophenol exhibited increased oxygen uptake following ischaemia although this was significant in the SLM population only after carnitine perfusion (Figure 4a,b). Lastly, both mitochondrial populations isolated from ischaemic hearts showed a trend ($P > 0.05$) towards a reduction in atractyloside-sensitive ADP uptake capability, a phenomenon partially prevented by carnitine perfusion (Figure 4c,d).

Effect of reperfusion To assess the possible time-dependent effects of reperfusion on mitochondrial function, mitochondria were harvested following both early (10 min) as well as prolonged (30 min) reperfusion periods. Figure 5 shows oxidative phosphorylation values with pyruvate-malate of mitochondria isolated after 10 min of reperfusion. Virtually no effect was seen with the exception of a significant reduction in RCI of SLM and its prevention by carnitine perfusion (Figure 5). Although a slight, similar reduction and prevention by carnitine was seen with glutamate-malate, the effects were not

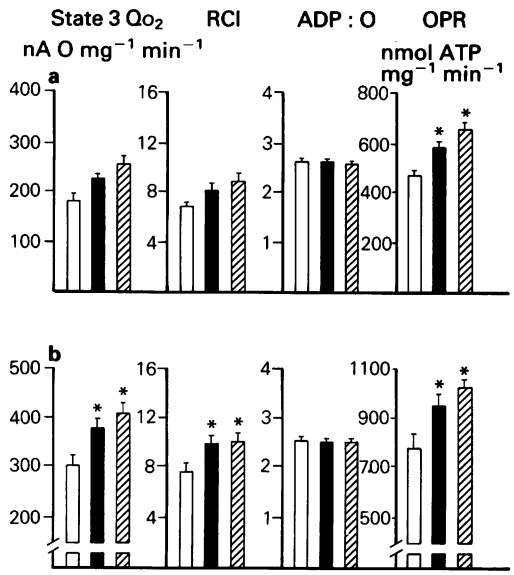

Figure 3 State 3 respiration (Qo_2), respiratory control index (RCI), ADP:oxygen ratio (ADP:O) and oxidative phosphorylation rate (OPR) with glutamate-malate as substrates for subsarcolemmal (a) and interfibrillar (b) mitochondria isolated from non-ischaemic hearts (open columns), or from hearts subjected to 30 min ischaemia without reperfusion in the absence (solid columns) or presence (hatched columns) of carnitine. Each column represents the mean with vertical bars indicating the s.e.mean of $n = 10$. * $P < 0.05$ compared with non-ischaemic values.

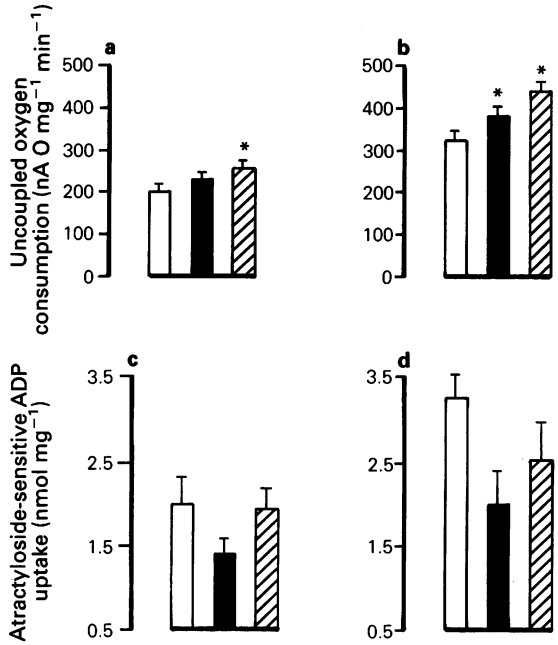

Figure 4 Uncoupled oxygen consumption (a and b) and adenine nucleotide translocase activity (c and d) for subsarcolemmal (a and c) and interfibrillar (b and d) mitochondria isolated from non-ischaemic hearts (open columns), or from hearts subjected to 30 min ischaemia without reperfusion in the absence (solid columns) or presence (hatched columns) of carnitine. Studies with uncoupled mitochondria were carried out in the presence of 2,4-dinitrophenol and glutamate-malate as substrates. Each column represents the mean with vertical bars indicating the s.e.mean of $n = 10$. * $P < 0.05$ compared with non-ischaemic values.

significant (data not shown). IFM, however, exhibited significantly elevated respiration rates when isolated from these hearts. Figure 6 shows uncoupled oxygen uptake (a,b) and atractyloside-sensitive-ADP uptake values (c,d) in mitochondria isolated from 10-min reperfused hearts. No effect on the former was evident. However, both populations demonstrated significantly depressed ADP uptake; with SLM, but not IFM, this depression was partially prevented by perfusing hearts with carnitine (Figure 6).

Oxidative phosphorylation data after 30 min of reperfusion are summarized in Figures 7 and 8. With both substrates, respiration was significantly depressed in both mitochondrial populations. The depression in respiration rates observed with pyruvate-malate was prevented by carnitine (Figure 7). With glutamate-malate (Figure 8), this effect of carnitine was evident only with IFM. Furthermore, this population demonstrated a significant, carnitine-reversible, inhibition in RCI when glutamate-malate was used as substrate (Figure 8).

After 30 min reperfusion, both mitochondrial populations demonstrated significantly depressed uncoupled oxygen uptake (Figure 9, top) which was partially prevented by carnitine. However, only with IFM did carnitine reverse this effect to values not significantly different from control. Atractyloside-sensitive ADP uptake (Figure 9, c,d) was significantly depressed in both populations of mitochondria isolated from hearts after 30 min reperfusion. Carnitine partially prevented this depression although values remained significantly reduced from control.

Discussion

In this study we have shown that 100 μM D,L-carnitine modestly protects the ischaemic and reperfused isolated rat heart. With respect to function, this protective influence was manifested by improved post-ischaemic ventricular contractile recovery and reduced resting tension although the latter was not statistically significant. Furthermore, carnitine

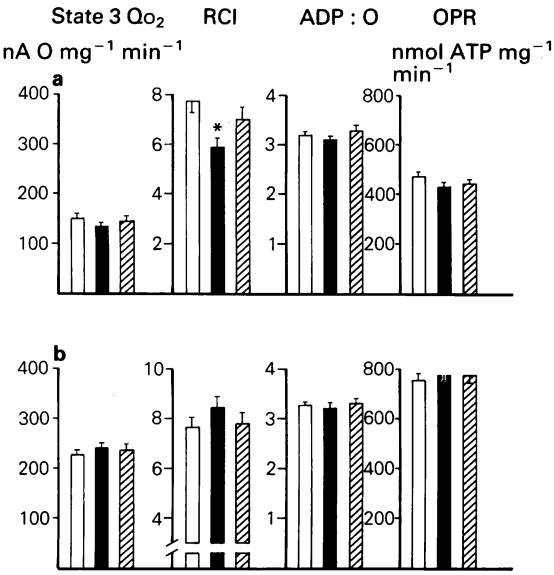

Figure 5 State 3 respiration (Qo_2), respiratory control index (RCI), ADP:oxygen ratio (ADP:O) and oxidative phosphorylation rate (OPR) with pyruvate-malate as substrates for subsarcolemmal (a) and interfibrillar (b) mitochondria isolated from non-ischaemic hearts (open columns), or from hearts subjected to 30 min ischaemia and 10 min reperfusion in the absence (solid columns) or presence (hatched columns) of carnitine. Each column represents the mean with vertical bars indicating the s.e.mean of $n = 10$. * $P < 0.05$ compared with non-ischaemic values.

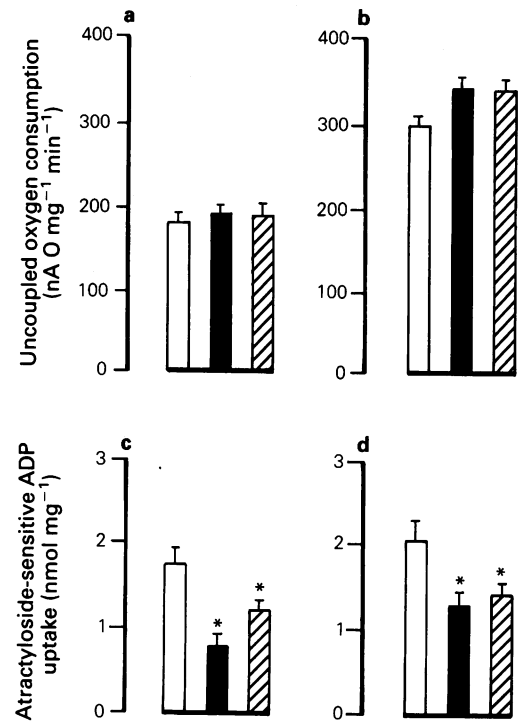

Figure 6 Uncoupled oxygen consumption (a and b) and adenine nucleotide translocase activity (c and d) for subsarcolemmal (a and c) and interfibrillar (b and d) mitochondria isolated from non-ischaemic hearts (open columns), or from hearts subjected to 30 min ischaemia and 10 min reperfusion in the absence (solid columns) or presence (hatched columns) of carnitine. Studies with uncoupled mitochondria were carried out in the presence of 2,4-dinitrophenol and glutamate-malate as substrates. Each column represents the mean with vertical bars indicating the s.e.mean of $n = 10$. * $P < 0.05$ compared with non-ischaemic values.

reduced the incidence of ventricular arrhythmias during both low-flow and reperfusion conditions. As discussed in detail below, mitochondrial function was also improved by carnitine treatment.

The effects of carnitine on the ischaemic heart are controversial. A number of studies have reported a protective influence of carnitine or its analogues on the hypoxic or ischaemic isolated heart (Fanelli, 1978; Liedtke & Nellis; 1979, Liedtke *et al.*, 1981; Paulson *et al.*, 1984; 1986). Others however, failed to demonstrate any ameliorating effect of carnitine with respect to contractile function, metabolism or arrhythmogenesis in the ischaemic heart (Neely *et al.*, 1979; Paulson & Shug, 1982; Fazekas *et al.*, 1986). A partial explanation for the discrepant results may be related to concentration of carnitine employed. For instance, *in vitro* studies showing a lack of protective effect of carnitine have employed concentrations in the millimolar range (Neely *et al.*, 1979; Paulson & Shug, 1982). Indeed, it has been demonstrated that millimolar concentrations of carnitine actually have a toxic influence and reduce ventricular recovery after reperfusion of the ischaemic heart (Hearse *et al.*, 1980). In initial experiments not reported in this study we found that carnitine concentrations of 200 μM or greater produced a marked elevation in coronary perfusion pressure indicative of coronary constriction and failed to improve contractile recovery or reduce the incidence of arrhytmias, thus confirming a critical concentration-dependency of this agent. The concentration used in the present study represents the lowest level devoid of coronary effects. The maximum protection was seen with carnitine present throughout the perfusion protocol, as reported here. A possible explanation for this is that adequate intracellular concentrations of carnitine

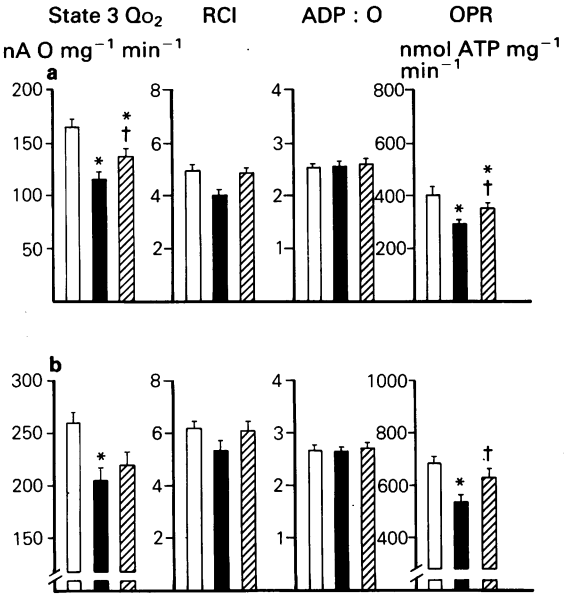

Figure 7 State 3 respiration (Qo_2), respiratory control index (RCI), ADP:oxygen ratio (ADP:O) and oxidative phosphorylation rate (OPR) with pyruvate-malate as substrates for subsarcolemmal (a) and interfibrillar (b) mitochondria isolated from non-ischaemic hearts (open columns), or from hearts subjected to 30 min ischaemia and 30 min reperfusion in the absence (solid columns) or presence (hatched columns) of carnitine. Each column represents the mean with vertcal bars indicating the s.e.mean of $n = 10$. * $P < 0.05$ compared with non-ischaemic values.

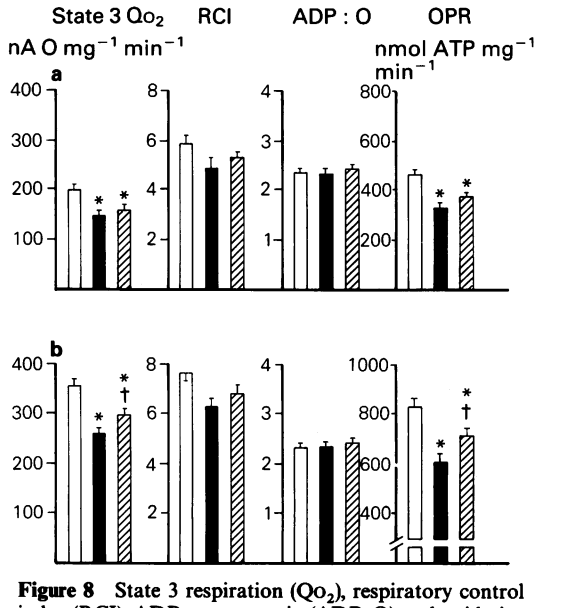

Figure 8 State 3 respiration (Qo_2), respiratory control index (RCI), ADP:oxygen ratio (ADP:O) and oxidative phosphorylation rate (OPR) with glutamate-malate as substrates for subsarcolemmal (a) and interfibrillar (b) mitochondria isolated from non-ischaemic hearts (open columns), or from hearts subjected to 30 min ischaemia and 30 min reperfusion in the absence (solid columns) or presence (hatched columns) of carnitine. Each column represents the mean with vertical bars indicating the s.e.mean of $n = 10$. * $P < 0.05$ compared with non-ischaemic values.

must be attained before a protective influence is observed. This may be particularly relevant in the present study where relatively low concentrations of the agent were used; normal physiological plasma levels of 44 μM (Neely *et al.*, 1982) have been reported. Thus the present study shows a physiologically relevant protective influence of carnitine.

The detailed assessment of mitochondrial function was based firstly on the well known importance of carnitine transport to cardiac mitochondrial fatty acid oxidation (Opie, 1979). A complex, and as yet unresolved issue is whether mitochondrial dysfunction participates in cardiac injury associated with ischaemia or reperfusion. Although mitochondrial injury in the ischaemic and reperfused myocardium has been reported, others have demonstrated either normal (Rouslin, 1987) or even stimulated (Pelikan *et al.*, 1987) mitochondrial respiration after ischaemia. Discrepancies in terms of mitochondrial function following ischaemia may reflect isolation artifacts following whole-heart treatment or a failure to separate the relative effect of pathological insult on the two identifiable mitochondrial populations which have been shown to possess distinct biochemical properties (Palmer *et al.*, 1977). In an effort to delineate more precisely the role of mitochondrial function to the carnitine-induced protection we undertook two primary approaches. First, mitochondria were harvested at the end of three specific points of the perfusion sequence, that is at the termination of ischaemia, after early (10 min) reperfusion, and finally after 30 min reperfusion. Secondly, we assessed possible differences in the response of two different mitochondrial populations.

Ischaemia without subsequent reperfusion failed to depress oxidative phosphorylation and indeed a stimulation in respiration rates was routinely observed particularly in the presence of carnitine. This stimulation may be due to compensatory mechanisms of some mitochondrial enzyme systems as previously suggested (Pelikan *et al.*, 1987). For instance dephosphorylation or a small increase in intracellular Ca^{2+} may stimulate enzymes such as pyruvate dehydrogenase (Kerbey *et al.*, 1976). Fur-

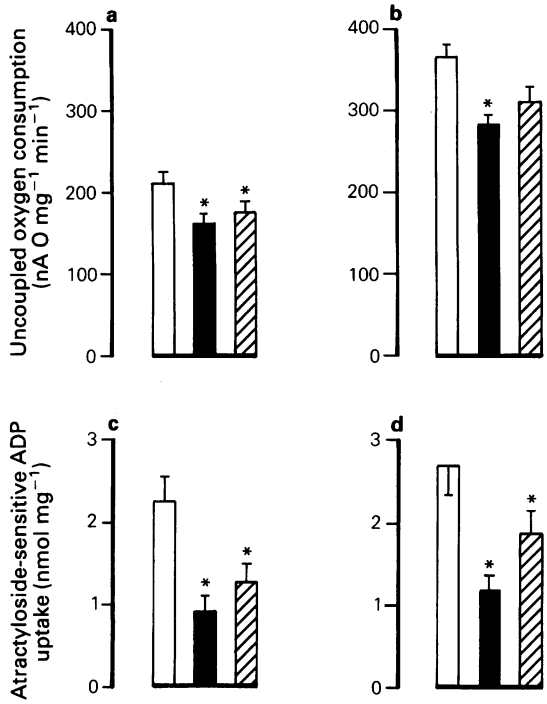

Figure 9 Uncoupled oxygen consumption (a and b) and adenine nucleotide translocase activity (c and d) for subsarcolemmal (a and c) and interfibrillar (b and d) mitochondria isolated from non-ischaemic hearts (open columns), or from hearts subjected to 30 min ischaemia and 10 min reperfusion in the absence (solid columns) or presence (hatched columns) of carnitine. Studies with uncoupled mitochondria were carried out in the presence of 2,4-dinitrophenol and glutamate-malate as substrates. Each column represents the mean with vertical bars indicating the s.e.mean of $n = 10$. * $P < 0.05$ compared with non-ischaemic values.

thermore, an increase in the creatine:creatine phosphate ratio, as would be expected under ischaemic conditions has also been shown to stimulate mitochondrial respiration (Jacobus & Diffley, 1986). Although the effect was not statistically significant a reduction in atractyloside-sensitive ADP uptake was noticeable in both mitochondrial populations isolated form ischaemic hearts which was reversed by carnitine. This observation suggests that the adenine nucleotide translocase is more sensitive to ischaemic insult than oxidative phosphorylation and that a reduction in the former is possible without any resultant depression in oxidative phosphorylation. This concept was reinforced by our observation that mitochondria isolated from hearts reperfused for 10 min demonstrated a significantly depressed ADP uptake in the absence of substantial changes in oxidative phosphorylation.

Defective function of mitochondria was evident after their isolation from hearts subjected to reperfusion. The more severe nature of mitochondrial injury after 30 min of reperfusion suggests that mitochondrial injury is not immediate as a consequence of reflow but instead progresses as reperfusion duration increases. These results also show equal sensitivity in the response of either SLM or IFM to 30 min reperfusion.

The results presented here show that the consequences of carnitine treatment on mitochondrial function post-reperfusion are extremely complex. The significance of the early depression in RCI after 10 min reperfusion and its prevention by carnitine is suggestive of a protective effect, however the failure to observe differences in contractile recovery after 10 min reperfusion questions whether this prevention was relevant to contractile recovery. Furthermore, our study shows that the marked inhibition of adenine nucleotide translocase activity after only 10 min reperfusion was not significantly affected by carnitine treatment. In contrast, the more pronounced depression in mitochondrial function after 30 min reperfusion was substantially affected by carnitine, primarily with respect to oxidative phosphorylation. Thus depression in OPR was in most cases significantly prevented by carnitine, suggesting that improved ATP repletion may play a part in the enhanced recovery. Although carnitine has been shown to preserve ATP content during myocardial ischaemia *per se*, following coronary artery ligation in dogs (Folts *et al.*, 1978) improved repletion of high energy phosphates has not been demonstrated. Indeed, it has been shown that carnitine can improve postischaemic left ventricular recovery in dogs through a mechanism independent of tissue ATP content (Silverman *et al.*, 1985). Thus it is evident that the precise role of adenine nucleotides in mediating the effects of carnitine on the ischaemic and reperfused heart requires further investigation.

In summary, our study shows that carnitine can offer modest improvement in ventricular recovery after reperfusion of ischaemic isolated rat hearts through a mechanism possibly mediated by improved mitochondrial function. This study also shows that carnitine can protect the isolated heart, in contrast to previous suggestions that the protective effects could not be observed in this model due to poor washout of the drug during ischaemia (Silverman *et al.*, 1985).

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Muscarinic receptors coupled to phosphoinositide hydrolysis and elevated cytosolic calcium in a human neuroblastoma cell line SK-N-SH

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1 The effects of the muscarinic agonist carbachol on phosphoinositide metabolism and its relationship to alteration of intracellular calcium were examined in SK-N-SH human neuroblastoma cells. Muscarinic receptors on these cells are coupled to phospholipase C and the myo [2-^{3}H]-inositol phosphates resulting from receptor activation of cells labelled with [^{3}H]-inositol accumulate rapidly. The breakdown of both inositol monophosphate ($InsP_1$) and inositol bisphosphate ($InsP_2$) is sensitive to lithium with inhibition of the latter only observed at higher concentrations of this ion.

2 Use of the calcium indicator dye Fura 2 revealed that carbachol stimulates a biphasic increase in intracellular calcium.

3 Carbachol was able to stimulate both [^{3}H]-inositol phosphate production and intracellular calcium levels with respective EC_{50} values of $15.9 \pm 1.0\,\mu M$ and $10.7 \pm 3.2\,\mu M$, indicating that no amplification occurs between these steps in the signal transduction pathway.

4 Inositol 1,4,5 trisphosphate ($Ins(1,4,5)P_3$) released $^{45}Ca^{2+}$ in a stereospecific and dose-related manner from intracellular stores of permeabilised cells.

5 These results suggest that this cell line may represent a useful model system to investigate receptor-mediated phosphoinositide metabolism and calcium homeostasis.

Introduction

Although it is now well established that several neurotransmitters stimulate phosphoinositide metabolism in the central nervous system, the recently discovered complexities of this metabolism (Nahorski, 1988; Irvine *et al.*, 1988) and calcium homeostasis cannot be easily delineated in cerebral preparations due to the cellular heterogeneity of the tissue. Indeed, there is still little evidence that inositol (1,4,5)-trisphosphate ($Ins(1,4,5)P_3$) can induce release of intracellular calcium in CNS neurones. To simplify interpretation of neurochemical data in cerebral tissue, several groups have recently used clonal neuroblastoma cell lines as a model homogeneous population of cells, as they express many characteristics of neuronal cells. The more commonly used cell lines are N1E-115 mouse neuroblastoma, which express a relatively low density of muscarinic (probably M_1) receptors linked to phosphoinositide metabolism (Fisher & Snider, 1987), and NG108-15 neurobastoma × glioma, which possess muscarinic receptors that inhibit adenylate cyclase (Harden *et al.*, 1988). However, preliminary investigations revealed that these cells displayed little or no production of inositol phosphates in response to muscarinic agonists (Baird & Nahorski, unpublished observations). In contrast the human cell line SK-N-SH, which was originally derived from bone marrow metasteses of a four year old girl (Biedler *et al.*, 1973), expresses a relatively high density of muscarinic receptors linked to phosphoinositide metabolism (Fisher & Snider, 1987; Fisher & Heacock, 1988). We have recently confirmed the relatively high density of M_3-muscarinic receptors on SK-N-SH cells by use of the radioligand [^{3}H]-N-methylscopolamine ([^{3}H]-NMS) (Lambert *et al.*, 1989) and here examine the linkage to phosphoinositide metabolism and calcium metabolism in this cell line. In previous measurements of phosphoinositide metabolism with these cells (e.g. Yu & Sadée, 1986; Fisher & Snider, 1987) only long periods of incubation with muscarinic agonists have been investigated and lithium has always been

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present. The use of lithium to inhibit dephosphorylation of inositol phosphates, and hence act as a means of amplification, has been widely used in many cells but may perturb the relative rates of phosphorylation/dephosphorylation of Ins(1,4,5)P_3 (Batty & Nahorski, 1987). A more accurate estimation of the initial rates of accumulation of the separate [^{3}H]-inositol phosphates is best attempted in its absence. In the present study, we provide evidence that these cells accumulate separate [^{3}H]-inositol phosphates in response to the muscarinic agonist carbachol. Carbachol was also able to elicit rapid increases in intracellular calcium, with similar EC_{50} values to its ability to stimulate [^{3}H]-inositol phosphate production, and Ins(1,4,5)P_3 could be demonstrated to release calcium from intracellular stores in permeabilised SK-N-SH cells.

Methods

[^3H]-*inositol phosphate determination*

SK-N-SH cells (obtained from the American Tissue Culture Collection, U.S.A.) were grown in Minimal Essential Medium supplemented with 10% foetal calf serum, 100 iu ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, 2 mM glutamine and 2.5 μg ml^{-1} Fungizone. Cultures were grown in 175 cm^2 tissue culture flasks (Gibco, U.K.) containing 50 ml of supplemented medium and maintained at 37°C in 5% CO_2/humidified air. Stock cultures were passaged every seven days (split ratio 1:3) and fed twice weekly. Experimental work was performed with cells of passages 50–70. Cells were seeded into 6-well plates at a density of 0.5 million cells per well. After 18–24 h myo-[2-^{3}H]-inositol (14.1 Ci $mmol^{-1}$; New England Nuclear), which had been cleaned by passing it through a small column of Dowex resin in the chloride form, was added to the monolayers of cells in Earles balanced salt solution (1.25 ml) containing amino acids. [^{3}H]-inositol was present at a final concentration of 1 μCi ml^{-1}. After at least a 24 h labelling period, the cells were washed with Krebs-Ringer Bicarbonate solution (KRB). Carbachol (1 mM) was added for the desired incubation period in KRB (1 ml). After termination of the reaction with cold perchloric acid (10% v/v), the monolayers of cells were removed with a rubber policeman. Following centrifugation the [^{3}H]-inositol phosphates were neutralised with Freon/octylamine (Downes *et al.*, 1986) and separated by Dowex AG-X resin in the Formate form (200–400 mesh) as described previously (Baird & Nahorski, 1986) and counted for radioactivity. When levels of $InsP_5$/$InsP_6$ were estimated these were eluted with 2 M ammonium formate/0.1 M formic acid, whilst inositol tetrakisphosphate ($InsP_4$) was eluted with 1 M ammonium formate/0.1 M formic acid. Where total [^{3}H]-inositol phosphates were measured the incubations were terminated by use of cold methanol and the chloroform was added to the suspension of cells after removal from the well, as described previously (Baird *et al.*, 1983). When present, atropine was added (at 37°C) 15 min before the addition of carbachol.

Determination of intracellular calcium concentration

Cells from near confluent flasks were scraped off and resuspended in KRB containing the calcium indicator dye Fura 2 AM (5 μM) for 45 min at 37°C. After loading, the cells were washed and resuspended in fresh KRB at room temperature. This regime reduced dye leakage. Intracellular calcium was measured in a 3 ml suspension of Fura 2-loaded SK-N-SH cells at 37°C in polypropylene cuvettes containing a magnetic stirrer bar. Fluorescence was measured in a Perkin-Elmer LS5B spectrofluorimeter. The excitation wavelengths were 340 and 380 nm with emission at 509 nm. The time taken to drive between 340 and 380 nm excitation intensities was 7.5 s. Intracellular calcium concentration was calculated from the fluorescence ratio at 340/380 nm according to Grynkiewicz *et al.* (1985). Maximum and minimum fluorescence values were determined by use of Triton X-100 (0.1%) and EGTA (3.0–4.5 mM) respectively.

Ins(1,4,5)P_3 induced calcium release

The method used was as described previously (Strupish *et al.*, 1988). Briefly, cells were permeabilised with saponin (100 μg ml^{-1}) for one minute and then suspended in a cytosol-like buffer containing oligomycin (2 μg ml^{-1}) and 2 μCi $^{45}Ca^{2+}$ ml^{-1} (free calcium concentration 200–300 nM) for 20 min, to load actively non-mitochondrial stores with $^{45}Ca^{2+}$. Aliquots (100 μl) of cell suspension were added to 100 μl of buffer containing varying concentrations of synthetic D/L-Ins(1,4,5)P_3 (kindly provided by Dr B.V.L. Potter, Department of Chemistry, Leicester University). After a one minute incubation the cells were centrifuged through silicone oil mixture (Dow-Corning 556/550,3:2, v/v). Following removal of buffer and oil the pellet was dissolved in Lumasolve (100 μl) (May & Baker, Dagenham, Essex) and then assayed for radioactivity.

Data analysis

The EC_{50} (concentration of drug producing 50% of maximal stimulation) and IC_{50} (concentration of

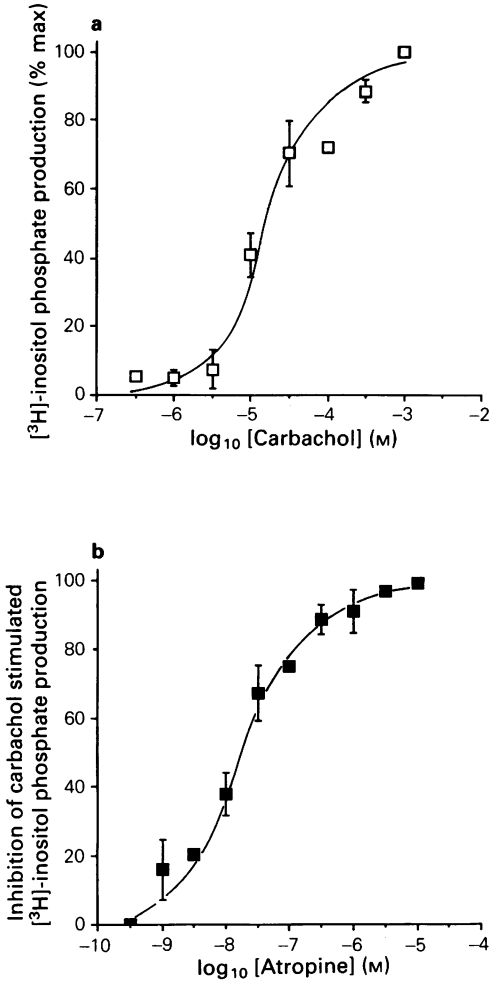

Figure 1 (a) The effects of a range of carbachol concentrations on production of [^{3}H]-inositol phosphates expressed as % of the response obtained with 1 mM carbachol (1160 ± 312 d.p.m. basal subtracted). (b) The inhibitory effect of atropine on carbachol (1 mM) stimulated [^{3}H]-inositol phosphate production (which amounted to 1539 ± 67 d.p.m., basal subtracted). In both cases the cells were stimulated for 30 min in the presence of lithium (10 mM; Na^+ concentration adjusted accordingly) and basal values of 760 ± 106 d.p.m. (a) and 859 ± 67 d.p.m. (b) were subtracted. Data are mean with vertical lines indicating s.e.mean (n = 3 in a) or range (n = 2 in b).

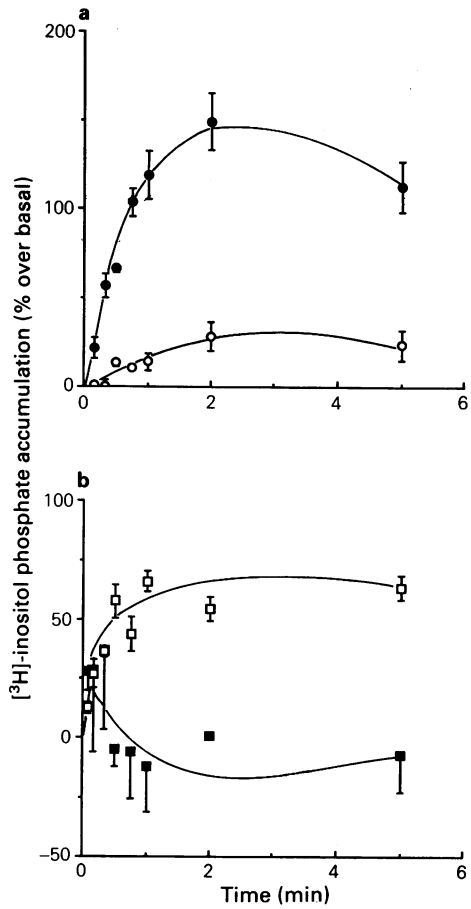

Figure 2 Time course of accumulating [^{3}H]-inositol phosphates in SK-N-SH cells in response to carbachol (1 mM). Cells were pre-incubated with myo-[2-^{3}H]-inositol and the [^{3}H]-inositol phosphates assayed as described. Data shown are the mean of at least three separate determinations performed using triplicate samples; vertical bars show s.e.mean. (Basal values were as follows: $InsP_1$ (○) 675 ± 54, $InsP_2$ (●) 174 ± 11, $InsP_3$ (□) 122 ± 8, $InsP_4$ (■) 142 ± 14 d.p.m.)

drug producing 50% inhibition) values were obtained by computer-assisted curve fitting by use of ALLFIT (De Lean *et al.*, 1978). K_i values for atropine were calculated according to Cheng & Prussoff (1973). Statistical significance was assessed by Student's t tests and differences were considered significant when $P < 0.05$.

Results

The effects of muscarinic receptor activation on [^{3}H]-inositol phosphate production

Carbachol stimulated the production of total [^{3}H]-inositol phosphates in a concentration-dependent

manner, maximum stimulation occurred in response to 1 mM carbachol (Figure 1a). The EC_{50} value for carbachol-stimulated [^{3}H]-inositol phosphate production was $15.9 \pm 1.0\,\mu$M (mean ± s.e.mean, $n = 3$). The muscarinic receptor antagonist atropine caused a dose-dependent inhibition of 1 mM carbachol stimulated total [^{3}H]-inositol phosphate production (Figure 1b), with a K_i of 0.27 ± 0.06 nM (mean ± range, $n = 2$). In other experiments the production of the separate inositol phosphates was examined. The data in Figure 2 show that following the addition of carbachol, [^{3}H]-inositol trisphosphate ([^{3}H]-$InsP_3$) increased to 57% after 30 s ($P < 0.05$) and remained stable for the following 5 min ($P < 0.05$). [^{3}H]-inositol bisphosphate ([^{3}H]-$InsP_2$) was maximal after 1 min increasing 149% ($P < 0.05$) and then decreasing slowly. [^{3}H]-inositol monophosphate ([^{3}H]-$InsP_1$) increased slowly reaching 42% over basal after 5 min. Levels of [^{3}H]-$InsP_4$ and higher inositol phosphates did not change significantly.

In order to compare the ability of lithium to influence the accumulation of the separate [^{3}H]-inositol phosphates, KRB containing 10 mM Li^+ (Na^+ concentration adjusted accordingly) was used. The results in Figure 3 show that carbachol increased [^{3}H]-$InsP_2$ and [^{3}H]-$InsP_1$ to 1140% and 406% after 30 min respectively ($P < 0.05$). Dose-response profiles to lithium show that the concentration of lithium needed to produce 50% of the maximal accumulation of [^{3}H]-$InsP_1$ was 0.60 ± 0.12 mM whilst that of [^{3}H]-$InsP_2$ was not complete even at 10 mM (mean value from two experiments ± range; data not shown). [^{3}H]-$InsP_3$ values were similar to those in the absence of lithium, although a small increase in [^{3}H]-$InsP_4$ levels to 23% above basal after 10 min was observed. No significant increase in higher [^{3}H]-inositol phosphates (likely to comprise of $InsP_5$ and $InsP_6$ eluted with 2.0 M ammonium formate/0.1 M formic acid) were observed, although the basal radioactivity in this fraction increased markedly after 24 h pre-incubation with myo-[2-^{3}H]-inositol in comparison with a 2 h preincubation (data not shown).

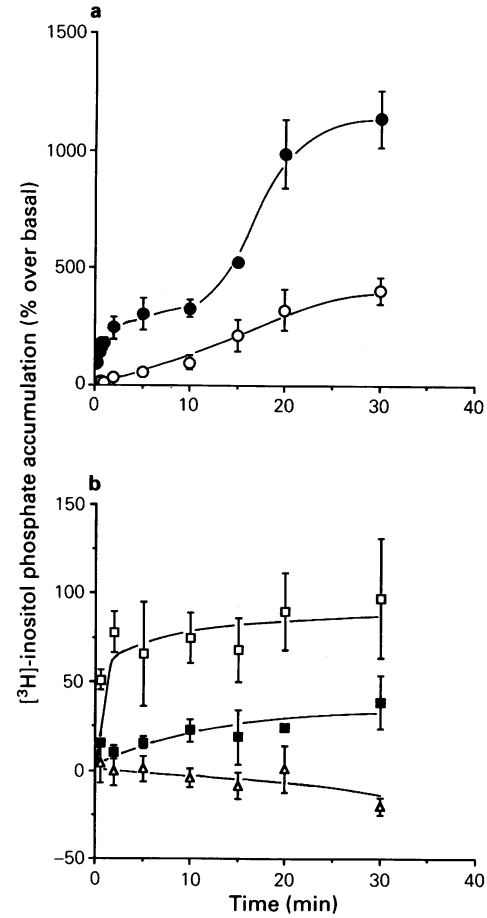

Figure 3 Time course of accumulating [^{3}H]-inositol phosphates in SK-N-SH cells in response to carbachol (1 mM) in a solution of KRB containing lithium at a concentration of 10 mM, with the Na^+ concentration adjusted accordingly. Cells were pre-incubated with myo-[2-^{3}H]-inositol and the [^{3}H]-inositol phosphates assayed as described. Data shown are the mean of at least three separate determinations performed using triplicate samples; vertical bars show s.e.mean. (Basal values were as follows: $InsP_1$ (○) 1026 ± 256, $InsP_2$(●) 208 ± 52, $InsP_3$ (□) 138 ± 17, $InsP_4$ (■) 106 ± 16, $InsP_5$ (△) 587 ± 164 d.p.m.)

Effect of carbachol on intracellular calcium

Addition of carbachol (1 mM) to suspensions of Fura 2-loaded cells caused a rapid initial spike in intracellular calcium, maximal after 15 s. This peak declined slowly to reach a new plateau phase above basal values (Figure 4a). Carbachol caused a dose-related increase in peak intracellular calcium, maximum stimulation occurring at 1 mM carbachol (Figure 4b). The EC_{50} value for carbachol-stimulated increase in intracellular calcium was $10.7 \pm 3.2\,\mu$M ($n = 4$). The actions of carbachol were shown to be atropine-sensitive with maximal inhibition occurring at 1 μM (data not shown).

Ins(1,4,5)P_3-induced calcium release from permeabilised cells

To assess in a more direct way whether Ins(1,4,5)P_3 could release calcium in these cells, the effects of D/L-Ins(1,4,5)P_3 in saponin permeabilised SK-N-SH cells loaded with $^{45}Ca^{2+}$ were also examined. D/L-Ins(1,4,

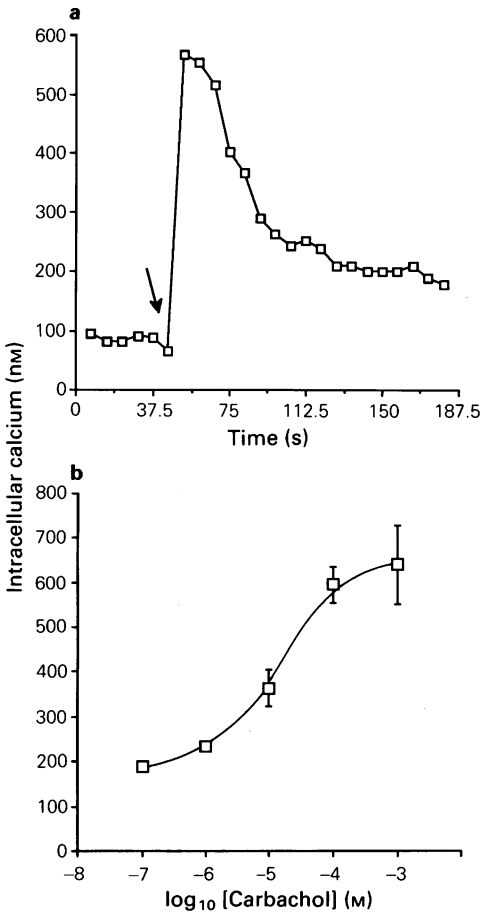

Figure 4 Upper panel (a) shows the effect of carbachol, 1 mM (indicated by the arrow) on intracellular calcium levels in Fura 2-loaded SK-N-SH cells. Fluorescence levels were measured at both 340 and 380 nm and the ratio was used to determine the concentration of intracellular calcium as described. The graph shown is typical of at least three others. Panel (b) shows the dose-response relationship of peak intracellular calcium levels to carbachol concentration. Data are the mean from 4 separate experiments; vertical bars show s.e.mean.

5)P_3 was able to release rapidly 50–60% of the $^{45}Ca^{2+}$ accumulated, with maximum and half-maximum release occurring at 10 and 1 μM, respectively. The synthetic unnatural L-isomer was ineffective at all concentrations used (Figure 5).

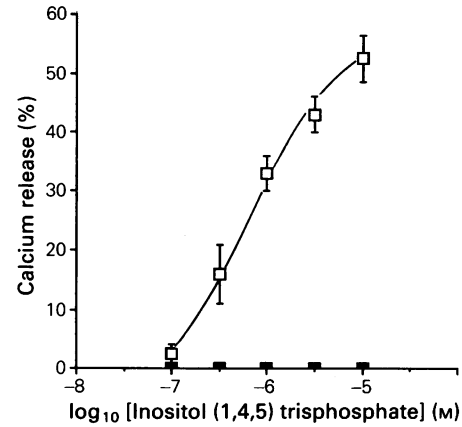

Figure 5 The effects of synthetic D/L-Ins(1,4,5)P_3 (□) and L-Ins(1,4,5)P_3 (■) added to permeabilised SK-N-SH cells loaded with $^{45}Ca^{2+}$. Data are the mean of three to six separate determinations; vertical bars show s.e.mean.

Discussion

Stimulation of muscarinic receptors on SK-N-SH neuroblastoma cells resulted in the rapid accumulation of the various [^{3}H]-inositol phosphates, which confirms and extends the previous studies of Yu & Sadée (1986), Fisher & Snider (1987) and Fisher & Heacock (1988). These cells clearly display a coupling of muscarinic receptors to phospholipase C, although in contrast to results obtained in cerebral cortical tissue (Batty & Nahorski, 1985; 1987) little $InsP_4$ appears to accumulate. It is difficult to interpret this latter data without further analysis of the proportions of the Ins(1,4,5)P_3 and inositol 1,3,4 trisphosphate (Ins(1,3,4)P_3) isomers in these experiments. For example, it remains possible that there is substantial flux through the tris-tetrakisphosphate pathway with little accumulation of [^{3}H]-InsP_4. Analysis of the isomeric composition of [^{3}H]-InsP_3 has not been performed here because of the relatively low extent of labelling of [^{3}H]-InsP_3 in these experiments. However, from our present understanding of receptor-mediated phosphoinositide metabolism it would seem certain that from the present results, at least initially, stimulated production of [^{3}H]-Ins(1,4,5)P_3 occurred. Whether this proceeds via dephosphorylation to inositol 1,4 bisphosphate (Ins(1,4)P_2) and/or phosphorylation to inositol 1,3,4,5 tetrakisphosphate (Ins(1,3,4,5)P_4) remains to be established. It is interesting to note that by use of the phenotypically stable SH-SY5Y cells derived from the parent SK-N-SH cells, mass measurements of Ins(1,4,5)P_3 have been performed and show that carbachol stimulates peak production (16 fold) after 10 s and then falls rapidly to a second steady state maintained above basal (Lambert & Nahorski, unpublished observations).

Many studies investigating phosphoinositide

metabolism have utilised the ability of lithium to inhibit the inositol phosphatases (see Berridge, 1984). The data in Figure 3 clearly show the ability of lithium to inhibit the hydrolysis of $InsP_1$ and $InsP_2$. The greater increase seen with 10 mM lithium on $InsP_2$ accumulation, over that seen with $InsP_1$, could relate to a major inhibition of $InsP_2$ breakdown and thus limit the accumulation of $InsP_1$. These data may also suggest that the major source of $InsP_1$ is from a lithium sensitive dephosphorylation of $InsP_2$. Since the dephosphorylation of Ins(1,4)P_2 to Ins(4)P_1 is the only known bis-phosphatase that is lithium-sensitive (see Nahorski, 1988), this again emphasizes the major route of Ins(1,4,5)P_3 metabolism in these cells is dephosphorylation to Ins(1,4)P_2 and probably Ins(4)P_1. The shape of the time courses for [^{3}H]-$InsP_1$ and [^{3}H]-$InsP_2$ in the presence of lithium are also of interest, in that the effects appear to be biphasic. This could relate to the uncompetitive inhibition of the phosphatases by lithium, with the degree of inhibition relating to the amount of substrate (Gee *et al.*, 1988). The lack of lithium-induced accumulation of [^{3}H]-$InsP_3$ may indicate that little Ins(1,3,4)P_3 is being formed, since at least one degradation route of this metabolite has been shown to be sensitive to lithium (Gee *et al.*, 1988). Furthermore, as Ins(1,3,4)P_3 is formed as a product of the action of the 5-phosphatase on $InsP_4$ (Batty & Nahorski, 1985), this would again emphasise that little $InsP_4$ was being produced from Ins(1,4,5)P_3 under these conditions. In view of the known calcium mobilising properties of Ins(1,4,5)P_3 and the accumulation of $InsP_3$ observed in this study, the effects of carbachol on intracellular calcium in SK-N-SH cells loaded with the calcium indicator dye Fura 2 were also evaluated. Carbachol was able to produce rapid and marked increases in levels of intracellular calcium (Figure 4), presumably due to release from an Ins(1,4,5)P_3-sensitive store. These studies have been repeated with the SH-SY5Y clone in which the time taken to change between wavelengths has been reduced to 3.8 s. Under these conditions we see at least one point on the upstroke of the calcium spike, peaking at 8.7 s (Lambert & Nahorski, 1989) and, by use of a single wavelength (340 nm), we have confirmed a similar time to reach a maximum level (unpublished observations). It is interesting to note that the production of total inositol phosphates (Figure 1a) and the elevation of intracellular calcium (Figure 4b) displayed similar EC_{50} values for carbachol, suggesting that receptor-mediated activation of phosphoinositide hydrolysis is tightly coupled to the mobilization of intracellular calcium in these cells.

Although analysis of [^{3}H]-Ins(1,4,5)P_3 was not undertaken in this study, it would seem probable in view of the results in permeabilised cells (Figure 5) that Ins(1,4,5)P_3 produced, as a consequence of muscarinic receptor activation, releases calcium from intracellular stores. These results are in good agreement with previous work from this laboratory with GH_3 anterior pituitary cells and 3T3 fibroblasts, where we have shown (Strupish *et al.*, 1988) that the mobilization of calcium from intracellular pools is stereospecific, with the D-isomer of Ins(1,4,5)-P_3 being active whilst the L-isomer and Ins(1,3,4)P_3 were inactive. Indeed D-Ins(1,4,5)P_3 had 2000 fold greater affinity than L-$InsP_3$ to receptor sites in cerebellum (Willcocks *et al.*, 1987). Thus, SK-N-SH cells display ATP-dependent high affinity uptake of $^{45}Ca^{2+}$ into non-mitochondrial stores which can be released by D-Ins(1,4,5)P_3.

In conclusion, we have established that the human neuroblastoma SK-N-SH cell line possesses muscarinic receptors coupled to phosphoinositide metabolism. The resulting inositol phosphates are associated with an increase in intracellular calcium. Addition of Ins(1,4,5)P_3 to permeabilised cells can release stored calcium, strongly suggesting that this is a physiological effect of the muscarinic receptor-stimulated formation of inositol phosphates and specifically Ins(1,4,5)P_3. Thus, SK-N-SH cells appear to provide a useful model neuronal system with which to define the regulation of muscarinic cholinergic post receptor events. However, this cell line has been shown to be phenotypically unstable, the population being plastic between both neuronal and epithelial cells types (Ross *et al.*, 1983). Recent work from this laboratory shows exclusive expression of muscarinic receptors on the more phenotypically stable neuronal subclone SH-SY5Y (Lambert *et al.*, 1989) and work currently in progress with this subclone may allow a further evaluation of the relationship between receptor occupancy, phosphoinositide metabolism and calcium homeostasis.

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Characterization of the prostanoid receptor profile of enprostil and isomers in smooth muscle and platelets *in vitro*

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1 Enprostil is composed, in approximately equal proportions, of 4 allenic isomers which are prostanoids structurally related to prostaglandin E_2 (PGE_2). The isomers are denoted as RS-86505-007, RS-86812-007 which are in the 'natural' **R** and **S** configuration (with respect to PGE_2) and RS-86505-008 and RS-86812-008 which are in the 'unnatural' **R** and **S** configuration. In the present study we have characterized their activity at prostanoid receptors, *in vitro*.

2 Enprostil acted as a highly potent ($-\log EC_{50} = 8.30 \pm 0.08$; mean ± s.e.mean, $n = 6$) EP_3 receptor agonist in the guinea-pig vas deferens, although no activity was observed at guinea-pig tracheal EP_2 receptors at concentrations up to and including 10 μM. Attempts to study the action of enprostil at EP_1 receptors were complicated by a general increase in the spontaneous activity of the guinea-pig isolated ileum. This response was stereospecific (i.e. observed, with the 'natural' **R** and **S** isomers only) and was not mediated through EP_1, FP or TP receptors.

3 Enprostil also exhibited a potent agonist effect at FP and TP receptors in the rat colon and guinea-pig aorta ($-\log EC_{50}$ values = 7.34 ± 0.11 and 6.54 ± 0.07, mean ± s.e.mean, $n = 4$–8 respectively). No activity at concentrations up to and including 10 μM was observed at DP or IP receptors in the guinea-pig platelet mediating inhibition of ADP-induced aggregation.

4 A similar profile was observed with the 'natural' **R** and **S** allenic isomers of enprostil (RS-86505-007 and RS-86812-007, respectively); RS-86505-007 was between 4 and 10 fold more potent than the racemic enprostil. The 'unnatural' allenic **R** and **S** isomers of enprostil were much less potent than enprostil, with the latter being virtually inactive.

5 Enprostil and the 'natural' **R** and **S** isomers, therefore, were EP_3, FP and TP agonists, being most potent at the EP_3 receptor. The preferred configurations for these receptors appears to be the **R**, and to a lesser extent the **S**, form of the natural allenic isomer. The effect of enprostil at EP_1 receptors was not characterized in view of the presence of excitatory EP_3 receptors in the guinea-pig ileum. These data were in accordance with the pharmacological activity of enprostil, including inhibition of gastric acid secretion (possibly EP_3) and diaorrhea (possibly TP).

Introduction

A number of synthetic prostaglandins are in clinical development with regard to their use in the treatment of peptic ulcer disease (see Bauer *et al.*, 1987 for review). Enprostil (Gardrin: methyl (±)-7-[(1**R***, 2**R***, 3**R***)-3-hydroxy-2-[(E)-3**R***)-3-hydroxy-4-phenoxy-1-butenyl]-5-oxocyclopentyl]-4,5,heptadienoate) is a racemic mixture of 4 isomers structurally related to prostaglandin E_2 (PGE_2) (Carpio *et al.*, 1987). It has been shown to elicit potent inhibitory effects on gastric acid secretion, both *in vitro* (Soll *et al.*, 1986) and *in vivo* (Roszkowski *et al.*, 1986), and possess efficacy in the treatment of ulcerative disorders of the gastrointestinal tract (McColl *et al.*, 1986; Thomas *et al.*, 1986; Lauritsen *et al.*, 1987). The exact mechanism by which these effects are mediated remains to be elucidated although, due to its structural similarity to PGE_2, an interaction at a PGE-sensitive receptor has been inferred (Roszkowski *et al.*, 1986).

In support of this hypothesis, enprostil has been previously shown to displace [^{3}H]-PGE_2 specific binding from sites present on membranes from isolated parietal cells, which suggests that it may possess affinity for PGE-sensitive receptors (Woo *et*

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al., 1986). However, whilst a binding site is present which exhibits saturable, stereoselective, high affinity binding for the E series of prostaglandins (Soll *et al.*, 1986), the prostanoid receptors present on the parietal cell have yet to be classified on a functional basis. Competition radioligand binding studies (see Robertson, 1986 for review) with prostaglandins may be ambiguous with regard to degree of specific binding to physiologically relevant receptors (Coleman *et al.*, 1985). Beinborn *et al.* (1985) have shown that prostanoid binding to mucosal cell membranes can be simulated by experimental artifacts.

An alternative approach is to use functional tissue responses to study prostanoid receptors. These have been classified (Kennedy *et al.*, 1982; Coleman *et al.*, 1985; Coleman, 1987) according to the scheme summarised in Table 1. The appropriate synthetic agonists and, where available, synthetic antagonists are also shown. The scheme has proved to be of use in our laboratory in the profiling of a number of synthetic prostanoids including misoprostol, fenprostalene, and RS-61756-007 (Eglen & Whiting, 1988; 1989). The latter two compounds are structurally similar to enprostil.

Enprostil consists of four epimers (which in this study are denoted as RS-86505-007, RS-86812-007, RS-86505-008 and RS-86812-008; Figure 1); two of which are in the 'natural' configuration and two are in the 'unnatural' configuration with respect to the structure of PGE_2. The aim of the present study was to characterize the profile of enprostil, and its constituent epimers at the prostanoid receptor subtypes as defined in Table 1. In this respect, Coleman *et al.* (1988) have obtained preliminary data showing that enprostil acted as a potent EP_1, EP_3, FP and TP agonist.

An abstract of this work has been previously presented to the British Pharmacology Society meeting, London, December, 1988 (Eglen *et al.*, 1989).

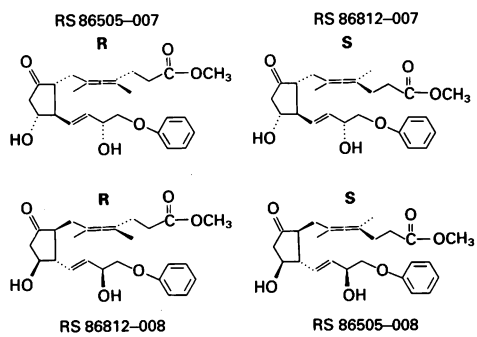

Figure 1 Structures of the 4 isomers of enprostil. (R) and (S) denote the absolute configurations.

Table 1 Classification of prostanoid receptors (after Kennedy *et al.*, 1982; Coleman *et al.*, 1985)

Receptor	*Natural prostaglandin*	*Synthetic agonist*	*Synthetic antagonist*
DP	PGD_2	BW 245C	BW A868C
EP[a]	PGE_1/PGE_2	Sulprostone	AH 6809
FP	$PGF_{2\alpha}$	Fluprostenol	NA
IP	PGI_2	Iloprost	NA
TP	TxA_2	U46619	SQ 29,548

[a] EP receptors presently form 3 subtypes, which are denoted as EP_1, EP_2 and EP_3 (Coleman *et al.*, 1987). These are characterized as shown below.

Receptor	*Natural prostaglandin*	*Synthetic agonist*	*Synthetic antagonist*
EP_1	PGE_1/PGE_2	Sulprostone	AH 6809
EP_2	PGE_1/PGE_2	Butaprost	NA
EP_3	PGE_1/PGE_2	Sulprostone	NA

NA – not available.

Methods

Smooth muscle preparations

All experiments were conducted on smooth muscles isolated from guinea-pigs (male Dunkin-Hartley, 300–350 g) or rats (male, Sprague-Dawley, 200–250 g) previously killed by CO_2 asphyxiation. Platelet-rich plasma was prepared from freshly obtained blood (withdrawn by cardiac puncture) from guinea-pigs previously anaesthetised with sodium pentobarbitone (60 mg kg^{-1}, i.p.). The preparations used to study each prostanoid receptor are summarised in Table 2 and detailed methodology has been described recently (Coleman, 1987; Eglen & Whiting, 1988). The tissues were transferred to warm (37°C) physiological salt solutions (pH 7.4) containing indomethacin (1 μM), for further preparation. Guinea-pig proximal ileum and rat ascending colon (Eglen & Whiting, 1988) were gently flushed intraluminally and suspended under 1.0 g tension in Tyrode physiological salt solution. Guinea-pig trachea, previously cut into zig-zag strips (Emmerson & MacKay, 1971), and rings of guinea-pig thoracic aorta were suspended under 1.0 g tension in Krebs physiological salt solution. Guinea-pig vas deferens were prepared according to the method of Hedquist & von Euler (1972) and suspended between parallel platinum electrodes in Krebs physiological salt solution, under 0.5 g tension.

All smooth muscles were allowed 60 min to equilibrate, during which time the bathing fluid was replaced every 15 min. After this period, KCl (50 mM) was added to all tissues, with the exception of the

Table 2 Prostanoid receptors and bioassays employed in the present study

Receptor	*Bioassay*
DP	Guinea-pig platelet
EP_1	Guinea-pig ileum
EP_2	Guinea-pig trachea
EP_3	Guinea-pig vas deferens
FP	Rat colon
IP	Guinea-pig platelet
TP	Guinea-pig aorta

See also Kennedy *et al.*, 1982; Coleman, 1987; Eglen & Whiting, 1988 for references and reviews

vas deferens, for a 5 min period in order to obtain an estimate of the viability of the tissues. The tissues were then washed and allowed to return to baseline tension levels. Phenoxybenzamine (1 μM) was then added to all tissues with the exception of the trachea and vas deferens for a period of 15 min. The tissues were then washed twice over the following 10 min period. Atropine (1 μM) was then added to all preparations, with the exception of the trachea and vas deferens, for the duration of the experiment. Contractile responses were determined in the guinea pig ileum (EP_1), rat colon (FP), and guinea-pig aorta (TP). Previous studies (Eglen & Whiting, 1988) have shown that the guinea-pig trachea, and rat colon also possess EP_1 and TP receptors through which contractile responses are mediated. Consequently, the EP_1 and TP antagonists, AH 6809 (3 μM) and SQ 29,548 (1 μM) respectively, were routinely added in studies with these tissues in order to exclude activity at these receptors. The pA_2 values for AH 6809 and SQ 29,548 at EP_1 and TP receptors have been shown (Eglen & Whiting, 1988) to be 7.4 and 8.3, respectively.

In studies with the trachea, relaxations due to EP_2 receptor stimulation were determined in tissues precontracted with carbachol (10 μM). Agonists were added once a sustained contracture was attained. EP_3 receptor function was studied by determining the inhibition of the height of the 'twitch' contractions of the vas deferens, which were elicited by field stimulation. A period of 15 min was allowed in order to attain reproducible contractions to the stimulation. The stimulation-parameters were trains of pulses at 2 min intervals (supramaximal voltage, 5 Hz, 1 ms duration).

In some studies, the effects of the antagonists AH 6809 or SQ 29,548 were measured to determine the role of EP_1 or TP receptors, respectively. The equilibration period with these antagonists was 60 min. Only one concentration of antagonist was applied to each tissue, and control studies, undertaken in parallel, were used to correct for potential changes in sensitivity of the preparations.

Platelet studies

The method used was that described by Eglen & Whiting (1988). Briefly, platelet rich plasma (PRP) was prepared from freshly obtained guinea-pig blood, by centrifugation at 200 *g* for 10 min. Platelet poor plasma (PPP) was prepared by centrifuging at 900 *g* for 2 min. The TP antagonist, SQ 29,548 (1 μM) was added routinely to the PRP to exclude any proaggregant effects of agonists due to TP receptor stimulation (Eglen & Whiting, 1988). Adenosine 5'-diphosphate (ADP, 2 μM) was then added to elicit reversible platelet aggregation. Agonists were added 1 min before the ADP in order to determine inhibitory effects on the subsequent aggregation response. Previous studies (Hamid-Bloomfield & Whittle, 1986; Eglen & Whiting, 1988) have shown that both IP and DP receptors mediate inhibition of ADP-induced aggregation in guinea-pig platelets. IP receptors were distinguished from DP receptors in this preparation, by using 10 μM AH 6809. At this concentration, AH 6809 acts as a competitive antagonist of DP receptors, but not IP receptors, and exhibits a pK_B value of 5.7 (Eglen & Whiting, 1988).

Measurement and analysis of results

Smooth muscles All agonists were added on a cumulative basis, using incremental concentrations spaced at 0.5 log intervals. Successive concentrations were added once a sustained response was obtained in response to the previous agonist concentration. The exceptions to this protocol were the ileal studies, in which non-cumulative concentration-response curves were constructed in order to prevent desensitization. Only one agonist was applied to each tissue, the responses being normalized against the maximum response elicted by a full prostanoid agonist i.e. EP_1 – 16,16 dimethyl PGE_2, EP_2 – PGE_2, EP_3 – sulprostone, FP – $PGF_{2\alpha}$, TP – U46619.

All tissue responses were determined as changes in isometric tension (mg), recorded by either a Hugo Sachs K30 or Grass FT03 force transducer and displayed on either a Graphtec-Watanabe or Beckman Dynograph R311 recorder, respectively. The responses were calculated as percentage of the maximal response and the potencies were derived by calculation of the EC_{50}, by means of non-linear iterative curve fitting procedures (Parker & Waud, 1971). In the antagonist studies, the dissociation constants were estimated from a single concentration of

antagonist by use of the relationship: $-K_B$ = [antagonist]/concentration-ratio − 1 (Furchgott, 1972).

Platelet studies Agonists were added 1 min before the ADP and the extent of platelet aggregation was determined by measuring changes in light transmission (measured in arbitrary units) with a Chrono-log Whole-Blood aggregometer and recorded by a flat bed recorder. The responses were calculated as the percentage inhibition of the aggregation response to ADP, and the EC_{50} value was then calculated as described above.

Physiological salt solutions

The composition (in mM) of the physiological salt solutions used were as follows: Krebs solution: NaCl 118.4, KCl 4.9, $MgSO_4 \cdot 7H_2O$ 1.2, KH_2PO_4 1.2, glucose 11.1, $NaHCO_3$ 25.0 and $CaCl_2 \cdot 6H_2O$ 1.1.

Tyrode solution: NaCl 136.9, KCl 2.7, $MgCl_2 \cdot 6H_2O$ 1.1, $NaH_2PO_4 \cdot 2H_2O$ 0.4, glucose 5.6, $NaHCO_3$ 11.9 and $CaCl_2 \cdot 6H_2O$ 1.8.

All solutions were gassed with 5% CO_2/95% O_2 and indomethacin (1 μM) was present throughout the experiments.

Drugs

The following drugs were used: PGD_2, PGE_1, PGE_2, 16,16 dimethyl PGE_2, PGI_2, U46619 (9,11-methanoepoxy PGH_2), $PGF_{2\alpha}$ (Cayman Chemical Co.), BW 245C (5-(6-carboxyhexyl)-1-(3-cyclohexyl-3-hydroxypropyl) hydantoin; Burroughs Wellcome), sulprostone, enprostil (methyl (±)-7-[(1**R***, 2**R***, 3**R***)-3-hydroxy-2-[(E)-3**R***)-3-hydroxy-4-phenoxy-1-butenyl]-5-oxocyclopentyl]-4,5, heptienoate), and the isomers, RS-86505-007, RS-86505-008, RS-86812-007 and RS-86812-008 (IOC, Syntex Research, Palo Alto); AH 6809 (6-isopropoxy-9-oxoanthene-2-carboxylic acid, Glaxo); SQ 29,548 (15-[1α2β(5Z),3β, Hα]-7-[3-[2-(phenylamino)-carbonyl]hydrazino]-methyl]-7-oxobicyclo[2.2.1]-hept-2-yl]-5-hept-enoic acid, Squibb); carbachol, indomethacin, atropine (Sigma) and phenoxybenzamine (SK & F).

Stock solution of prostanoids (usually 3×10^{-2} M) were prepared in ethanol and stored at −20°C. Upon use, these solutions were diluted with H_2O. Specific details for the preparation and stability of the various compounds used in this study are described elsewhere (Coleman, 1987).

Results

In agreement with previously published data (Coleman, 1987; Eglen & Whiting, 1988; 1989) the majority of tissues responded to the prostanoid agonists by exhibiting concentration-dependent contractions. As previously shown (Eglen & Whiting, 1988; Hedquist & von Euler, 1972) the guinea-pig trachea and field stimulated vas deferens exhibited concentration-dependent relaxations and inhibition of twitch responses, respectively.

Agonists

The potencies, as denoted by the −log EC_{50} values, for both standard natural and synthetic prostanoids are shown in Table 3. These are also described below:

EP_1 receptors (guinea-pig ileum) The rank order of agonist potency was 16,16 dimethyl PGE_2 > PGE_1 > PGI_2 > PGE_2 > $PGF_{2\alpha}$ ≫ U46619 = BW 245C = 0. All these compounds, with the exception of U46619 and BW 245C, acted as full agonists. U46619 and BW 245C, at concentrations of up to 30

Table 3 Potencies (−log EC_{50}) of standard prostaglandins at receptors in various tissues

	EP_1 (*Ileum*)	EP_2 (*Trachea*)	EP_3 (*Vas deferens*)	*FP* (*Colon*)	*TP* (*Aorta*)
PGE_1	7.81 ± 0.08	6.57 ± 0.06	8.46 ± 0.11	6.23 ± 0.07	NR
PGE_2	6.84 ± 0.12	7.43 ± 0.09	7.70 ± 0.06	7.21 ± 0.12	< 5.0
DME_2	8.96 ± 0.03	5.45 ± 0.14	8.84 ± 0.03	6.93 ± 0.11	6.44 ± 0.08
$PGF_{2\alpha}$	6.21 ± 0.05	NR	6.16 ± 0.06	8.02 ± 0.09	6.20 ± 0.03
U46619	NR	NR	NR	NR	7.44 ± 0.12
Sulprostone	NR[a]	5.84 ± 0.05	9.32 ± 0.07	5.21 ± 0.11	NR
BW 245C	NR	NR	NR	NR	NR
PGI_2	7.34 ± 0.05	5.32 ± 0.08	5.52 ± 0.08	5.12 ± 0.13	NR

Values are mean −log EC_{50} ± s.e.mean, n = 4–8. DME_2 – 16,16 dimethyl PGE_2; NR – no response at the concentrations studied (1 nM–30 μM).

[a] An increase in spontaneous activity was observed which persisted after washout. The variability of this response was difficult to quantify (Figure 2), and no −log EC_{50} value was calculated.

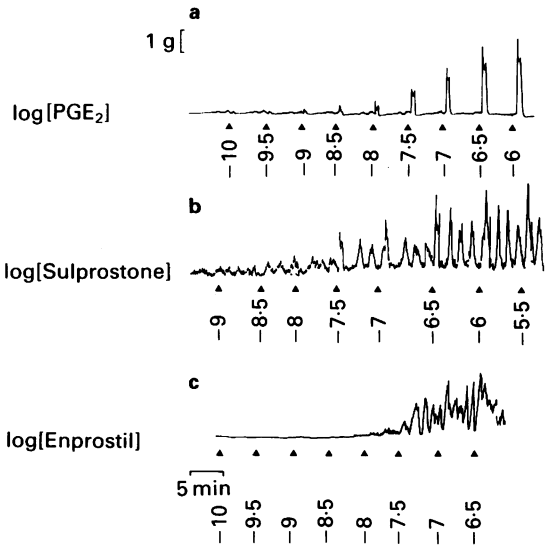

Figure 2 Responses of guinea-pig isolated ileum to prostaglandin E_2 (PGE_2) (a), sulprostone (b) and enprostil (c). Agonists were added for 30 s on a 5 min dose cycle in a non-cumulative fashion.

μM had no agonist activity. The contractile responses, with the exception of sulprostone, were qualitatively similar, in that clearly defined contractions were observed which did not persist when the bathing solution was replaced (Figure 2). However, the responses to sulprostone consisted of an increase in the spontaneous activity of the preparation and these responses did persist after the bathing solution was replaced. The magnitude of this activity appeared to be concentration-dependent, but due to the extreme variability of the responses, no accurate measurements could be performed and the $-\log EC_{50}$ values were not calculated. These responses are shown in Figure 2, together with responses to PGE_2 for comparative purposes. The responses to sulprostone were unaffected by the inclusion of either the EP_1 antagonist, AH 6809 (3 μM) or the TP antagonist, SQ 29,548 (1 μM; data not shown). No contractile responses were observed in this preparation to either U46619 or BW 245C at the concentrations studied (1 nM–30 μM).

EP_2 receptors (guinea-pig trachea) The rank order of agonist potency at receptors mediating relaxation of this preparation was $PGE_2 > PGE_1 >$ sulprostone $>$ 16,16 dimethyl $PGE_2 = PGI_2$. All compounds acted as full agonists. No relaxant response was observed in this preparation to either BW 245C, $PGF_{2\alpha}$ or U46619 at the concentrations studied (1 nM–30 μM).

EP_3 receptors (guinea-pig vas deferens) The rank order of agonist potency at receptors inhibiting the twitch response to electrical stimulation was sulprostone $>$ 16,16 dimethyl $PGE_2 > PGE_1 > PGE_2 > PGE_{2\alpha} > PGI_2$. All compounds acted as full agonists. The responses to all these agonists were unaffected by inclusion of 3 μM AH 6809 (data not shown). No inhibitory responses were observed to U46619 or BW 245C at the concentrations studied (1 nM–30 μM).

FP receptors (rat colon) The rank order of agonist potency was $PGF_{2\alpha} > PGE_2 >$ 16,16 dimethyl $PGE_2 > PGE_1 >$ sulprostone $= PGI_2$. These compounds acted as full agonists whilst U46619 and BW 245C were inactive at the concentrations used (1 nM–30 μM).

TP receptors (guinea-pig aorta) The rank order of potency observed in this preparation was U46619 $>$ 16,16 dimethyl $PGE_2 > PGF_{2\alpha} > PGE_2$. All compounds, with the exception of PGE_2, acted as full agonists. At 3.2×10^{-5} M PGE_2 elicited a contractile response which was $28 \pm 3\%$ (mean $\pm$ s.e.mean, $n = 6$) of the maximal response. PGE_1, sulprostone PGI_2 and BW 245C were inactive at the concentrations studied (1 nM–30 μM).

The rank order of potency of the above compounds are in accordance with the hypothesis that the responses observed are due to EP_1, EP_2, EP_3, FP and TP stimulation, respectively (Coleman *et al.*, 1987; Eglen & Whiting, 1988).

Enprostil and isomers

The potencies of enprostil and the 4 epimers at the various prostanoid receptors are shown in Table 4. Enprostil exhibited responses in the ileum that were clearly different in nature from those of other EP_1 agonists such as PGE_1 or PGE_2 but most closely resembled those observed to sulprostone (see above). These responses are shown in Figure 2 and in Table 4 have been denoted as 'no response' for the reasons described below. The increase in spontaneous activity was also highly variable, and persisted after washout. Some degree of concentration-dependence was observed, but no accurate quantitation could be performed and, therefore, the interaction of enprostil at ileal EP_1 receptors was difficult to quantify. The responses in the ileum were also observed with the 'natural' **R** and **S** isomers but were not observed with the 'unnatural' **R** and **S** isomers. The increase in spontaneous activity was unaffected by the inclusion of either the TP antagonist, SQ 29,548 (1 μM) or the EP_1 antagonist, AH 6809 (3 μM; data not shown).

Neither enprostil nor any isomers acted as EP_2 receptor agonists, since no relaxations of the precon-

Table 4 Potencies ($-\log EC_{50}$) of enprostil and isomers at prostanoid receptors in various tissues

	EP_1 (*Ileum*)	EP_2 (*Trachea*)	EP_3 (*Vas deferens*)	*FP* (*Colon*)	*TP* (*Aorta*)
Enprostil	NR[a]	NR	8.30 ± 0.08	7.34 ± 0.11	6.54 ± 0.07
RS-86505-007	NR[a]	NR	8.86 ± 0.03	7.41 ± 0.10	7.43 ± 0.05
RS-86812-007	NR[a]	NR	8.18 ± 0.05	7.57 ± 0.08	6.86 ± 0.12
RS-86505-008	NR	NR	6.76 ± 0.11	6.46 ± 0.03	NR
RS-86812-008	NR	NR	<6.0	NR	5.39 ± 0.14

Values are mean $-\log EC_{50}$ ± s.e.mean, $n = 4$–8. PGE_2 and sulprostone were standard agonists at the EP_3 receptor ($-\log EC_{50}$ values = 7.70 and 9.32), $PGF_{2\alpha}$ was the standard agonist at FP receptors ($-\log EC_{50} = 8.02$), whilst U46619 was the standard agonist at TP receptors ($-\log EC_{50} = 7.44$). NR – no response.

[a] Indicates no clear sustained contractile response at the concentrations studied (1 nM–10 μM), although an increase in spontaneous activity was observed.

tracted guinea-pig tracheal preparations were observed at the concentrations studied (10 nM–10 μM). However, potent inhibitory effects on the twitch response in the vas deferens were observed. The rank order of potency at EP_3 receptors in this tissue was RS-86505-007 > enprostil = RS-86812-007 > RS-86505-008 > RS-86812-008.

At FP receptors mediating contractions of the rat colon, enprostil, RS-86505-007 and RS-86812-007 were equipotent. In contrast, RS-86505-008 was approximately 10 fold less potent, whilst no response was observed to RS-86812-008 at the concentrations studied (1 nM–10 μM).

At TP receptors mediating guinea-pig aortic contractions, the order of potency was RS-86505-007 > RS-86812-007 ≥ enprostil ≫ RS-86812-008. RS-86505-008 was inactive at the concentrations studied (1 nM–10 μM). The responses to these agonists at TP receptors were sustained for 30 min after addition, and persisted after extensive washing of the tissue.

Platelet studies

PGI_2 and BW 245C acted as potent and selective IP and DP receptor agonists ($-\log EC_{50}$ values = 9.3 ± 0.08 and 8.6 ± 0.13, mean ± s.e.mean, $n = 8$, respectively). The responses to BW 245C, but not PGI_2, were shifted in a parallel fashion to the right in the presence of AH 6809 and the calculated $-\log K_B$ was 5.3. Neither enprostil nor any of the isomers studied (under the conditions of TP blockade by 1 μM SQ 29,548) inhibited ADP-induced aggregation, at the concentrations studied (1 nM–10 μM).

Discussion

Enprostil is a 4,5 allenic 16 phenoxy prostaglandin, with structural similarities to PGE_2 (Carpio *et al.*, 1987). It has been previously shown to displace [^{3}H]-PGE_2 from binding sites in isolated parietal cells (Woo *et al.*, 1986), although the activity at prostanoid receptors in functional bioassays has not been fully studied. We have undertaken such a study and also assessed the activity of each of the 4 isomers of enprostil. Preliminary data by Coleman *et al.* (1988) and Reeves *et al.* (1988) have shown that it exhibits EP_1, EP_3, FP and TP agonist activity.

The data obtained with the standard prostanoids were in good agreement with those in the literature (Kennedy *et al.*, 1982; Coleman *et al.*, 1985; 1988; Coleman, 1987; Eglen & Whiting, 1988). Sulprostone was the most potent agonist studied, at EP_3 receptors, but was less potent at both EP_1 and EP_2 receptors in agreement with previous findings (Coleman *et al.*, 1988). AH 6809 has been shown to antagonise responses to all of these agonists (with the exception of sulprostone) at ileal EP_1 receptors, but not at EP_3 receptors in the vas deferens (Coleman *et al.*, 1987; Eglen & Whiting, 1988; this study). However, definitive evidence of the three putative subtypes awaits the development of selective EP_2 and EP_3 antagonists.

In the presence of EP_1 and TP blockade, the rat colon was highly sensitive to FP receptor stimulation by $PGF_{2\alpha}$ as previously shown (Eglen & Whiting, 1988). Finally, the guinea-pig aorta has been shown to possess TP receptors (Eglen & Whiting, 1988; 1989) and in accordance with these studies, it was highly sensitive to the effects of the TP agonist, U46619 (Coleman *et al.*, 1981).

The high potency of the selective DP agonist BW 245C (Hamid & Whittle, 1985; Giles *et al.*, 1989) at receptors inhibiting guinea-pig platelet aggregation is in accordance with the presence of DP receptors in this tissue (Hamid-Bloomfield & Whittle, 1986; Eglen & Whiting, 1989). The similarity in pK_B values for AH 6809 when either PGD_2 (5.7, Eglen & Whiting, 1988) or BW 245C (5.3, this study) was used, also suggested that these agonists interacted at the same receptor. The lack of TP activity of BW

245C renders this a more useful pharmacological tool than PGD_2 with which to classify DP receptors, and the development of BW A868C (Giles *et al.*, 1989) as a potent and selective DP antagonist has allowed further characterization of this receptor subtype. The high potency of PGI_2 and lack of antagonism by AH 6809 also indicate the presence of IP receptors in this tissue (Hamid-Bloomfield & Whittle, 1986; Eglen & Whiting, 1988), although no selective IP antagonist has been identified to date.

Enprostil potently inhibited twitch responses in the field stimulated vas deferens, indicating that it possessed EP_3 agonist properties (Coleman *et al.*, 1987). Since no relaxations of the precontracted guinea-pig trachea were observed, it probably lacks EP_2 receptor activity. It was not possible to characterize definitively the activity of enprostil on EP_1 receptors with the guinea-pig ileum. Enprostil did not exhibit single uniphasic contractile responses of the guinea-pig ileum, although these characteristics were observed with EP_1 agonists such as PGE_1, PGE_2 or 16,16-dimethyl PGE_2 (Eglen & Whiting, 1988; this study). A series of spontaneous contractions were observed which persisted even after washout of enprostil. Since this activity was not observed with either U46619 or $PGF_{2\alpha}$, it is unlikely that they can be ascribed to TP or FP receptor stimulation, respectively. In addition, the lack of effect of the EP_1 and TP antagonists, AH 6809 and SQ 29,548, suggested that the response was not mediated through either EP_1 or TP receptors respectively. Coleman *et al.* (1988) have shown an interaction of enprostil with EP_1 receptors in the guinea-pig fundus. Attempts to duplicate these data in our hands proved problematic due to the additional presence of FP and TP receptors (Dong *et al.*, 1986; R.M. Eglen unpublished observations).

The lack of a response in the ileum to the 'unnatural' **R** and **S** isomers of enprostil indicated a stereospecific interaction at a receptor mediating these responses. Sulprostone, which was also a potent EP_3 receptor agonist, elicited similar responses and similar findings have been observed with two prostanoids structurally related to enprostil, RS-61756-007 (Eglen & Whiting, 1989) and fenprostalene (Eglen & Whiting, 1988). One possible explanation is that these responses may be due to the presence of excitatory EP_3 receptors in this tissue. Similar conclusions have been reached by Poll *et al.* (1988) and Lawrence & Jones (1989) with the guinea-pig ileum. In agreement with these workers, we conclude that the guinea-pig ileum is an unsuitable preparation with which to study agonists with mixed EP_1 and EP_3 receptor activity, such as sulprostone and enprostil. Enprostil, therefore, possesses activity at EP_3 receptor, but not at EP_2 receptors. Its ability to stimulate EP_1 receptors (as found by Coleman *et al.*, 1988) remains to be studied further. Enprostil also acted as an agonist at both TP and FP receptors, a finding in agreement with the work of Coleman *et al.* (1988). The compound was approximately 10 fold less potent than U46619 at TP receptors, and approximately 3 fold less potent than $PGF_{2\alpha}$ at FP receptors. Enprostil lacked activity at platelet DP and IP receptors. Enprostil, therefore, possesses the following rank order of activity at prostanoid receptors: $EP_3 > FP > TP \gg = EP_2 = DP = IP$. A structurally related prostanoid to enprostil, RS-61756-007 (Eglen & Whiting, 1989), has recently been shown to possess a highly potent and selective action at TP receptors. Fenprostalene, which is also structurally related to enprostil, also acts as a FP and TP receptor agonist (Eglen & Whiting, 1988). No data are available with regard to the EP_3 activity of fenprostalene and RS-61756-007.

A similar pattern of activity was observed with both the **R** and **S** 'natural' isomers of enprostil, in that both stimulated EP_3, FP and TP receptors, whilst lacking activity at EP_2, DP and IP receptors. No definitive conclusions could be made with regard to EP_1 receptor activity. The most potent compound evaluated in the present study was the natural **R** epimer of enprostil (RS-86505-007) which was approximately 4 and 10 fold more potent than racemic enprostil at the EP_3 and TP receptors respectively. The activity of the **R** and **S** unnatural epimers was much less than enprostil, with the **S** epimer being virtually devoid of any activity. These data clearly suggest that it is the **R** and to a lesser extent the **S** configuration of the 'natural' epimer which is preferred for stimulation of the EP_3 and TP receptor.

How does the prostanoid receptor profile of enprostil relate to its gastric antisecretory activity? The rank order of affinity of prostanoid agonist binding to porcine mucosal cell membranes has been shown (Beinborn *et al.*, 1988) to be similar to the rank order of agonist potency defined in functional assays by Coleman *et al.* (1985) for the presence of an EP receptor. Similar findings have been obtained in functional studies by Reeves & Stables (1985) who used isolated parietal cells and gastric muscosal sheets, respectively. However, further characterization of the EP receptor subtype present on the parietal cells is complicated by the existence of three putative subtypes of this receptor, denoted as EP_1, EP_2 and EP_3 (Coleman *et al.*, 1987; Table 1). Reeves *et al.* (1988) have shown that the rank order of agonist potencies at EP receptors inhibiting acid secretion in rat isolated gastric mucosa, was similar to the rank order of potency at EP_3 receptors inhibiting electrically evoked twitch contractions of the guinea-pig vas deferens. The postulate that EP_3 receptors mediate inhibition of gastric acid secretion

is also supported by data showing that a number of synthetic prostaglandins, which are potent inhibitors of acid secretion, also appear to be potent EP_3 agonists (W.L. Smith & R.M. Eglen, unpublished observations).

Some workers have proposed that TP receptors may also play a role in the inhibition of gastric acid secretion (see Reeves & Stables, 1985 for references), although part of the effect of TP agonists appears to be indirect (Reeves & Stables, 1987). However, the evidence is controversial since other studies have shown that TP agonists are either ineffective (Tao & Wilson, 1984) or, when given by close injection into the gastric artery, can cause gastric mucosal damage (Espulugues & Whittle, 1988). Endogenous thromboxane A_2 may, therefore, be a local mediator of gastric injury. U68215 is a PGI_2 analogue which has been shown to inhibit acid secretion *in vivo* (Robert *et al.*, 1985). U68215 is an IP receptor agonist (R.M. Eglen, unpublished observations) which may suggest a role for IP receptors in the inhibition of gastric acid secretion (Beinborn *et al.*, 1985). However, PGI_2 and U68215 can also act as EP receptor agonists (Dong *et al.*, 1986; R.M. Eglen, unpublished observations) and this property may mediate their actions in the stomach. Taken together, it is likely that the activity of enprostil at EP_3 receptors is responsible for its effects on gastric acid secretion (Roszkowski *et al.*, 1986), although the properties mediated through TP receptors may play a role in the diarrheogenic activity (Clayton *et al.*, 1988; Smith *et al.*, 1988).

In conclusion, the data confirm preliminary findings by Coleman *et al.* (1988) and Reeves *et al.* (1988) showing that enprostil is a potent EP_3 agonist, but additionally possesses activity at FP and TP receptors. It lacks activity at EP_2, DP and IP receptors. The 'natural' **R** isomer is more potent than the racemic enprostil, whilst both 'unnatural' isomers lack significant activity. The effect of enprostil and epimers, at EP_1 receptors is difficult to characterize, in view of the possible presence of excitatory EP_3 receptors in the ileum.

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A phorbol diester-induced enhancement of synaptic transmission in olfactory cortex

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1 Extracellular field synaptic potentials were recorded from pial surface slices of guinea-pig olfactory cortex maintained *in vitro*.

2 Phorbol 12,13-dibutyrate (0.1–10 μM) enhanced the amplitude of the evoked potential (by 51.2 $\pm$ 10.4% with 1 μM) in normal solution. When the evoked potential was partially depressed by Cd, Co, Mn or a reduced Ca concentration, phorbol 12,13-dibutyrate (1 μM) induced a much larger enhancement of the evoked potential (196.5 $\pm$ 24.4% increase). Phorbol 12,13-diacetate and mezerein had similar effects but were less potent. 4β-Phorbol (10 μM) had no effect.

3 The diacylglycerol analogues, dioctanoylglycerol (100–1000 μM), 1-oleoyl-2-acetylglycerol (100–500 μM) or diolein (100 μM) had no effect on the evoked potentials, either alone or in the presence of Cd.

4 The isoquinolinylsulphonamide inhibitor (H-7) of protein kinase C slightly enhanced the e.p.s.p. and had no effect on the potentiation produced by phorbol ester. Another protein kinase C inhibitor, acridine orange (100–1000 μM), had no effect on the action of phorbol ester.

5 These results show that transmitter release, as at other synapses, is enhanced by phorbol esters but Ca did not potentiate this action. The pharmacological profile of the effect on transmitter release differed from that of protein kinase C in cell-free preparations and therefore it is unclear whether protein kinase C was involved in the present study.

Introduction

Intracellular messengers mediate the actions of several transmitters and humoral agents. One of these messenger systems involves the breakdown of phosphatidylinositol giving 1,4,5-inositol triphosphate and *sn*-1,2-diacylglycerols (see for example, Berridge, 1986; Kaczmarek, 1987). The diacylglycerol is believed to activate the phosphorylating enzyme protein kinase C which in turn has various actions on intracellular proteins including some of those comprising the receptor- or voltage-activated ion channels in the plasma membrane. Several agents which influence this messenger system have been studied in some detail in nervous tissue. Some phorbol esters are activators of protein kinase C, and because of their high potency, their resistance to breakdown and their ability to penetrate the cell membrane, they have been used extensively for experimental studies of this enzyme. Most of these studies have been confined to postsynaptic structures and information about the central nerve terminals is rather scant. Thus at present it is unclear whether nerve terminals contain any of the enzymes or binding proteins associated with the cyclic nucleotide and lipid mediators. However, several workers have shown that phorbol esters enhance transmitter release from the neuromuscular junction (Publicover, 1985; Caratsch *et al.*, 1986; Eusebi *et al.*, 1986; Haimann *et al.*, 1987; Murphy & Smith, 1987; Shapira *et al.*, 1987) and from synapses of brain (Zurgil & Zisapel, 1985; Malenka *et al.*, 1987). Since phorbol diester binding sites and immunoreactive protein kinase C are particularly concentrated in the synaptic layer of the olfactory cortex (Mochly-Rosen *et al.*, 1987; Worley *et al.*, 1987), it seemed worthwhile to study the effect of protein kinase C activators in this brain area.

We show here that phorbol esters, but not diacylglycerol analogues and several other substances which are considered to act on protein kinase C, potentiate transmitter release from the olfactory cortex. The phorbol ester effects are potentiated under conditions which reduce Ca-influx.

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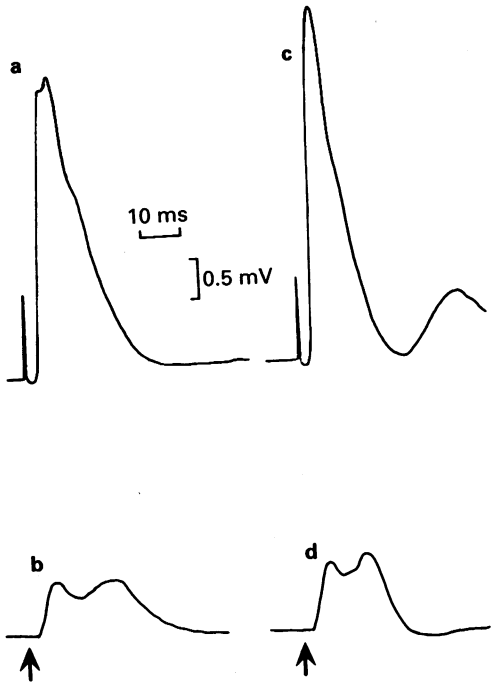

Figure 1 Effect of phorbol diester on single excitatory synaptic potentials recorded from the pial surface of a slice of guinea-pig olfactory cortex. In (a) and (c), a supramaximal stimulus was applied to the lateral olfactory tract (stimulus 30 V and 0.1 ms duration, marked by the stimulus artefact) and a small stimulus in (b) and (d) (2 V and 0.02 ms marked by the arrows under the trace). For records (a) and (b), the slice was bathed in normal solution. Records (c) and (d) were obtained 30 min after the addition of 1 μM phorbol 12,13-dibutyrate to the bathing solution.

Methods

Pial surface slices (500 μm thick) of periamygdaloid area of the olfactory cortex were cut from freshly excised brains from rats or guinea-pigs and placed on a nylon mesh within a recording bath having an effective volume of 0.5 ml (Scholfield, 1980). Krebs solution (50 ml) equilibrated with 95% O_2/5% CO_2 was recirculated through the bath at 5 ml min^{-1}. With concentrated solutions of expensive drugs, 10 ml instead of 50 ml of solution was recirculated. To reduce the amount of tissue, the slices were trimmed to leave a pial surface area of 2 × 6 mm and included the periamygdaloid cortex and lateral olfactory tract (LOT). The caudal part of the LOT was continually stimulated with supramaximal stimuli of 0.2 ms duration (normally 20–30 V at 0.05 Hz) via a pair of tungsten 125 μm diameter wires with lacquer insulation. When the preparation had equilibrated following experimental manipulation, a pair of stimuli separated by 40 ms was administered. Synaptically evoked potentials were recorded from the pial surface via a single glass micropipette filled with Krebs solution. This was connected via a silver electrode to a capacitively coupled amplifier having an input resistance of 10^{10} ohm and time constant of 3 s.

The β-phorbol esters, phorbol 12,13-dibutyrate, phorbol 12-myristate,13-acetate, phorbol monobutyrates and phorbol 12,13-diacetate were dissolved in 100% ethanol or 100% dimethylsulphoxide (DMSO) at a concentration of 1 mM immediately before experimentation. DMSO had no effect alone up to 0.1% (v/v). Appropriate volumes of these solutions were added directly to the medium superfusing the slice. The highest concentration of ethanol when added alone had no effect on the evoked responses. The diacylglycerols were added to DMSO or ethanol and the mixture placed in an ultrasonicating bath for 30 min. In two experiments with dioctanoylglycerol, the low toxicity detergent, Pluronic (0.4–1 mg ml^{-1}), was added to help disperse the DMSO suspension. Mezerein was dissolved directly into DMSO. Comparison of potentials before and after drug effects were statistically tested by Student's t test (paired t test with data from the same slices, unpaired with different slices). Multiple measurements were summarised as a mean ± standard error of the mean (s.e.mean). When changes were measured, the changes in individual experiments were first calculated then these values averaged.

Drugs and solutions

The normal solution used had the following composition (mM): Na 144, K 5.9, Ca 2.5, Mg 1.3, Cl 128, HCO_3 25, D-glucose 10. Phosphate was omitted to avoid precipitation of Cd, Co and Mn.

β-Phorbol 12,13-dibutyrate, 1-oleoyl-2-acetylglycerol, diolein, β-phorbol 12-acetate,13-myristerate, β-phorbol 12,13-acetate, 1-(5-isoquinolinylsulphonyl)-2-methylpiperazine (H-7), acridine orange and 4β-phorbol were obtained from Sigma Chemical Co., Poole, Dorset, dioctanoylglycerol, ethylene glycol dioctanoate and pluronic detergent from Molecular Probes, Oregon, U.S.A. and β-phorbol 12-acetate,13-myristerate and mezerein from LC Services Corporation, 165, New Boston Street, Woburn, MA 01801, U.S.A.

Results

Stimulation of the presynaptic input to the olfactory cortex, the lateral olfactory tract (LOT) evoked a negative synaptic potential from the pial surface of the olfactory cortex. This is a monosynaptic poten-

tial resulting from the release of excitatory transmitter of unknown nature from *en passant* synapses along unmyelinated axons derived from the LOT (Scholfield, 1980). On adding phorbol 12,13-dibutyrate (Figure 1), the amplitude of this monosynaptically evoked potential increased over a 30 min period and then remained elevated (Figure 2). In 18 preparations, 0.1 μM phorbol dibutyrate increased the evoked potential from 2.28 ± 0.26 mV to 3.77 ± 0.38 mV i.e., a $69 \pm 7\%$ increase ($P < 0.0005$) and at 1 μM in 11 slices, it was increased from 2.87 ± 0.31 mV to 4.25 ± 0.51 (a $51 \pm 10\%$ increase, $P < 0.0005$).

There was no recovery of the evoked potential on prolonged (300 min) washing out of the phorbol diester (Figure 2). With much prolonged use, the amplitude of the response tends to decline very slowly making it difficult to discern any long term effects. However, after an 18 h washout from 0.1 μM phorbol dibutyrate, a reapplication of this substance produced an elevation of the evoked potential which was similar to that in slices without prior phorbol ester treatment. This observation suggests that at least some recovery from the action of phorbol ester had occurred.

In the olfactory cortex, several synaptic responses are elicited with LOT stimulation. Two monosynaptic potentials are apparent as pial surface depolarizations. The longer latency response arises from fibres less superficial to the pia than the earlier potential (Gilbey & Wooster, 1979). It was also enhanced by phorbol ester (Figure 1). Figure 1 also shows an increase of a longer latency negative wave. This wave results from feed-back excitation of transmitter release from the axons of the LOT (Pickles & Simmonds, 1976). In this particular experiment, very little of the wave was apparent before phorbol ester.

With an increased synaptic response, the true effect may be much larger if the amplitude of the potential had reached a ceiling. If true, this limitation might arise in two ways: (a) the enhancement may be underestimated because the relationship between transmitter concentration at the postsynaptic membrane and the postsynaptic depolarization is a non-linear function of the postsynaptic input resistance (Scholfield 1978a,b); (b) with each presynaptic volley, the amount of transmitter available for release is limited. In our normal solution, transmitter output is almost maximal thus with each stimulus, there is little extra transmitter available for release by any agent enhancing transmitter release.

To test the first possibility, the stimulus voltage was reduced so that only a small proportion of the presynaptic axons were activated. The stimulus was adjusted so that the amplitude of the evoked potential was 10–20% of that with supramaximal stimulation. Phorbol dibutyrate (0.1 or 1 μM) enhanced the evoked potential by a proportionately similar amount to that seen with supramaximal stimulation (Figure 1).

Another way of reducing synaptic activity is by depressing transmitter release in all presynaptic axons by reducing Ca influx into the nerve terminals. Of the Ca channel blockers, Cd is particularly effective in this preparation (Kuan & Scholfield, 1986; Scholfield, 1988). A Cd concentration was selected (2–50 μM) which depressed the evoked response to about 30% of that in normal solution and Figure 3 shows such an experiment. The proportional enhancement of the e.p.s.p. was much greater than in

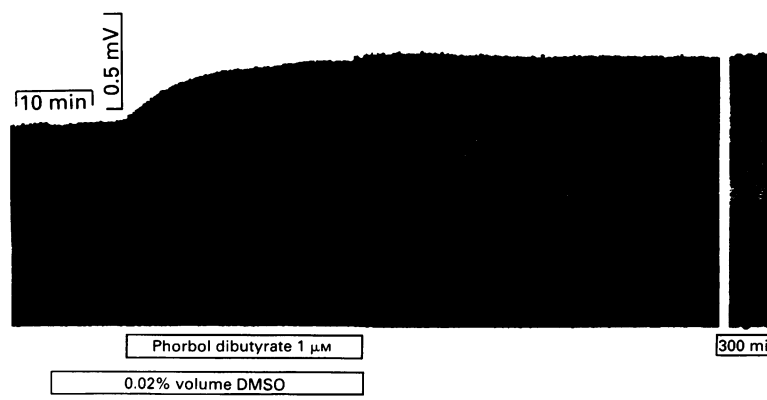

Figure 2 Record using a sample-and-hold amplifier showing the time-course for the action of phorbol 12,13-dibutyrate in normal solution. The phorbol ester was applied for a period of 30 min and then washed out for 330 min. The last section of the record marked '300 min' is a period where the chart recorder speed and stimulus rate was reduced from 0.05 to 0.001 Hz.

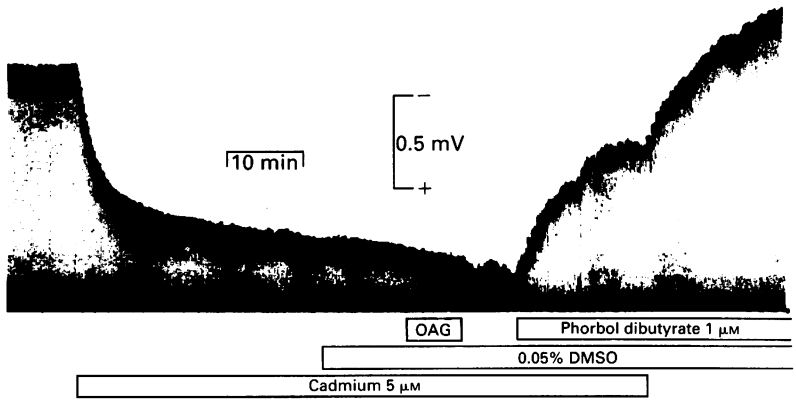

Figure 3 Sample-and-hold showing the influence of Ca-channel blockade on phorbol diester action. A preparation was bathed sequentially in the solutions indicated in the boxes below the record. The concentration of 1-oleoyl-2-acetylglycerol (OAG) was 100 μM.

normal solution (compare the effect in Figure 1c and Figure 2). In 24 slices, 1 μM phorbol dibutyrate increased the evoked potential from 1.02 $\pm$ 0.15 mV to 2.85 $\pm$ 0.41 mV (197 $\pm$ 24% increase, $P < 0.0005$). When the Cd was removed after phorbol ester action, the e.p.s.p. increased to a level greater than that in normal solution (Figure 3). In 6 slices, phorbol dibutyrate was tested at 0.1 μM in the presence of Cd (2–10 μM) and this gave a 114 $\pm$ 68% increase in the evoked potential. The subsequent raising of the phorbol dibutyrate concentration to 1 μM further increased the evoked potential so that its amplitude was 231 $\pm$ 85% of that in Cd alone ($P = 0.03$ for the further increase).

A similar potentiation in the effect of 1 μM phorbol dibutyrate was also noted when the evoked potential was depressed by Mn (1–2 mM: the evoked potential was increased by 186 $\pm$ 45% in 9 slices) or by 2 mM Co (a 131 and a 187% increase in 2 slices).

In 5 slices, dose-response curves to Cd^{2+} were plotted in the presence and absence of phorbol ester (Figure 4). The shape of the curve was unchanged by phorbol diester although the effectiveness of Cd was reduced by 20% (the concentration producing a 50% depression in normal solution was 11.2 μM compared to 13.4 μM in phorbol ester, values significantly different at $P < 0.05$).

The Ca concentration was reduced from the normal 2.5 mM to 1.0 mM and this reduced the amplitude of the evoked potential from 2.75 $\pm$ 0.53 to 1.49 $\pm$ 0.32 mV ($P < 0.0005$). Phorbol dibutyrate (1 μM) increased the amplitude of the evoked potential in 1.0 mM Ca from 1.49 $\pm$ 0.32 to 2.98 $\pm$ 0.63 mV (an increase of 98 $\pm$ 19%, $n = 5$, $P = 0.007$). This effect was significantly greater than that in slices bathed in normal solution ($P = 0.02$). In another 5 slices the Ca was reduced to 0.5 mM and the amplitude of the evoked potential was reduced from 1.39 $\pm$ 0.21 in normal solution to 0.30 $\pm$ 0.01 mV. Phorbol dibutyrate 1 μM increased the amplitude from 0.30 $\pm$ 0.01 to 0.79 $\pm$ 0.13 mV (an increase of 192 $\pm$ 42% which was a significantly greater effect, $P = 0.001$, than that increase for slices in normal 2.5 mM Ca solution).

When double stimuli were applied, the second response was normally greater than the first conditioning response (for example in normal solution, the second response was enhanced by 27 $\pm$ 7% compared to the first in 29 slices, $P < 0.05$). When a phorbol ester (0.1 or 1 μM) was added to preparations bathed in normal solution, this facilitation

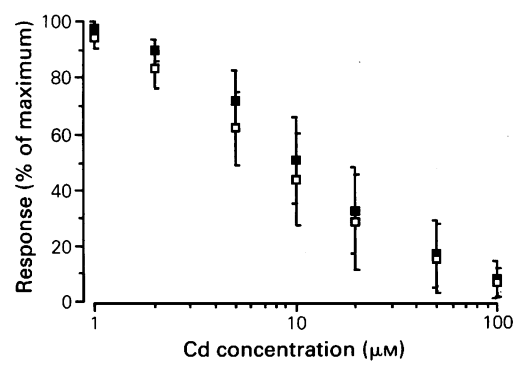

Figure 4 Dose-response curves of the amplitude of the evoked potential against the Cd concentration in normal solution (□) and after equilibration with 1 μM phorbol dibutyrate (■) in 5 slices. The ordinate scale shows the amplitude of the evoked potential in the respective bathing solution as a percentage of that before adding Cd. The large error bars resulted from differences of Cd sensitivity between slices.

was no longer apparent: the second response was instead depressed. In the same 29 slices this second response was reduced by 51 ± 7% ($P < 0.0005$).

Action of other phorbol esters

Mezerein (3 μM), phorbol 12,13-diacetate (10 μM) and phorbol 12-myristate,13-acetate (0.1–3 μM) had similar effects on the evoked potentials to those produced by phorbol dibutyrate.

The phorbol base (10 μM) had no effect on the evoked potential over 100 min periods ($n = 3$) nor did it influence the enhancement of the phorbol diester (1 μM) when it was subsequently added. Both phorbol monobutyrates were tested at 1 μM in the presence of Cd and were without effect.

Action of protein kinase C inhibitors

If phorbol esters were acting via protein kinase C, then their actions should be reversed by any substance inhibiting the enzyme. One such compound is H-7 (1-(5-isoquinolinylsulphonyl)-2-methylpiperazine) (Hidaka & Hagiwara, 1987); this was applied at a concentration of 100 μM in normal solution for 15 min in 3 slices and in the presence of Cd in another 3 slices (100 μM), prior to the application of phorbol dibutyrate (1 μM). In normal solution, H-7 alone slightly enhanced the evoked potential (9.0 ± 1.1%) but the phorbol ester still produced an enhancement (37.3 ± 14.1%) which was not significantly different from the enhancement without H-7. In the presence of Cd, H-7 again enhanced the evoked response (8.3 ± 7.2%) and subsequent addition of 1 μM phorbol dibutyrate still produced an enhancement (76.7 ± 11.5%) (unpaired t test to assess inhibition:- $0.05 < P \leqslant 0.1$).

Acridine orange has also been shown to inhibit protein kinase C (Hannun & Bell, 1988) and this was tested in similar manner to H-7 in 3 preparations. In normal solution acridine orange had a slight depressant effect on the evoked response (6.0 ± 1.2%, $P = 0.05$ in normal solution and 34.7 ± 9.7%, $P = 0.07$, in Cd). When phorbol dibutyrate was added, the evoked potential increased in normal solution by 40.0 ± 9.2% and in Cd by 153.7 ± 35.3% (these changes were not significantly different from those seen without acridine orange).

Action of diacylglycerols

The following lipids were tested on the synaptic response both in normal solution and in Cd: dioctanolyglycerol (100–1000 μM) had little consistent effect (see Figure 3) (a 8.3 ± 8.8% depression; (not significant), 1-oleo-2-acetylglycerol (100–500 μM) produced a 14.0 ± 5.0% increase (not significant) and diolein (100 μM) produced a 24.5 ± 10.2% increase ($P = 0.07$).

The effect of temperature

Most experiments were conducted at 24–25°C. Membranes undergo phase changes at different temperatures and since exogenously applied protein kinase C activators need to cross the membrane to cause the hydrophobic association between the membrane and protein kinase C, it seemed worthwhile to repeat some experiments at 36–37°C. Phorbol dibutyrate (1 μM) produced the same enhancement of the evoked potentials alone or in the presence of Cd at it did at 24–25°C (6 slices). Dioctanoylglycerol (500 μM) had no effect at the elevated temperature (3 slices).

Action of diacylglycerol kinase inhibitor

Ethylene glycol dioctanoate has been shown to inhibit the enzyme which breaks down physiologically produced diacylglycerol (Bishop *et al.*, 1986). Ethylene glycol dioctanoate (500 μM) itself produced a small rise in the amplitude of evoked potentials in the presence of Cd:- 16.6 ± 3.4% increase ($P = 0.0015$). In the presence of the same concentration of ethylene glycol dioctanoate, dioctanoylglycerol (500 μM) had an erratic effect on the evoked potential (a 21 ± 25% increase).

Discussion

The phorbol esters tested in the present study clearly enhanced synaptic transmission in the olfactory cortex of rat and this accords with the observations of workers studying the effects of these substances at other synapses (see Introduction).

Site of action

Two observations point to a predominantly presynaptic action. Firstly, if the effect was postsynaptic, one might expect phorbol diester to have had the same effect on both responses to a pair of stimuli (Richards, 1972). The observation that the first was clearly enhanced whereas the second response was little affected indicated a presynaptic action. Secondly, the effect of phorbol diester was greatly enhanced in Cd^{2+}. The Ca currents in the presynaptic axons are particularly sensitive to Cd^{2+} compared to Ca-channels of the postsynaptic neurones in the same preparation (Kuan & Scholfield, 1986; Scholfield, 1988): Cd^{2+} has little effect on postsynaptic conductances at the concentrations effective presynaptically.

Mechanism of action

It is generally assumed that phorbol diester specifically activates protein kinase C in a manner similar to the physiological activator, diacylglycerol. This substance was not tested because it is known to penetrate membranes poorly and to be broken down by diacylglycerol kinase. However, the membrane permeant analogues of this compound used by other workers failed in the present study to mimic the effect of phorbol esters on the evoked potential. Negative observations such as this can rarely lead to a definitive conclusion, but the large amounts of the different diacylglycerol analogues tested, the comparatively high potency of the phorbol diester and the small diameter of the presynaptic axons favouring intracellular penetration would seem to imply a genuine resistance of this preparation to the action of these substances. This failure of the diacylglycerols might result from diacylglycerol kinase activity but application of an inhibitor of this inactivating enzyme did not have the same effect as phorbol ester. Most workers using intact cell preparations study phorbol diesters and very few have included diacylglycerols in their studies. Thus there is the possibility that the poor effect of diacylglycerol analogues is generally a more widespread phenomenon than the published reports suggest. This raises the possibility that the phorbol esters were acting in some way not involving protein kinase C. The idea has some support from the observations of those workers who have described a dissociation between the actions of diacylglycerol analogues and phorbol esters or who have described an activation of phosphorylation by phorbol esters which did not involve protein kinase C (Moon *et al.*, 1984; Kreutter *et al.*, 1985; Gschwendt *et al.*, 1986; Malviya *et al.*, 1986; Kiss & Luo, 1986). An alternative hypothesis arises from the work of Ohno *et al.* (1987) and Nishizuka (1988) who have described three or more types of protein kinase C: perhaps the nerve terminals of the olfactory cortex contain a protein kinase C isozyme showing some resistance to the diacylglycerol analogues.

Three observations indicate that the larger effect of phorbol ester in low calcium or Cd concentration was not the result of the reduced e.p.s.p. *per se*: (i) the effect with submaximal stimuli; (ii) the larger effect in 0.5 than in 1 mM Ca; (iii) the time course for the onset in action of phorbol dibutyrate (0.1 or 1 μM) was the same irrespective of Ca concentration and the presence or absence of inorganic Ca-channel blockers. If the capacity of the synaptic response had been limiting and the molecular action of phorbol ester independent of Ca, one would have expected the effect on the electrical recordings to have been completed within 5 min when the ceiling had been reached although the molecular action of phorbol ester would continue. Thus the action of phorbol esters in this preparation does appear to be inversely dependent on calcium.

Several authors have described a Ca-dependence for the action of phorbol esters on synaptic transmission (Zurgil & Zisapel, 1985; Eusebi *et al.*, 1986) and this accords with the known Ca requirement for the activation of protein kinase C (Takai, 1979; Nishizuka, 1984). In the present study Ca appeared to antagonize the action of phorbol ester. There are two possible causes of this: (i) phorbol ester, either directly or via an intermediary, is activating or facilitating the opening of Ca-channels (DeReimer *et al.*, 1985; Rane & Dunlap, 1986; Gleason & Flaim, 1986; Strong *et al.*, 1987; Spedding, 1987) when the action potential invades the nerve terminals. (ii) Ca is in some way interfering with the action of phorbol ester. From the present results, these possibilities cannot be reconciled.

These results show that the same phorbol esters which activate protein kinase C act on the presynaptic axons to enhance transmitter release. At present, the mechanism of action is unclear, although the results give reason to suspect a protein kinase C involvement.

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Electrophysiological effects of bepridil and its quaternary derivative CERM 11888 in closed chest anaesthetized dogs: a comparison with verapamil and diltiazem

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1 The electrophysiological effects of bepridil, its quaternary derivative, CERM 11888 (methyl-pyrrolidinium bromide) (both 2.5 mg kg^{-1} i.v.) and those of verapamil and diltiazem (0.2 mg kg^{-1} i.v.) were studied in closed chest anaesthetized dogs at doses used in clinical studies.

2 The four drugs caused a bradycardia with the following order of potency: bepridil > CERM 11888 > diltiazem > verapamil.

3 All the compounds slowed conduction in the AV node, increased the refractory period (RP) and decreased Wenckebach rates with the following order: verapamil ≫ diltiazem > bepridil > CERM 11888.

4 Verapamil and diltiazem did not affect conduction or the RP in atria while bepridil weakly slowed the former and markedly increased the latter. CERM 11888 caused a lengthening of RP but this was a delayed effect.

5 In the ventricle, bepridil and CERM 11888 caused a small increase in the QRS and a more pronounced increase in the RP. Both compounds increased QTc but did not modify HV. Verapamil and diltiazem had no significant effects at the ventricular level.

6 Our results confirm that the main sites of action of calcium antagonists are the SA and AV nodes. Bepridil has a broader spectrum of activity and also acts at the atrial and ventricular levels. A comparison of the effects of bepridil with those of its quaternary derivative suggests the involvement of an intracellular action in the electrophysiological effects of bepridil.

Introduction

Bepridil is an antianginal drug (Cosnier *et al.*, 1977; Alpert *et al.*, 1985) having a long plasma half-life (Benet, 1985) which should allow it to be effective as a once-a day monotherapy for stable angina pectoris (Sharma *et al.*, 1988). In addition to the common property of calcium antagonists to inhibit the slow calcium current, bepridil also inhibits sodium channels (Schwartz *et al.*, 1985; Sperelakis, 1987). The combination of these two effects may explain its additional antiarrhythmic properties in animal experiments (Marshall *et al.*, 1984; Flaim & Cummings, 1986) and its efficacy against both supraventricular (Rowland *et al.*, 1985) and ventricular (Levy *et al.*, 1984) arrhythmias in man. However, bepridil also enters cells (Sperelakis, 1987) and is able to modulate intracellular calcium movements, even in intact tissues (Lynch *et al.*, 1985a,b; 1986; Leboeuf *et al.*, 1987; 1988). In the present study, we have determined if such intracellular actions of bepridil could be involved in its electrophysiological effects in closed-chest anaesthetized dogs, by comparing the effects of bepridil with those of its quaternary derivative (methyl-pyrrolidinium bromide) CERM 11888. CERM 11888 would be expected to penetrate into cells to a lesser extent or at a much reduced rate than the parent drug bepridil. CERM 11888 has been shown to have an approximately similar potency to bepridil on sarcolemmal calcium and sodium channels (Wahler *et al.*, 1986; Schaeffer *et al.*, 1986; Leboeuf *et al.*, unpublished results). Thus in the present study the effects of bepridil and CERM 11888 were compared to those of two classical calcium antagonist drugs: verapamil and diltiazem (see Figure 1, for the different chemical

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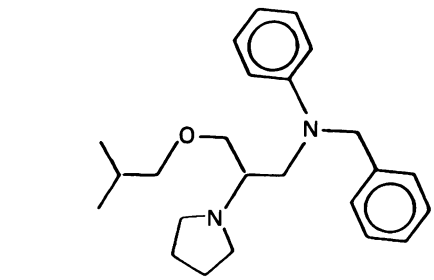
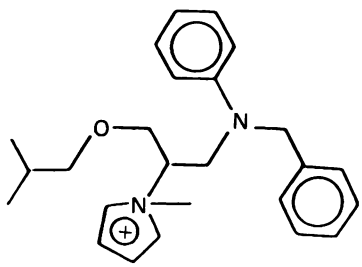


Bepridil



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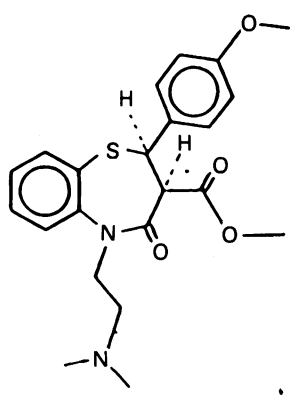

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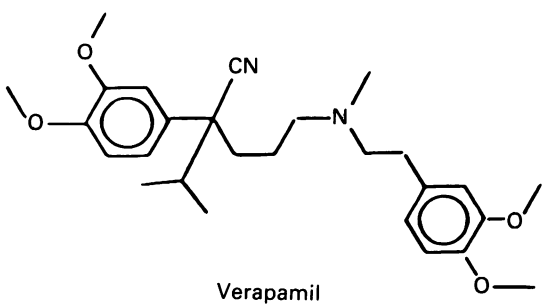

Verapamil

Figure 1 Chemical structures of bepridil and its quaternary derivative CERM 11888 and of verapamil and diltiazem.

structures of these compounds) at doses used in clinical studies and for which comparative haemodynamic data exist in anaesthetized dogs (see Ponsonnaille *et al.*, 1982; Beaughard *et al.*, 1986).

Methods

Forty beagle dogs of either sex (12 to 16 kg) were anaesthetized with alpha chloralose ($100\,mg\,kg^{-1}$ i.v.) and sodium pentobarbitone ($5\,mg\,kg^{-1}$ i.v.). The trachea was cannulated and the animal respired with room air by means of a Minerve respirator. An electrocardiogram (ECG, Lead II) was recorded by use of subcutaneous electrodes. Three endocavitary electrodes (Plastimed 6 F) were positioned with the aid of an image intensifier. The first, a bipolar electrode, was positioned via the left femoral artery in the root of aorta at the level of the aortic cusps to record His-bundle electrogram (Scherlag *et al.*, 1968). The second, a quadripolar electrode, was positioned in the right atrium via the right jugular vein and used for atrial pacing and endocavitary recording of the atrial electrogram. The third electrode (bipolar) was placed in the right ventricle (via left femoral vein) for ventricular pacing. Blood pressure was measured with a Bell and Howell pressure transducer connected to the right femoral artery. Blood gases (Po_2, Pco_2) and pH were determined with an automatic analyser (Corning) and respiration was adjusted to obtain arterial blood gases (Po_2 80–100 mmHg, Pco_2 35–40 mmHg) and pH (7.4 ± 0.5) within the physiological range. Records were made on a four-channel recorder (Beckman R 511 A) at a paper speed of either $200\,mm\,s^{-1}$ for determining the different intervals of conduction or $100\,mm\,s^{-1}$ for refractory period determination.

Parameters studied

Heart rate, systolic (SAP) and diastolic (DAP) arterial pressure were assessed at baseline (Time 0) and 1, 5, 20, 35 and 50 min after the beginning of the infusion. The other electrophysiological parameters were measured at Time 0 and 5, 20, 35 and 50 min post-infusion. Interventricular conduction time (QRS) and repolarization (QT) were measured during spontaneous sinus rate. QTc was calculated by use of Bazett's formula ($QT/\sqrt{RR}$ Bazett, 1920) although, even in man, its ability to predict accurately the effects of drugs on repolarization is limited, particularly at low and high heart rates (Milne *et al.*, 1980; Rickards & Norman, 1981; Staniforth, 1983). Nevertheless it was felt justified to calculate QTc in this way, avoiding the 1 min post-drug time point when very often reflex tachycardia was evident, since it is a parameter frequently quoted

in pharmacological and clinical studies (see Brugada *et al.*, 1988; Zehender *et al.*, 1989). Furthermore under the present experimental conditions and when using a fixed pacing rate it was not always possible to measure QT because the stimulus artifact often arrived before the end of the T wave in cases where QT was lengthened by drugs. Finally the fact that the baseline sinus rates were similar in the different groups of animals used in these studies lends a better predictive value to these QTc measurements. The sinus recovery time (SRT), intraauricular (St-A), intranodal (AH) and His-Purkinje (HV) conduction times and atrial, AV node and ventricular refractory periods were determined during either fixed atrial or ventricular pacing (20% above the baseline spontaneous sinus rate). SRT was assessed during atrial pacing (during a 1 min period) using the atrial electrogram and was defined as the interval between the last auricular response elicited by stimulation and the first spontaneous one. The corrected SRT was measured according to the method of Narula *et al.* (1972) as the difference between the sinus pause duration and the spontaneous cycle length. The refractory periods of the atrium, AV node and ventricle were measured according to the extra-stimulus technique during atrial or ventricular pacing. After every 8th paced beat induced by stimulus S_1, an extrastimulus (S_2) with the same intensity (2 × threshold) and duration (2 ms) was delivered by a stimulator (Medtronic 5325) at a decreasing S_1–S_2 interval (at 10 ms steps) until S_2 no longer evoked a propagated response (R_2). There was a 3 s interval between each successive step. The effective refractory period (ERP) was defined as the greatest S_1–S_2 interval not followed by an R_2. The functional refractory period (FRP) was the smallest value of R_1 R_2 for any S_1 S_2. For the AV junctional refractory periods the reference was the atrial R_1–R_2 interval rather than S_1–S_2 interval. Anterograde and retrograde Wenckebach rates were determined under atrial and ventricular pacing, respectively, by increasing progressively the rate of pacing until AV conduction block appeared.

Protocol

Dogs were divided into five groups (A, B, C, D, E) each of eight animals. Each group received a single drug concentration in a 10 ml volume injected over 1 min. Similar sequences of events were performed before and after drug application. First, without pacing, spontaneous sinus rate, QRS, QT and SAP and DAP were determined followed by a 1 min atrial pacing to obtain paced AH and HV, A-St and, at the end of pacing, SRT. Then, under similar conditions, atrial effective and functional refractory periods were determined (A-ERP, A-FRP respectively) followed by the assessment of anterograde Wenckebach rate (A-Wck). At this time, ventricular pacing permitted measurement of ventricular effective and functional refractory periods (V-ERP and V-FRP respectively) followed by the retrograde Wenckebach rate (R-Wck).

Group A received physiological saline, group B: bepridil (2.5 mg kg^{-1}), group C: CERM 11888 (2.5 mg kg^{-1}), group D: verapamil (0.2 mg kg^{-1}) and group E: diltiazem (0.2 mg kg^{-1}). The doses of bepridil, verapamil and diltiazem are the mean doses commonly used in intravenous studies in man (Singh *et al.*, 1985a).

Drugs used

Bepridil monohydrochloride, monohydrate and CERM 11888, methyl pyrolidinium bromide salt were synthesized in the Department of Chemistry RL-CERM. Verapamil (base) was purchased as Isoptine Injectable from Laboratoire Biosedra France and diltiazem hydrochloride from Sigma Laboratories. The required amount of verapamil was made up to 10 ml using a 0.9% NaCl solution (Iso-Physio, UVA) and bepridil, CERM 11888 and diltiazem were dissolved directly in 10 ml of the same solution.

Statistical analysis

Differences between the changes in the various parameters from baseline values induced by the drugs and those observed in the control group of dogs were analysed by use of Student's t test for independent series.

Results

In the control group of animals, no significant changes in the various electrophysiological parameters were seen throughout the entire experimental period and the mean changes of the parameters in the control group was around 5% with the exception of cSRT which showed relatively large changes with time (time 0 = 86 ± 10 ms, 20 min = 115 ± 21 ms, 50 min = 95 ± 24 ms). Analysis of variance of this data showed that these changes were not significant ($P \simeq 0.3487$).

Effects of compounds on blood pressure

Initial systolic blood pressures in the 5 groups of animals (A, B, C, D and E) used in these experiments were 157 ± 7, 173 ± 7, 143 ± 7, 165 ± 6, and 145 ± 12 mmHg, respectively.

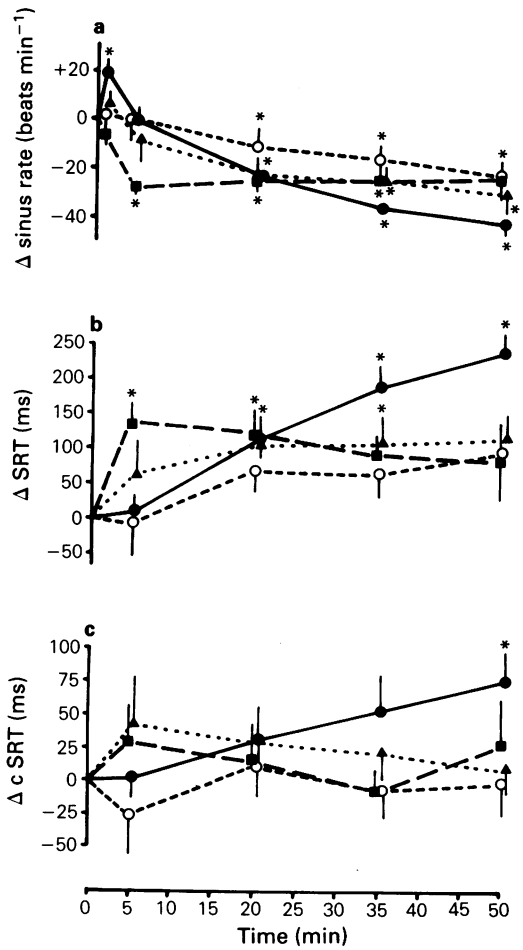

Figure 2 Time course of the effects of bepridil, CERM 11888, verapamil and diltiazem on sinus node function. Changes in spontaneous sinus rate (a), sinus recovery time (SRT) (b) and corrected sinus recovery time (cSRT) (c) with time (in min) caused by bepridil (■), CERM 11888 (●), verapamil (○) and diltiazem (▲). Bepridil and CERM 11888 were given at a dose of 2.5 mg kg^{-1} i.v. while verapamil and diltiazem were given at a dose of 0.2 mg kg^{-1} i.v. Abscissa scales represent time after the beginning of the 1 min infusion of the compounds. Note that the effect of bepridil on spontaneous sinus rate was rapid compared to the other compounds. Symbols are the same in all the other figures. * $P < 0.05$, compared to the control group of animals by Student's t test for independent series. Vertical lines represent s.e.mean.

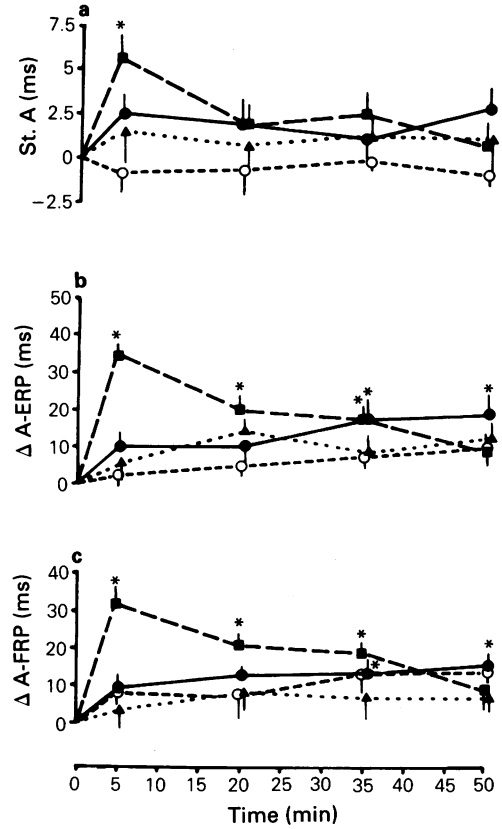

Figure 3 Time course of the effects of bepridil (■), CERM 11888 (●), verapamil (○) and diltiazem (▲) at the atrial level. Changes in intra-atrial conduction time (St.A) (a), atrial effective refractory period (A-ERP) (b) and atrial functional refractory period (A-FRP) (c) caused by the different compounds. Note that bepridil was the sole compound to have a marked effect on these parameters while the significant effect of CERM 11888 was delayed compared to it. * $P < 0.05$, compared to the control group of animals by Student's t test for independent series. Vertical lines represent s.e.mean.

Bepridil, CERM 11888, verapamil and diltiazem all caused a transient hypotension at the end of injection which returned to baseline values after 5 min for bepridil but required around 10 to 15 min for the other compounds. At time 1 min, SAP and DAP were lowered respectively by 10.5 and 23.5% by bepridil, 19 and 30% by CERM 11888, 17 and 28% by verapamil and 10 and 23% by diltiazem.

Effects of compounds on sinus node function

Baseline spontaneous sinus rates of groups A, B, C, D, E were 137 ± 9, 141 ± 7, 155 ± 6, 139 ± 8 and 153 ± 7 beats min^{-1} respectively. All compounds caused significant bradycardia (Figure 2a). However, the effect of bepridil was maximal after 5 min whilst

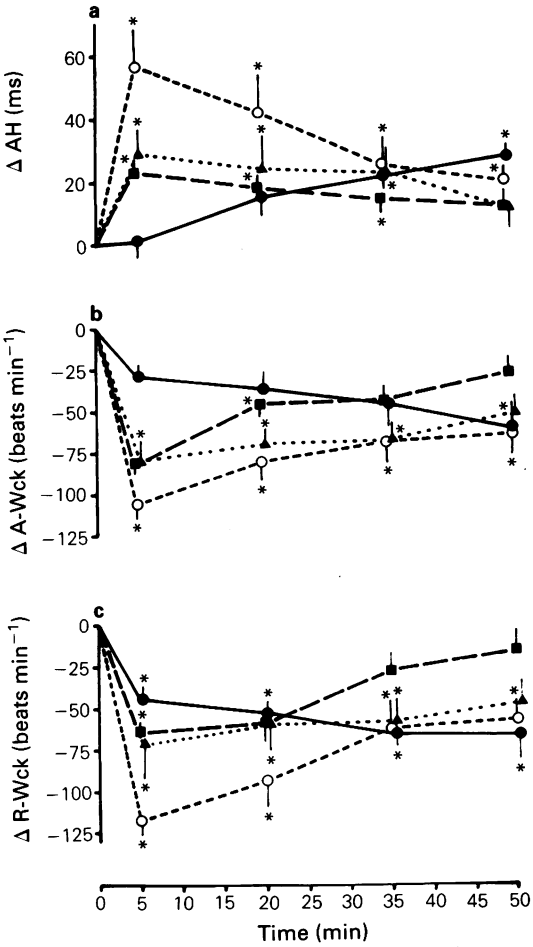

Figure 4 Time course of the effects of bepridil (■), CERM 11888 (●), verapamil (○) and diltiazem (▲) on AV node function. Changes in intranodal conduction time (AH) (a), anterograde Wenckebach rate (A-Wck) (b) and retrograde Wenckebach rate (R-Wck) (c) caused by the different compounds. Note that on these parameters verapamil was much more potent than the other compounds. * $P < 0.05$, compared to the control group of animals by Student's t test for independent series. Vertical lines represent s.e.mean.

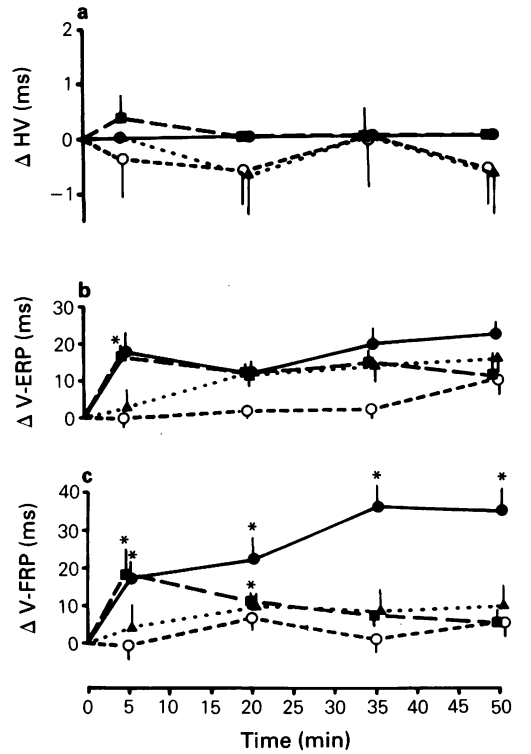

Figure 5 Time course of the effects of bepridil (■), CERM 11888 (●), verapamil (○) and diltiazem (▲) at the ventricular level. Changes in His-Purkinje conduction time (HV) (a), ventricular effective refractory period (V-ERB) (b) and ventricular functional refractory period (V-FRP) (c) elicited by the different compounds. It should be noted that although the effects of bepridil, unlike that of CERM 11888, on V-ERP (5 min) reached statistical significance from control values, there was no significant difference between the effects of bepridil and CERM 11888 at this time point. * $P < 0.05$, compared to the control group of animals by Student's t test for independent series. Vertical lines represent s.e.mean.

that of the other compounds only appeared after 20 min. At 1 and 5 min, there was a significant difference between the effects of bepridil and those of its quaternary derivative which was the only drug to cause a significant, but transient, tachycardia at the end of infusion (see Figure 2a).

Bepridil, CERM 11888 and diltiazem lengthened SRT while verapamil did not significantly modify this parameter (Figure 2b). The effects of bepridil and CERM 11888 were greater than those of diltiazem on this parameter and, as with the bradycardiac effect, that of CERM 11888 was delayed compared to bepridil. Only CERM 11888 significantly increased cSRT (Figure 2c).

Atrial actions of compounds

Bepridil slightly, but significantly, increased intra-auricular conduction time (Figure 3a) and markedly lengthened effective (Figure 3b) and functional (Figure 2c) refractory periods of the atria. CERM 11888 only slightly affected and diltiazem and verapamil had no significant effect on these parameters.

Effects of compounds on AV node function

Verapamil had a marked and long-lasting effect on atrioventricular conduction time (Figure 4a) while diltiazem and bepridil caused significant but less lengthening of AH at a fixed pacing rate. Again, compared to bepridil, the effects of CERM 11888 were delayed. The same potency order for the compounds was seen on anterograde (Figure 4b) and retrograde (Figure 4c) Wenckebach rates. Under these experimental conditions we rarely obtained the effective refractory period of the AV node because it was shorter than the atrial period. However, in the dogs where the baseline refractory period of the AV node was able to be determined, verapamil caused the most marked lengthening ($\simeq +100$ ms) in this parameter while the effects of diltiazem and bepridil were about 50% of verapamil.

Ventricular actions of compounds

As shown in Figures 5 and 6, verapamil and diltiazem had no effects on the electrophysiological parameters of the ventricle. Bepridil and CERM 11888 slightly increased QRS interval (Figure 6a) and effective refractory period (Figure 5b), had no effect on HV interval (Figure 5a), lengthened QT (Figure 6b), QTc (Figure 6c) and the functional refractory period (Figure 5c); CERM 11888 had the longest duration of action.

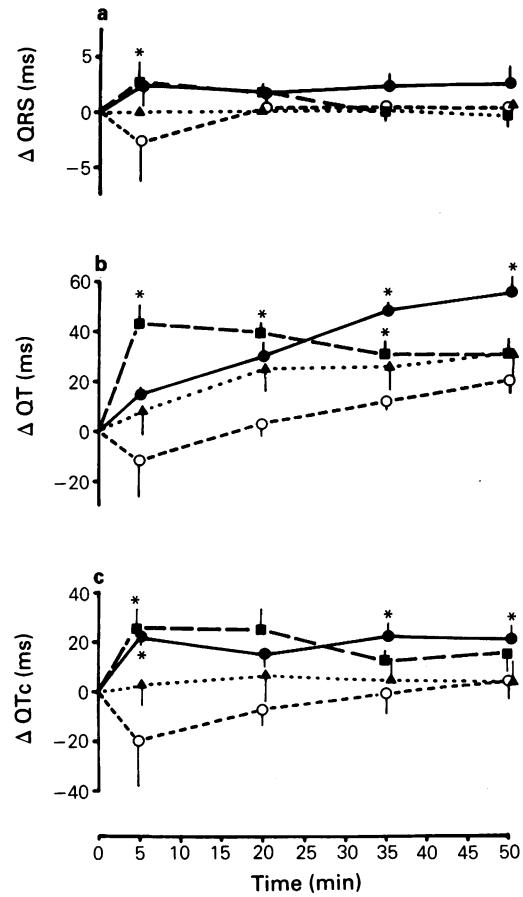

Figure 6 Time course of the effects of bepridil (■), CERM 11888 (●), verapamil (○) and diltiazem (▲) on repolarization. Changes in interventricular conduction time QRS (a), QT (b) and corrected QT (c) values elicted by the compounds. There was no significant difference between the effects of bepridil and those of CERM 11888 on QRS. Note that only these two compounds significantly increased QT and QTc intervals. * $P < 0.05$, compared to the control group of animals by Student's t test for independent series. Vertical lines represent s.e.mean.

Discussion

In the myocardium excitation is mediated by two major inward currents. In 'slow structures', such as the sinus and AV nodes, it is mediated mainly by a slow inward calcium current. In 'fast structures', such as the atria, ventricles and the His-Purkinje system, excitation is mediated by a fast inward sodium current (see Fleckenstein, 1985). In this study, we have shown that in good agreement with their relatively selective action on slow calcium channels (Fleckenstein, 1985; Singh *et al.*, 1985a; Sperelakis, 1987), verapamil and diltiazem at the dose levels used (0.2 mg kg^{-1} i.v.), only modified the rate of conduction and the refractory periods of the 'slow structures' and had no significant effects upon 'fast structures'. Thus both compounds elicited long-lasting bradycardia, markedly increased the intranodal conduction time (AH) and reduced anterograde and retrograde Wenckebach rates. The fact that verapamil was more active than diltiazem in these respects is probably explained by the fact that verapamil is somewhat more potent (2–3 times) than diltiazem as a calcium channel blocker *in vitro* (Lamar *et al.*, 1983; Zsoter & Church, 1983). These findings are similar to those found by other workers in the dog (Millard *et al.*, 1982; Singh *et al.*, 1982) and are probably related to their direct effects upon the sinus and AV nodes (Taira, 1979; Kawai *et al.*, 1981).

Similar effects on 'slow structures' have also been demonstrated with bepridil *in vivo* (Beaughard *et al.*, 1982; Kawada *et al.*, 1983) and, *in vitro* studies in cardiac muscle have shown that this compound preferentially inhibits slow calcium currents (Labrid *et al.*, 1979; Yatani *et al.*, 1986). Such an action could

explain the effect of bepridil on 'slow structures' observed in the present study. These experiments have also shown that the quaternary ammonium derivative of bepridil, CERM 11888, has effects upon 'slow structures' but these were somewhat weaker, and more delayed in onset, than those induced by bepridil. *In vitro* experiments have shown that CERM 11888 has an equivalent calcium channel blocking potency to bepridil (Wahler *et al.*, 1986; Schaeffer *et al.*, 1986), although recent results have indicated that CERM 11888 is some 2–3 times less potent than bepridil at vascular calcium channels (John and Massingham, unpublished results). Further experiments are required to resolve this discrepancy.

In addition to its effects upon slow calcium channels, it is well known that bepridil (Labrid *et al.*, 1979; Yatani *et al.*, 1986) and CERM 11888 (Leboeuf unpublished results) can also inhibit fast sodium channels at concentrations only slightly higher than those affecting the slow calcium channel. Our results also provide *in vivo* evidence that bepridil and CERM 11888 can act upon 'fast structures' to increase refractory periods in atria and ventricles and to slow atrial and intraventricular conduction times.

Comparison of the effects of bepridil and CERM 11888 on the slow action potential has suggested that they act at a common site on the slow calcium channel, probably at the exterior of the cell. Bepridil, however, may also possess a second site of action inside the cell, which may not be readily accessible to its quaternary derivative, CERM 11888 (see Wahler *et al.*, 1986). In the present experiments the effects of bepridil on sinus node, atrial refractory periods and AV node were more marked and significantly different from the effects of CERM 11888, which again suggests additional sites of action for the parent drug. Bepridil has been shown to enter readily and to accumulate in cells (Cramb & Dow, 1983; Pang & Sperelakis, 1983; 1984), whereas CERM 11888, being a charged molecule, would presumably enter cells less readily than the parent molecule. In agreement with this assumption, we have recently shown that bepridil, but not CERM 11888, decreased the amplitude of caffeine-induced contractions in ferret heart trabeculae *in vitro*, responses which are known to be mediated by the intracellular release of calcium from sarcoplasmic reticulum (SR) stores (Leboeuf *et al.*, 1987; Chapman & Tunstall, 1988). It is also interesting to note that the relative importance of calcium release in the excitation-contraction coupling mechanism is much more important in atria than in the ventricle (Fabiato & Fabiato, 1978; Bers, 1985). An action of bepridil on calcium released from the SR might, in part, explain why in these experiments the electrophysiological effects of bepridil are more manifest on supraventricular compared to ventricular structures (see also Sassine *et al.*, 1984). A decreased intracellular release of calcium could modify the kinetics of sarcolemmal calcium channels, the amplitude of the calcium-activated K^+ current and Na^+-Ca^{2+} exchange (see Noble, 1984), which may lead to a lengthening of the action potential and an increase in the refractory period. Studies performed in rabbit heart have shown that bepridil causes a greater lengthening of atrial than ventricular action potentials (Winslow *et al.*, 1986). Thus an intracellular action may also be involved in the effects of bepridil on refractory periods and repolarization of the ventricle but these are weaker than the effects seen in atria perhaps, as suggested previously, because calcium-induced calcium release is less important in this structure. Finally, the effects of diltiazem, verapamil and bepridil in this closed-chest, anaesthetized dog model are very similar to those obtained with these compounds in the clinical situation (Ponsonnaille *et al.*, 1982; Singh *et al.*, 1985a,b) in agreement with findings of other workers (Brugada *et al.*, 1988). Thus, this model may be useful for the evaluation and extrapolation of the electrophysiological effects of cardioactive drugs to man.

In conclusion, these results have shown that bepridil has a broader electrophysiological profile than the classical calcium antagonists verapamil and diltiazem. In this respect bepridil is unique. Such properties could account for the activity of bepridil against both supraventricular and ventricular arrhythmias (Fauchier *et al.*, 1985; Levy *et al.*, 1984; Rowland *et al.*, 1985) in addition to its pronounced antianginal properties (Alpert *et al.*, 1985). Comparison of bepridil with its quaternary ammonium derivative, CERM 11888, indicated that intracellular effects, in addition to an action upon slow calcium and fast sodium channels, may be responsible for this unique pharmacological profile of bepridil.

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Endothelium-dependent vasodilator effects of platelet activating factor on rat resistance vessels

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1 To elucidate the mechanisms of the powerful and long-lasting hypotension produced by platelet activating factor (PAF), its effects on perfusion pressure in the perfused mesenteric arterial bed of the rat were examined.

2 Infusion of PAF (10^{-11} to 3×10^{-10} M; $EC_{50} = 4.0 \times 10^{-11}$ M; 95%CL = $1.6 \times 10^{-11} - 9.4 \times 10^{-11}$ M) and acetylcholine (ACh) (10^{-10} to 10^{-6} M; $EC_{50} = 3.0 \pm 0.1 \times 10^{-9}$ M) produced marked concentration-dependent vasodilatations which were significantly inhibited by treatment with detergents (0.1% Triton X-100 for 30 s or 0.3% CHAPS for 90 s).

3 Pretreatment with CV-6209, a PAF antagonist, inhibited PAF- but not ACh-induced vasodilatation.

4 Treatment with indomethacin (10^{-6} M) had no effect on PAF- or ACh-induced vasodilatation.

5 These results demonstrate that extremely low concentrations of PAF produce vasodilatation of resistance vessels through the release of endothelium-derived relaxing factor (EDRF). This may account for the strong hypotension produced by PAF *in vivo*.

Introduction

Platelet activating factor (PAF, acetyl glyceryl ether phosphorylcholine) has been shown to produce strong and long-lasting hypotension in various animal species, e.g. normotensive and spontaneously hypertensive rats, rabbits, guinea-pigs, and dogs (Tanaka *et al.*, 1983). This action of PAF is thought to be endothelium-dependent (Kamitani *et al.*, 1984; Kasuya *et al.*, 1984a,b; Shigenobu *et al.*, 1985; 1987). In a previous study (Shigenobu *et al.*, 1987), we found that relatively low concentrations of PAF (10^{-9}–10^{-7} M) produced endothelium-dependent relaxation of the rat aorta in the presence of bovine serum albumin. This vasodilator action of PAF at low concentrations might be the cause of its hypotensive action *in vivo*. While the aorta will offer a resistance to flow, it is obvious that the contribution of vessels of smaller diameter to peripheral vascular resistance is much greater. In this regard, the mesenteric circulation of the rat receives approximately one-fifth of the cardiac output (Nichols *et al.*, 1985) and, thus, regulation of this bed may make a significant contribution towards systemic blood pressure and circulating blood volume. In the present study, therefore, we examined the effect of PAF on the resistance vessels of the rat mesenteric vascular bed and found that extremely low concentrations (10^{-11} to 3×10^{-10} M) can produce endothelium-dependent vasodilatation.

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Methods

Perfusion of the mesenteric arterial bed

Male Wistar rats (190–360 g) were killed by decapitation. The mesenteric arterial bed was removed and perfused according to the method described by McGregor (1965), with various modifications. Briefly, the abdominal cavity was opened by midline incision, and the entire mesenteric arterial bed and associated intestinal tract was removed from the rat. The mesenteric artery and vein were tied off close to the caecum, and the remaining intestine was separated from the vascular bed along the intestinal wall. A cannula of PE90 tubing was inserted approximately 1.5 cm into the superior mesenteric artery from its junction with the aorta and tied firmly. The isolated

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preparation was placed in an organ bath and perfused through the cannula with Krebs-Henseleit solution by a peristaltic pump (Tokyo Rikakikai, Model MP-3) at a rate of 5 ml min^{-1}. The Krebs-Henseleit solution was composed (in mM) of NaCl 118.0, KCl 4.7, $CaCl_2$ 1.8, $MgSO_4$ 1.2, $NaHCO_3$ 25, dextrose 11.1 and 0.25% bovine serum albumin. The perfusate was maintained at 37°C and gassed constantly with 95% O_2 and 5% CO_2. Changes in perfusion pressure were monitored with a pressure transducer (Nihon Kohden, Model AP-2001) and recorded on a pen recorder (Yokogawa, Model 3021). After an equilibration period of 60 min, the perfusion circuit was changed to a closed system, i.e., the perfusion solution was collected in a second bath and recirculated into the mesenteric arterial bed. The total volume of the closed system was 50 ml, and agents were administered via the second bath. The mesenteric arterial bed was precontracted with 10^{-5} M methoxamine. Acetylcholine (ACh) was administered cumulatively, but PAF was infused in single doses to avoid tachyphylaxis.

Removal of endothelium by detergents

The endothelium was removed by intraluminal perfusion with Krebs-Henseleit solution containing detergents (0.1% Triton X-100 for 30 s or 0.3% CHAPS for 90 s). After a re-equilibration period of 30 min, responses to ACh or PAF were examined.

Statistics

Since ACh was administered cumulatively and PAF was infused in single doses, EC_{50} data for ACh are presented as the mean ± s.e.mean and EC_{50} data for PAF and CV-6209 are presented as 95% confidence limits (CL) of EC_{50} according to the method of Litchfield & Wilcoxon (1949). Significance of differences was determined with Student's non-paired *t* test.

Drugs

PAF (1-O-alkyl-2-acetyl-*sn*-glyceryl-3-phosphorylcholine; Bachem, Switzerland) and CV-6209 (2-[N-acetyl-N-(2-methoxy-3-octadecylcarbamoyloxypropoxycarbonyl)aminomethyl]-1-ethylpyridinium chloride; Takeda, Osaka, Japan) were dissolved in ethanol and stored at −20°C. On the day of use, the ethanol was dried under a stream of nitrogen gas and the drugs dissolved in Krebs-Henseleit solution containing 0.25% bovine serum albumin (Fraction V, Sigma, St. Louis, U.S.A.). Sonication for 30 s ensured that the drugs were completely dissolved. Methoxamine hydrochloride, indomethacin, 3-[(cholamidopropyl)-dimethyl-ammonio]-1-propanesulphonate-(CHAPS), Triton X-100 (Sigma) and acetylcholine hydrochloride (Daiichi, Tokyo, Japan) were dissolved in 0.9% saline.

Results

Perfused mesenteric arterial bed of rat

The basal perfusion pressure of the rat mesenteric arterial bed was 51.4 ± 1.2 mmHg ($n = 10$). Perfusion with methoxamine (10^{-5} M) increased the perfusion pressure to 118.4 ± 6.1 mmHg ($n = 10$).

Relaxation in response to ACh

In the perfused mesenteric arterial bed preconstricted with methoxamine (10^{-5} M), infusion of increasing

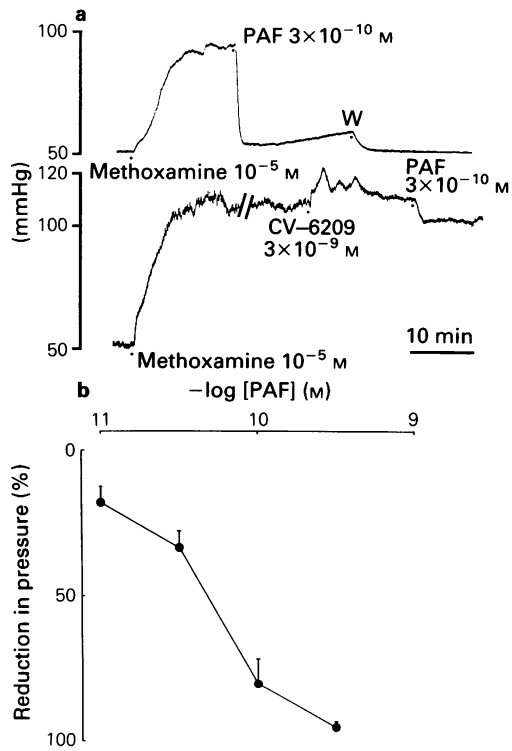

Figure 1 Effects of PAF on the perfusion pressure of the methoxamine (10^{-5} M)-constricted mesenteric vascular bed. (a) Upper panel: relaxation induced by PAF (3×10^{-10} M). Lower panel: effects of the PAF-antagonist, CV-6209 (3×10^{-9} M), on the relaxation induced by PAF (3×10^{-10} M). (b) Concentration-response curve for the relaxation produced by PAF (10^{-11} to 3×10^{-10} M) in the methoxamine (10^{-5} M)-constricted mesenteric vascular bed. Each point is the mean and vertical bars represent the s.e.mean from 5 experiments.

concentrations of ACh (10^{-10} to 10^{-6} M) produced concentration-dependent vasodilatations. The EC_{50} concentration was $3.0 \pm 0.1 \times 10^{-9}$ M ($n = 6$) and the maximal relaxation induced was $95.5 \pm 8.6\%$.

Relaxation in response to PAF

PAF (10^{-11} to 3×10^{-10} M) produced concentration-dependent, long-lasting relaxation of the methoxamine (10^{-5} M)-constricted mesenteric arterial bed (Figure 1). The EC_{50} value for PAF was 4.0×10^{-11} M (95% CL = 1.6×10^{-11}–9.4×10^{-11} M, $n = 5$) and the maximal relaxation induced was $95.5 \pm 8.6\%$. CV-6209 (3×10^{-11}–3×10^{-9} M), a PAF antagonist, inhibited PAF (3×10^{-10} M)-induced vasodilatation in a dose-dependent manner. The IC_{50} ($n = 5$) value for CV-6209 was 1.0×10^{-10} M (95% CL = 3.1×10^{-10}–2.8×10^{-9} M, $n = 5$). CV-6209 (3×10^{-9} M) had no effect on ACh-induced relaxation (data not shown). Infusion of lysoPAF (3×10^{-10} M) had no effect on the perfusion pressure of the mesentery (data not shown).

Effects of detergents on responses to ACh

Perfusion of the mesentery with detergents (0.1% Triton X-100, $n = 5$; 0.3% CHAPS, $n = 3$) produced a slight but significant increase in methoxamine (10^{-5} M)-induced vasoconstriction (data not shown). Both detergents significantly suppressed the vasorelaxant response to ACh (10^{-7}–10^{-4} M) (Figure 2). Pretreatment with indomethacin (10^{-6} M) did not affect ACh-induced relaxation (data not shown).

Effects of detergents on responses to PAF

PAF (3×10^{-10} M)-induced vasodilatation was significantly inhibited by pretreatment of mesentery with Triton X-100 (0.1%, $n = 5$) or CHAPS (0.3%, $n = 3$). In contrast, pretreatment with indomethacin (10^{-6} M) had no effect on PAF-induced vasodilatation (Figure 3).

Discussion

Since Furchgott & Zawadzki (1980) demonstrated the obligatory role of endothelium in vascular relaxation by ACh, many studies have suggested that endothelium-derived relaxing factor (EDRF) is released from endothelial cells in response to a large number of agonists (Furchgott, 1984). In the present study with perfused resistance vessels, ACh produced vasodilatation in a concentration-dependent manner and the vasorelaxant responses were significantly suppressed by perfusion with detergents such as CHAPS or Triton X-100. Since the detergents are thought to destroy the endothelial cells only, these results confirmed that ACh-induced vasodilatation was mediated through release of EDRF. Moreover, in agreement with recent findings by Randal & Hiley (1988) and Tesfamariam & Halpern (1988), these detergents enhanced the ability of the vascular smooth muscle to contract in response to methoxamine, an α-adrenoceptor agonist.

Infusions of extremely low concentrations of PAF (10^{-11} to 3.1×10^{-10} M) produced a marked and long-lasting vasodilatation which was significantly suppressed by treatment with detergents (CHAPS or

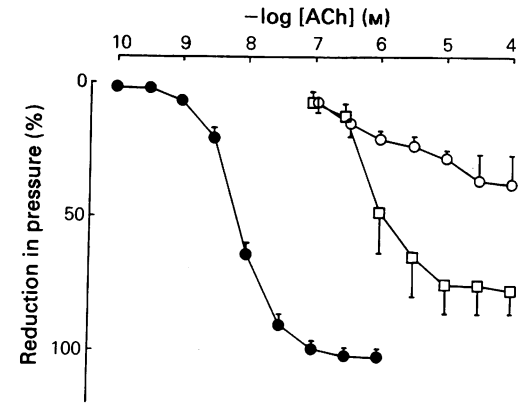

Figure 2 Effects of detergents on acetylcholine (ACh)-induced relaxation of the methoxamine (10^{-5} M)-constricted mesenteric vascular bed. Concentration-response curves are shown for ACh-induced vasodilatation before (●) and after treatment with 0.3% CHAPS (□) or 0.1% Triton X-100 (○). Each point is the mean and vertical bars represent the s.e.mean from 5 experiments.

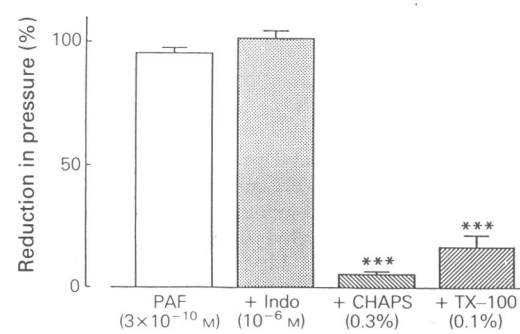

Figure 3 Effects of indomethacin (Indo, 10^{-6} M, $n = 4$) and detergents (0.3% CHAPS, $n = 3$; 0.1% Triton X-100, $n = 5$) on the relaxation induced by PAF (3×10^{-10} M) in the methoxamine (10^{-5} M)-constricted mesentery. Each column is the mean and vertical bars represent the s.e.mean. *** $P < 0.001$.

Triton X-100). These data strongly suggest the possible involvement of the endothelium in the relaxation induced by PAF.

CV-6209, a PAF antagonist, inhibited PAF-induced but not ACh-induced vasodilatation in a concentration-dependent manner. Specific antagonism by CV-6209 has already been obtained with respect to PAF-induced hypotension or platelet aggregation (Terashita *et al.*, 1987). An accumulating body of evidence suggests that hypotension resulting from endotoxin challenge is due to the endogenous release of PAF from endothelial cells (Camussi *et al.*, 1983), leukocytes (Demopoules *et al.*, 1979), macrophages (Mencia-Huerta & Benveniste, 1979; Camussi *et al.*, 1983) and platelets (Chingard *et al.*, 1979). Indeed, PAF antagonists can reverse established endotoxin-induced hypotension (Terashita *et al.*, 1985; Handley *et al.*, 1985a,b). From the above data and the results of the present study, one possible explanation for endotoxin-induced hypotension may be that the release of PAF occurs, which then binds to its receptors located on the endothelial cells, stimulating production of EDRF.

Prostaglandin I_2 a powerful vasodilator is synthesized by the endothelial cells (Moncada *et al.*, 1977). However, Furchgott's group showed that ACh-induced, endothelium-mediated vasodilatation was not modified by the cyclo-oxygenase inhibitor, indomethacin. We found that the endothelium-dependent vasodilatation induced by PAF was not inhibited by indomethacin, ruling out the involvement of vasodilator prostanoids. Furthermore, PAF at a low concentration showed neither chronotropic nor inotropic effects on the guinea-pig isolated right and left atria, respectively (Tanaka *et al.*, 1983; Shigenobu *et al.*, 1989), excluding the possible involvement of cardiac depression in the PAF-induced hypotension.

In conclusion, we demonstrated that extremely low concentrations of PAF produce long-lasting vasodilatation in a resistance vessel of the mesenteric vasculature. Moreover, we showed that this PAF-induced vasodilatation is mediated by a vasodilator substance released from endothelial cells (EDRF) which is not a prostaglandin. Since the PAF-induced endothelium-dependent relaxation observed in the present study was elicited at low concentrations and was long-lasting, it may be the main mechanism by which PAF induces hypotension *in vivo*.

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Temporal changes in the calcium-dependence of the histamine H_1-receptor-stimulation of cyclic AMP accumulation in guinea-pig cerebral cortex

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1 2-Chloroadenosine (2CA) causes a maintained rise in adenosine 3':5'-cyclic monophosphate (cyclic AMP) content of guinea-pig cerebral cortical slices which is augmented by addition of histamine. We have investigated the temporal profile of the sensitivity of this response to calcium.

2 Rapid removal of extracellular calcium with EGTA (5 mM) at 2CA (30 μM)-induced steady state caused a slight increase in the cyclic AMP response to 2CA alone and completely abolished the augmentation produced by histamine (0.1 mM) added 20 min later. When EGTA was added only 2 min before histamine, the augmentation was reduced by 72%.

3 The calcium sensitivity of the histamine response was also indicated in studies in which EGTA was added 1 or 3 min after histamine at 2CA-induced steady state. Following addition of EGTA at either of these times, the augmentation was not maintained.

4 When calcium was rapidly removed with EGTA once a steady state level of cyclic AMP had been achieved with histamine, the augmentation response was maintained. This was despite the fact that EGTA had a similar effect on both extracellular free calcium and tissue calcium content when it was applied before or after histamine.

5 The 2CA response was augmented by phorbol esters (which mimic the actions of diacylglycerol) in a calcium-independent manner.

6 These results suggest that calcium is important for the initiation and early stages of the histamine-induced augmentation response. The apparent lack of calcium sensitivity of the response at later stages could mean that calcium is not involved in the maintenance of the response or that the intracellular machinery involved in the augmentation process becomes more sensitive to calcium as the response progresses, such that it becomes able to operate at a much lower level of intracellular calcium. A possible role for diacylglycerol in the maintenance of the response is discussed.

Introduction

Histamine can elevate adenosine 3':5'-cyclic monophosphate (cyclic AMP) levels in brain slices by two mechanisms. It can stimulate adenylate cyclase directly via histamine H_2-receptors. Alternatively, it can act through H_1-receptors to augment the cyclic AMP responses to H_2-, vasoactive intestinal peptide (VIP)- or adenosine A_2-receptor stimulation (Palacios *et al.*, 1978; Al-Gadi & Hill 1987; Hill *et al.*, 1981; Magistretti & Schorderet, 1985; Hollingsworth & Daly, 1985; Garbarg & Schwartz, 1988; Hill & Kendall, 1989). The augmentation of the adenosine response is rapid in onset and requires a maintained stimulation of H_1-receptors; addition of the H_1-receptor antagonist mepyramine causes a rapid fall in cyclic AMP levels to those obtained with adenosine alone (Donaldson *et al.*, 1988b). Kinetic studies have indicated that this augmentation is exerted via an action on adenylate cyclase (i.e. on the G_s regulatory protein or the catalytic unit) rather than by inhibition of the enzyme which breaks down cyclic AMP, phosphodiesterase (Donaldson *et al.*, 1988b).

Little is yet known about the intracellular mechanisms by which neurotransmitters indirectly potentiate cyclic AMP accumulation. However, the production of another second messenger seems to be

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involved since the effect is lost in membrane preparations (Hegstrand *et al.*, 1976; Green *et al.*, 1977; Coupet & Szuchs-Meyers, 1981; Green, 1983; Hill, 1987). It has been suggested that the products of inositol phospholipid breakdown, the inositol phosphates and diacylglycerol, promote increased activity of the cyclase via calcium mobilisation and protein kinase C activation, respectively (Schwabe *et al.*, 1978; Hollingsworth *et al.*, 1985; Danoff & Young, 1987). Histamine H_1-receptor stimulation is accompanied by an increased hydrolysis of inositol phospholipids in mammalian brain slices and hence can give rise to both an increase in intracellular calcium and the formation of diacylglycerol (Daum *et al.*, 1984; Donaldson & Hill, 1986; Hill, 1987). Phorbol esters, which mimic the action of diacylglycerol by activating protein kinase C, have been reported to mimic fully the effects of H_1-receptor stimulation in potentiating the cyclic AMP response to 2-chloroadenosine (2CA) in a synaptoneurosome preparation of guinea-pig cerebral cortex (Hollingsworth *et al.*, 1985). However, since the concentrations of phorbol ester used were much higher than those normally required to activate protein kinase C, the significance of this observation has been challenged (Danoff & Young, 1987).

An involvement of calcium in the augmentation response has been suggested, since a reduction in extracellular free calcium concentration prior to agonist addition has been shown to abolish the synergism between histamine and adenosine analogues in slices of guinea-pig cerebral cortex (Schwabe *et al.*, 1978). Furthermore, in rabbit cerebral cortex the indirect H_1-receptor-mediated cyclic AMP response has been found to be reduced in calcium-free Krebs or by addition of EGTA (Al-Gadi & Hill, 1987). In slices of guinea-pig hippocampus, the H_1-receptor-mediated augmentation of the direct H_2-receptor-induced cyclic AMP response appears to involve both calcium ions and diacylglycerol (Garbarg & Schwartz, 1988).

The rise in intracellular calcium in response to histamine H_1-receptor activation has been studied in some detail in both vascular and tracheal smooth muscle, with calcium-sensitive fluorescent dyes (Reynolds & Dubyak, 1986; Matsumoto *et al.*, 1986; Takuwa *et al.*, 1987; Kotlikoff *et al.*, 1987). Following H_1-receptor activation in these cells, there appears to be a large transient rise in intracellular calcium which peaks around 1–2 min then falls to a plateau somewhat above basal. The contractile response, however, remains constant despite the fall in intracellular calcium. This observation has led several authors to suggest that the initiation of the contractile response is dependent upon mobilization of calcium while contraction is maintained by the activation of protein kinase C (Park & Rasmussen, 1985; Takuwa *et al.*, 1987). If the rise in intracellular calcium following H_1-receptor activation in guinea-pig brain slices were also transient, then the calcium sensitivity of the response might be expected to change with time. In the present study we have therefore investigated in detail the temporal profile of the calcium sensitivity of the augmentation by histamine of the cyclic AMP response to 2CA in slices of guinea-pig cerebral cortex, by examining the effect of a sudden reduction in external free calcium (and associated change in intracellular calcium e.g. see Al-Gadi & Hill, 1987) with ethylene-glycol-*bis* (β-amino ethylether)N,N′-tetraacetic acid (EGTA) at various times before and after histamine addition at 2CA-induced steady state levels of cyclic AMP.

Methods

Measurement of [^{3}H]-cyclic AMP accumulation

Cyclic AMP accumulation was determined by the [^{3}H]-adenine prelabelling technique, essentially as described previously (Donaldson *et al.*, 1988b). Briefly, slices (300 × 300 μm) of guinea-pig cerebral cortex were labelled with [^{3}H]-adenine, washed three times with Krebs-Henseleit solution at 37°C and finally allowed to settle under gravity. Slices (1 ml packed volume) were transferred to a 20 ml glass vial containing 8 ml Krebs-Henseleit solution (calcium concentration 2.5 mM). Slices were stirred at a rate sufficient to prevent them settling. The H_2-receptor antagonist tiotidine (30 μM) was included in the incubation medium in order to eliminate the direct actions of histamine (acting via H_2-receptors). The direct cyclic AMP stimulus was provided by the stable adenosine analogue 2CA, and the effects of endogenous adenosine were eliminated by inclusion of adenosine deaminase (1.2 u ml^{-1}) in the incubation medium. At various times before and after EGTA addition, 300 μl aliquots of the slice suspension were taken for analysis of cyclic AMP. A concentration of 5 mM EGTA was used, since this concentration was calculated, using constants for Mg^{2+}, Ca^{2+}, and H^+ binding to EGTA given by Sillen & Martell (1964), to reduce extracellular free calcium to below 0.1 μM. Addition of 5 mM EGTA to Krebs solution causes a decrease in pH (from 7.5 to 6.7) because of H^+ release on calcium chelation. Consequently in all experiments it was added in combination with sufficient 1 M NaOH to maintain a pH of 7.5 in the incubation medium.

In experiments using calcium channel antagonists and some of the experiments using phorbol esters, incubations were performed in flat bottomed insert vials. In these experiments, 50 μl portions of gravity packed slices were added to 240 μl of Krebs medium

containing tiotidine (30 μM), calcium channel antagonists, phorbol esters or adenosine deaminase (1.2 u ml^{-1}) where appropriate. Tubes were gassed with 95% O_2:5% CO_2, capped and incubated for 10–20 min at 37°C in a shaking water bath. Agonist was added after this step in 10 μl of medium and the tubes gassed again and incubated for an additional 10 min.

Incubations or aliquots from them (300 μl) were stopped and cyclic AMP extracted by mixing with 200 μl ice cold 1 M HCl. The [^{3}H]-cyclic AMP was then isolated by sequential Dowex-alumina chromatography, as described previously (Donaldson *et al.*, 1988a). The recovery of [^{3}H]-cyclic AMP off the columns was corrected for by spiking the samples with 100 μl [^{14}C]-cyclic AMP (0.001 μCi, 0.24 μM).

Calcium content of the slices

The calcium content of guinea-pig cerebral cortical slices was monitored at various times before and after addition of EGTA (5 mM) by atomic absorption spectrophotometry (Pye-Unicam series 2). Aliquots (300 μl) of the slice suspension were added rapidly to 1 ml ice-cold calcium-free Krebs and the slices precipitated by spinning at 12,000 *g* in a Beckman Microfuge B for 5 s. The supernatant was discarded and the slices rapidly washed a second time in 1 ml ice-cold calcium-free Krebs. The tissue calcium was finally extracted in 250 μl of 5% trichloroacetic acid. The sample was diluted with 250 μl of a solution containing 200 mM NaCl, 200 mM KCl and 2% $LaCl_3$. Tissue debris was precipitated by brief centrifugation at 12,000 *g*. The calcium content of the supernatant was then analysed by atomic absorption spectrophotometry. Calcium standards were prepared in twice distilled water containing 100 mM KCl, 100 mM NaCl, 1% $LaCl_3$ and 2.5% trichloroacetic acid. The protein content of slices was determined by the method of Lowry *et al.* (1951).

In preliminary experiments, the effect on the tissue calcium content of including 1 mM EGTA in the ice-cold calcium-free wash buffer, was investigated. No significant difference was observed in the recorded tissue calcium content with the two different wash buffers. Consequently, EGTA was not included in the wash medium in all subsequent experiments.

Measurements of extracellular free calcium

Extracellular free calcium concentration was determined by use of a calcium electrode (Orion Research Inc., Cambridge, MA) with a silver/silver chloride reference electrode. Calcium standards were prepared over the range 2.5 mM to 10 μM in de-ionised twice-distilled water (further purified with an Elgastat UHQ) containing 140 mM NaCl, 5 mM KCl, 10 mM HEPES, pH 7.4. Zero calcium was determined in an aliquot of this buffer containing 5 mM EGTA. The standard curve (mV vs log [Ca]) was linear over this range. The response to a free calcium concentration below 0.1 μM was obtained with an aliquot of the same buffer containing 5 mM EGTA.

Data analysis

In time course studies, cyclic AMP was measured at single time points and the lines through the data points were drawn by inspection. Each experiment was repeated at least three times. The mean effect of each intervention is given in the text along with the s.e.mean. *n* in the text refers to the number of separate experiments. Concentration-response curves for phorbol esters were fitted by non-linear regression using the programme Graphpad. The equation fitted was:

$$\% \text{ of maximum response} = \frac{E_{max} \times D^n}{D^n + (EC_{50})^n}$$

where D is the concentration of phorbol ester, n is the Hill coefficient, EC_{50} is the concentration of phorbol giving 50% maximal response and E_{max} is the maximal stimulation.

Chemicals

Dowex 50W, H^+ form (200–400 mesh), 2-chloroadenosine, adenosine deaminase (type VI), neutral alumina (type WN-3), imidazole, mepyramine maleate, histamine dihydrochloride, ethyleneglycol-*bis*(β-amino ethylether)N,N′-tetraacetic acid (EGTA), 4β-phorbol 12-myristate 13-acetate, 4β-phorbol 12,13-dibutyrate, 4α-phorbol, nifedipine, nickel chloride, cadmium chloride, polymyxin B sulphate and H-7 (1-(5-isoquinolinylsulphonyl)-2-methyl-piperazine dihyrochloride) were purchased from Sigma. [8-^{3}H]-adenine (specific activity, 26 Ci $mmol^{-1}$) was obtained from Amersham International, and [8 – ^{14}C]-cyclic AMP (specific activity 42.4 mCi mol^{-1}) from Dupont-N.E.N. Tiotidine was a gift from I.C.I. PN-200-110 (isopropyl 4-(2,1,3,benzoxadiazol-4-yl)-1,4-dihydro-2,6-dimethyl-5-methyoxy-carbonyl-pyridine-3-carboxylate) was obtained from Sandoz and staurosporin from Boehringer Mannheim.

Results

Effect of EGTA

Addition of 30 μM 2CA to guinea-pig cerebral cortical slices resulted in an increase in cyclic AMP levels,

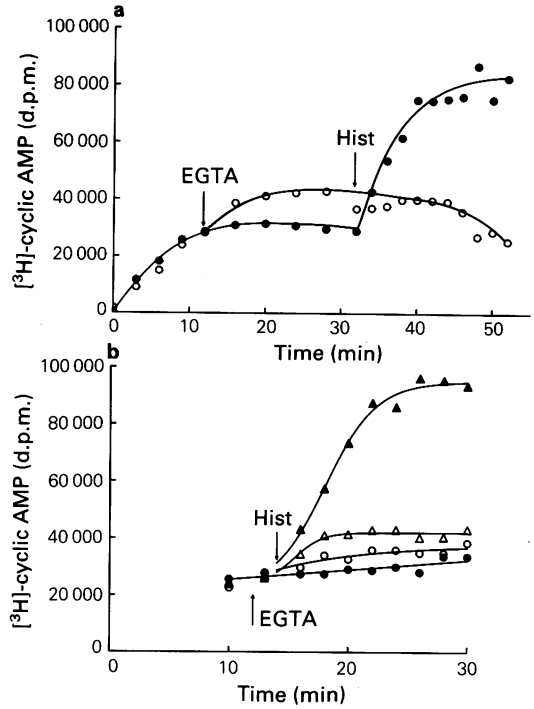

Figure 1 Effect of 5 mM EGTA on the cyclic AMP response to 2-chloroadenosine (2CA) (30 μM) and 0.1 mM histamine in guinea-pig cerebral cortical slices. (a) EGTA added 20 min prior to histamine at 2CA steady state: (●) control, (○) EGTA. 2CA was added at zero time. Histamine (Hist) was added 32 min after 2CA. (b) Effect of addition of EGTA 2 min prior to histamine at 2CA steady state. 2CA (30 μM) was added at zero time: (●) 2CA alone; (○) 5 mM EGTA added at 2CA steady state; (▲) 0.1 mM histamine added at 2CA steady state; (△) 5 mM EGTA added at 2CA steady state followed by 0.1 mM histamine 2 min later. Experiments (both a and b) were performed in the presence of adenosine deaminase (1.2 u ml^{-1}). The H_2-receptor antagonist tiotidine (30 μM) was also included in the incubation medium in order to prevent the direct actions of histamine via H_2-receptors (Donaldson *et al.*, 1988b). Data were obtained in single experiments. Similar results were obtained on (a) two or (b) five other occasions.

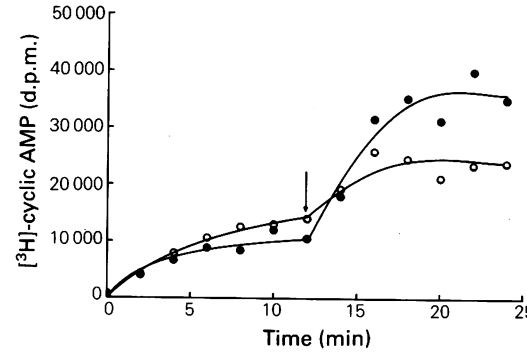

Figure 2 Comparison of the histamine-induced augmentation of the cyclic AMP response to 2-chloroadenosine (2CA) in guinea-pig cerebral cortical slices in calcium-containing (2.5 mM) and calcium-free Krebs: (●) calcium-containing; (○) calcium-free. 2CA (30 μM) was added at zero time and histamine (0.1 mM) 12 min later (at arrow). In both cases slices were prelabelled with [^{3}H]-adenine in calcium-containing Krebs as described under Methods. The H_2-receptor antagonist tiotidine (30 μM) and adenosine deaminase (1.2 u ml^{-1}) were included in the incubation medium. Data were obtained in a single experiment. Similar results were obtained on two other occasions.

which reached steady state within 10 min (46.1 ± 3.4 fold over basal, $n = 29$). EGTA (5 mM) had no significant effect on basal levels of cyclic AMP ($n = 6$) but elevated the cyclic AMP response to 2CA (30 μM), both when it was added 20 min or 2 min before 2CA (data not shown) and 12 min after (i.e. during 2CA-induced steady state) (Figure 1a). Addition of EGTA 2 min before or 12 min after 2CA (Figure 1a), resulted in an increase in the steady state level of cyclic AMP in the presence of 2CA of 1.61 ± 0.61 fold ($n = 3$) and 1.34 ± 0.06 fold ($P < 0.05$ paired t test, $n = 3$) over the response to 2CA in the absence of EGTA, respectively. When EGTA was added 20 min before 2CA, it produced a maximum elevation of the 2CA response of 1.35 ± 0.03 ($n = 3$, $P < 0.01$ paired t test). However, in this case a steady state level of cyclic AMP was not maintained in the presence of EGTA (data not shown).

Addition of 0.1 mM histamine to guinea-pig cerebral cortical slices at 2CA steady state, caused cyclic AMP levels to rise and achieve a new steady state, again within 10 min (3.70 ± 0.17 fold over the steady state response to 2CA, $n = 28$) (Figure 1). When 5 mM EGTA was added 2 min before histamine (0.1 mM) at 2CA steady state, histamine-induced augmentation was markedly reduced (by 71.7 ± 8.4% of the response in the absence of EGTA, $n = 6$, Figure 1b). If EGTA was added 20 min before the histamine ($n = 3$, Figure 1a), the histamine-induced augmentation was totally abolished.

These results suggested that the histamine response was in some way dependent on calcium. However, it is possible that these effects of EGTA on cyclic AMP accumulation may not be due to the chelation of calcium, but to another property of the EGTA. In order to investigate this possibility, we compared the histamine-induced cyclic AMP

response in normal Krebs (2.5 mM calcium) and in nominally calcium-free Krebs (50 μM calcium in the presence of slices, 5 μM calcium in the absence). In these experiments, slices were preincubated and labelled with [^{3}H]-adenine in normal Krebs buffer. The slices were then divided into two batches. One batch was washed and incubated in normal Krebs; the other was washed with calcium-free Krebs and the experiment carried out in this latter buffer. In calcium-free conditions the histamine-induced augmentation was reduced by 43.2 ± 1.5% ($n = 3$, Figure 2) whilst the response to 2CA alone was unaffected (Figure 2). These experiments therefore provided further evidence for a role for calcium in the augmentation response.

The next series of experiments investigated the effect of a sudden reduction in external calcium after the histamine had been added. In contrast to the results of experiments in which EGTA was added before histamine, addition of EGTA once a steady state level of [^{3}H]-cyclic AMP had been achieved with histamine (in the presence of 2CA) was without significant effect on the augmentation response (Figure 3a). Following EGTA addition, cyclic AMP levels fell by only 3.3 ± 2.3% ($n = 5$) over 10 min, even though a large significant fall ($P < 0.05$, 44.1 ± 4.5% over 10 min, $n = 3$) was obtained following addition of the H_1-receptor antagonist mepyramine (1 μM) (data not shown but see Donaldson *et al.*, 1988b).

In order to investigate at what point EGTA became ineffective in reducing the cyclic AMP response to histamine, we examined the effect of adding EGTA 1 min or 3 min after histamine (at 2CA steady state). At these times a new steady state level of cyclic AMP had not yet been achieved (see Figure 1). Following EGTA addition at either of these times, the augmentation was substantially reduced (Figure 3b) and although cyclic AMP levels continued to rise for 2–4 min after EGTA addition they then gradually fell towards basal ($n = 3$ in each case).

These results therefore suggested that calcium was important for the initiation and early stages of the augmentation response to histamine, but was less important for its maintenance, once a steady state had been attained. However, this apparent change in the calcium sensitivity of the response could be explained if EGTA was having different effects on calcium levels at the different time points. The effects of EGTA on both extracellular free calcium and tissue calcium content during cyclic AMP steady states in the presence of 2CA or 2CA plus histamine were therefore measured (Figure 4). The effect of EGTA on extracellular free calcium was apparently the same at the two steady states. In the presence of 2CA or 2CA plus histamine, EGTA addition caused

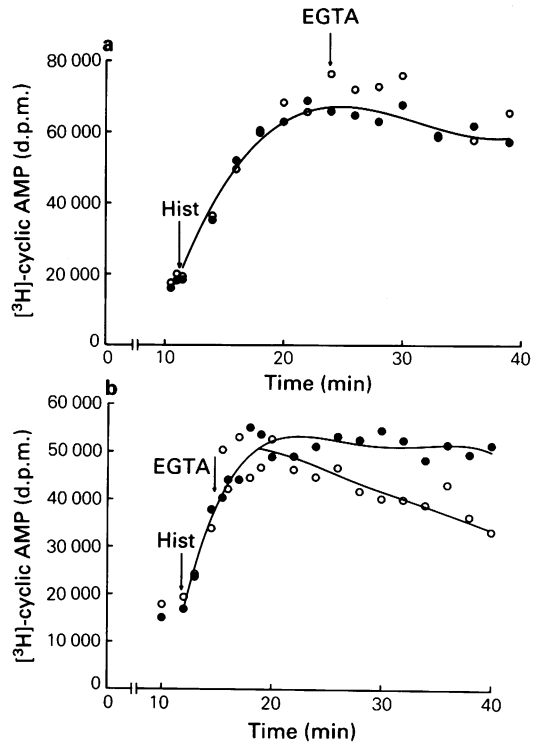

Figure 3 Effect of addition of EGTA (a) at histamine steady state or (b) 3 min after histamine (Hist) on the histamine-induced augmentation of the 2-chloroadenosine (2CA) cyclic AMP response in guinea-pig cerebral cortex: (●) control; (○) EGTA. 2CA (30 μM) was added at zero time. Histamine (0.1 mM) was added 12 min after 2CA. EGTA (5 mM) was added (a) 12 or (b) 3 min after histamine. The experiment was performed in the presence of adenosine deaminase (1.2 u ml^{-1}) and tiotidine (30 μM). Data were obtained in single experiments. Similar results were obtained on (a) four or (b) two other occasions.

extracellular free calcium to fall rapidly to below 10 μM ($n = 3$) (Figure 4a and b). EGTA also produced a rapid and large reduction in the tissue calcium content (Figure 4c and d). Addition of EGTA at either 2CA- or histamine-induced steady states caused tissue calcium content to fall by 50–70% ($n = 3$ in each case) within 2 min. A more detailed analysis of the fall in tissue calcium content over the first minute following EGTA addition revealed that calcium levels were unaffected for the first 20 s, but then fell rapidly over the next 20 s (33.3 ± 2.4% in 20 s, $n = 3$, data not shown). However, since the fall in tissue calcium content was similar at the two cyclic AMP steady states, it could not account for

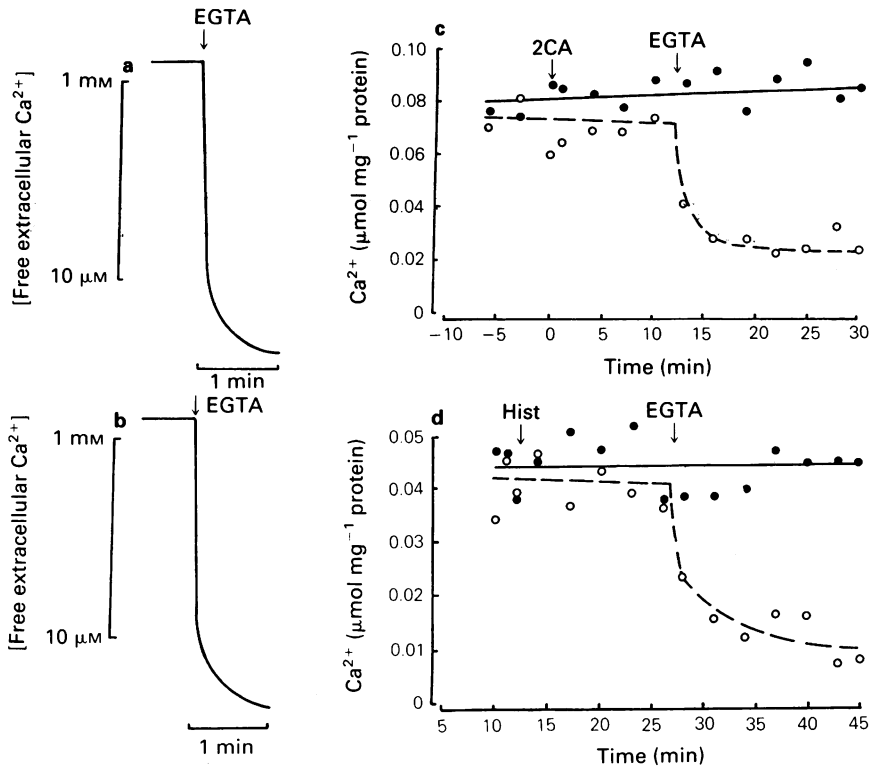

Figure 4 Effect of 5 mM EGTA on extracellular free calcium concentration (a and b) and tissue calcium content (c and d) in a suspension of guinea-pig cerebral cortical slices incubated with 2-chloroadenosine (2CA) or 2CA + histamine. In (a) and (c) EGTA was added at 2CA (30 μM) steady state (i.e. 12 min after 2CA). In (b) and (d) histamine (Hist, 0.1 mM) was added 12 min after 2CA and EGTA 12 (b) or 14 (d) min later (i.e. at histamine steady state): control (●); EGTA (○). Extracellular free calcium concentration was determined with a calcium electrode and tissue calcium content measured as described under methods. Data were obtained in single experiments but are representative of those obtained on two other occasions.

the apparent differences in calcium sensitivity at the different time points.

Calcium channel antagonists

In addition to chelating extracellular calcium, EGTA also had a rapid profound effect on tissue calcium content. It was therefore difficult to draw any conclusions as to the origin of the calcium involved in the histamine augmentation response from these experiments. We therefore performed a number of experiments using calcium channel antagonists to assess whether an influx of extracellular calcium was involved in the effect of histamine. An example of two of these experiments is shown in Table 1. The dihydropyridines, PN-200-110 and nifedipine were without significant effect on the cyclic AMP responses to 2CA (30 μM) and 2CA plus histamine (0.1 mM) ($n = 3$). However, the divalent cation nickel (1 mM) whilst having no significant effect on the cyclic AMP response to 2CA (30 μM) significantly reduced the histamine-induced augmentation (by 40.8 ± 2.6% of that in the absence of nickel, $n = 3$, $P < 0.01$, paired t test). Another divalent cation, cadmium (20 μM) also reduced the histamine response (by 29.6 ± 7.6%, $n = 3$). However, this concentration of cadmium also reduced the cyclic AMP response to 2CA to a similar extent (22.3 ± 3.5%, $n = 3$, $P < 0.05$, paired t test).

Effect of phorbol esters

The phorbol ester 4β-phorbol 12,13-dibutyrate (4βPDB) strongly augmented the maximal stimu-

Table 1 Effect of various calcium channel antagonists on the accumulation of cyclic AMP elicited by 2-chloroadenosine (2CA) (30 μM) or 2CA (30 μM) plus histamine (0.1 mM) in guinea-pig cerebral cortical slices

	Accumulation of [^{3}H]-cyclic AMP (d.p.m.) in the presence of	
	2CA	*2CA + histamine*
Experiment 1		
Control	23440 ± 901	78859 ± 11461
Nifedipine (1 μM)	23173 ± 906	87435 ± 2554
Nifedipine (10 μM)	25050 ± 515	91222 ± 3001
PN 200–110 (1 μM)	22484 ± 825	85563 ± 3658
PN 200–110 (10 μM)	24090 ± 702	84756 ± 3859
Experiment 2		
Control	36767 ± 675	140972 ± 6985
Cadmium (20 μM)	31125 ± 1673	131567 ± 6340
Nickel (1 mM)	34940 ± 1301	89789 ± 1322*
Cadmium (20 μM) + nickel (1 mM)	36751 ± 1664	89641 ± 3867*

Slices were incubated with antagonists for 10 min before the addition of agonist for a further 10 min as described under Methods. Control indicates the response to either 2CA or 2CA plus histamine in the absence of any calcium antagonist. Data represent the mean ± s.e.mean of three incubations in single experiments. *$P < 0.001$ compared to 2CA + histamine control (one-way analysis of variance). Similar results were obtained on two other occasions and the statistical data for the three paired experiments is given in the text.

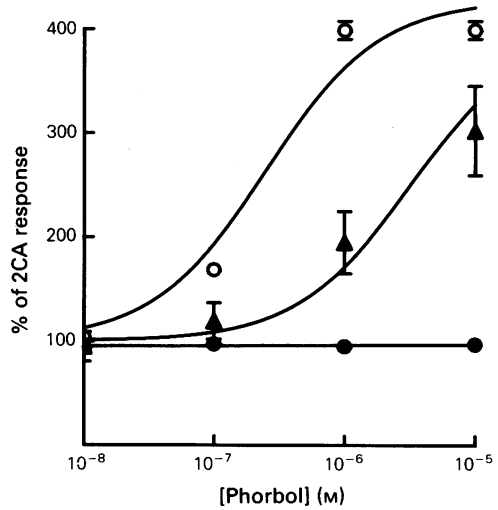

Figure 5 Effect of various phorbol esters on the stimulation of cyclic AMP accumulation elicited by 2-chloroadenosine (2CA) 30 μM in slices of guinea-pig cerebral cortex: (●) 4α-phorbol; (▲) 4β-phorbol 12-myristate 13-acetate (4β-PMA), (○)4β-phorbol 12,13-dibutyrate (4β-PDB). Slices were preincubated with phorbols for 20 min prior to addition of 2CA for a further 10 min. Data are expressed as % stimulation over the response to 2CA alone. Each point represents the mean of three incubations with s.e.mean shown by vertical bars. Where error bars are not shown, they are within the size of the symbol. Adenosine deaminase (1.2 u ml^{-1}) was included in the incubation medium.

lation of cyclic AMP accumulation elicited by 30 μM 2CA in a concentration-dependent manner (EC_{50} 2.3×10^{-7} M, Figure 5). Another phorbol ester, 4β-phorbol 12-myristate 13-acetate (4βPMA), also augmented the response to 2CA (30 μM) (Figure 5). A maximum response was not achieved with the highest concentration used (10 μM) so an EC_{50} value could not be calculated. This latter phorbol ester, however, appeared to be approximately 10 fold less potent than 4β-PDB (Figure 5). In contrast, the inactive phorbol ester 4α-phorbol had no stimulatory effect at concentrations up to 10 μM (Figure 5). All three phorbol esters were without effect on basal levels of cyclic AMP (data not shown).

The time course of the accumulation of cyclic AMP following addition of a low dose of 1 μM 4β-PMA at 2CA steady state is shown in Figure 6. This low dose was selected, since the need to avoid high doses and thus non-specific effects has been stressed (Nishizuka, 1984). Following addition of 4β-PMA, cyclic AMP levels rose to a new steady state (1.94 ± 0.12 fold over the response to 2CA

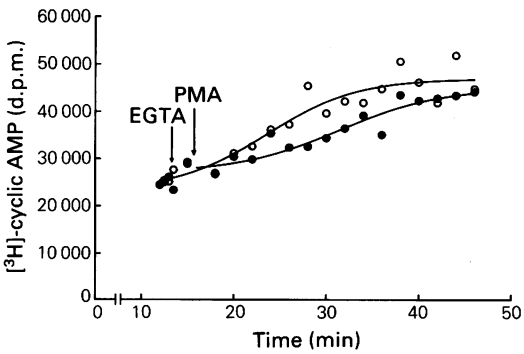

Figure 6 Effect of addition of 5 mM EGTA 2 min before 4β-phorbol 12-myristate 13-acetate (β-PMA) (1 μM) at 2-chloroadenosine (2CA) cyclic AMP steady state in slices of guinea-pig cerebral cortex. (●) 4β-PMA, (○) 4β-PMA + EGTA. 2CA (30 μM) was added at zero time. EGTA was added 12 min after 2CA and 4β-PMA 2 min after EGTA. The experiment was performed in the presence of adenosine deaminase (1.2 u ml^{-1}). Data were obtained in a single experiment. Similar results were obtained in 5 other experiments.

alone, $n = 8$) within 25 min. Similar results were obtained with 4β-PDB. Following addition of this latter phorbol (at a concentration of 0.1 μM) in the presence of 2CA, cyclic AMP levels rose to 1.94 ± 0.10 ($n = 3$) over the response to 2CA alone, again within 25 min. The time course of the augmentation produced by the phorbols was notably slower than that produced by histamine (Figure 1). In addition, the phorbol ester response was insensitive to removal of extracellular calcium with EGTA. Following addition of 5 mM EGTA 2 min before 4β-PMA at 2CA steady state, the augmentation produced by the phorbol ester was slightly increased (Figure 6, $n = 6$). This increase was however consistent with the elevation of the 2CA response by EGTA.

The possibility of using protein kinase C inhibitors to investigate the role of protein kinase C in the histamine-induced augmentation response was investigated. However, all the inhibitors used were unsatisfactory. Polymyxin B and H-7 showed no inhibition of the response to 1 μM 4β-PMA at concentrations up to 0.1 mM (data not shown). Staurosporin produced a very weak inhibition of the 4β-PMA (1 μM) response at concentrations of 0.1 μM and above. However, these concentrations of staurosporin also augmented the 2CA cyclic AMP response on their own (by 1.67 ± 0.17 fold, $n = 5$).

Discussion

Stimulation of H_1-receptors in guinea-pig cerebral cortical slices augmented the cyclic AMP response to 2CA, thus confirming the results of several previous studies (Daly, 1977; Hill *et al.*, 1981; Schwartz *et al.*, 1982; Hough & Green, 1984; Hollingsworth & Daly, 1985; Shonk & Rall, 1987; Donaldson *et al.*, 1988a,b; Hill & Kendall, 1989). The mechanism by which histamine produces this effect is uncertain. However, the products of the inositol phospholipid breakdown which occurs in response to H_1-receptor activation (Daum *et al.*, 1984; Carswell *et al.*, 1985; Claro *et al.*, 1986; Donaldson & Hill, 1986), have been implicated as mediators of this response (Schwabe *et al.*, 1978; Hollingsworth & Daly, 1985; Al-Gadi & Hill, 1987; Garbarg & Schwartz, 1988). The two products of inositol phospholipid hydrolysis, the inositol phosphates and diacylglycerol, are thought to mediate their effects by elevating intracellular calcium and activating protein kinase C respectively. The present study concentrates on the role of calcium in the augmentation response. In previous studies, histamine and adenosine have been added simultaneously under calcium-free conditions and the effects on the cyclic AMP accumulating after 10 min examined (Schwabe *et al.*, 1978; Al-Gadi & Hill, 1987). We have now investigated the time course of the effect of a sudden reduction in the external calcium concentration with EGTA, at various times before and after the addition of histamine at 2CA-induced steady state.

Addition of EGTA to guinea-pig cerebral cortical slices was found to produce a slight increase in the cyclic AMP response to 2CA, whether it was added 2 or 20 min before 2CA or at 2CA steady state. A similar elevation of the cyclic AMP response to adenosine in guinea-pig cerebral cortical slices by EGTA has been reported previously (Schwabe *et al.*, 1978). The reason for this is not known. One possible explanation of the effect of EGTA on the adenosine response could be that in the absence of extracellular calcium, the adenosine receptor undergoes a conformational change to a more active form.

The histamine-induced cyclic AMP response in cerebral cortical slices was much more dependent on calcium than the response to 2CA. When extracellular calcium was removed 20 min before histamine was added at 2CA steady state, the histamine-induced augmentation was completely abolished, while if EGTA was added just 2 min before histamine, a substantial reduction in the augmentation (72%) was still observed. These results therefore suggest that calcium has a role in the histamine-induced cyclic AMP response. However, it was difficult to determine from these experiments whether the calcium involved in the augmentation process was predominantly of an intra- or extracellular origin. Although EGTA was used to chelate extracellular calcium, it also had a profound effect on tissue calcium content. Reductions in the tissue calcium content were detectable after only 20 s and were greater than 30% within 1 min. The determination of tissue calcium is only a very crude method of assessing intracellular calcium levels and will partly reflect calcium binding to the extracellular membranes and entrapment of free calcium in the extracellular matrix within the slices. However, the fact that the inclusion of 1 mM EGTA in the wash solution did not reduce the measurements of tissue calcium content, would suggest that the calcium may be predominantly intracellular. Furthermore, the 20 s delay observed following addition of 5 mM EGTA before a measurable fall in slice calcium content was seen, also suggests that the calcium measured is predominantly intracellular.

In order to address the question of the origin of the calcium involved in the histamine response we performed a number of experiments using calcium channel antagonists to prevent any influx of extracellular calcium. Three different tyes of voltage-sensitive calcium channels have been defined by

electrophysiological and pharmacological techniques, the T, N and L channels (Hofmann *et al.*, 1987). T channels are blocked by 0.1 mM nickel (Hofmann *et al.*, 1987), L channels by the dihydropyridines (Triggle & Janis, 1987; Miller, 1987), whilst both N and L channels are blocked by 20 μM cadmium (Hofmann *et al.*, 1987). Furthermore millimolar concentrations of nickel have been reported to block a voltage-insensitive calcium channel responsible for refilling intracellular calcium stores, without affecting release of intracellular calcium (Hallam *et al.*, 1989; Carter *et al.*, 1988). Of the calcium channel antagonists tested, only Ni^{2+} (1 mM) produced a marked and selective attenuation of the H_1-receptor-mediated augmentation of cyclic AMP accumulation, although some 60% of the response to histamine remained in the presence of this antagonist. These results therefore suggest that the histamine-induced augmentation of the cyclic AMP response to 2CA in guinea-pig cerebral cortex is mediated predominantly by a release of stored intracellular calcium and is dependent to a lesser extent on influx of extracellular calcium. The small influx of calcium may enter via the voltage-independent channels responsible for refilling intracellular pools (Hallam *et al.*, 1989) or via the voltage-dependent T channels. It was not possible to eliminate the T channels as a specific antagonist for these channels was not available. These results are not inconsistent with data obtained by other workers in vascular and tracheal smooth muscle (Reynolds & Dubyak, 1986; Takuwa *et al.*, 1987). They discovered, using calcium-sensitive fluorescent dyes, that agonist-induced contraction is mediated by an initial mobilisation of intracellular calcium, followed by an influx of extracellular calcium.

Interestingly, when we studied the effects of EGTA addition after the histamine response was established, we discovered apparent differences in the calcium sensitivity of the response. When EGTA was added 1 or 3 min after histamine, the augmentation was not maintained. However, if EGTA was added once steady state had been achieved with histamine, it was apparently without effect. This was in marked contrast to the effect observed following addition of the H_1-receptor antagonist mepyramine at the histamine steady state (Donaldson *et al.*, 1988b and confirmed in the present study when the effects of EGTA and mepyramine additions were compared in the same experiment). Mepyramine addition caused cyclic AMP levels to fall to a level equivalent to that obtained with 2CA alone. Thus the apparent change in calcium sensitivity cannot be explained by a change at the receptor, since the H_1-receptor stimulus was still required to maintain the augmentation once steady state had been reached. The difference in calcium sensitivity could also be explained if EGTA were having different effects at the different time points. However, this seems unlikely since EGTA had similar effects on both extracellular free calcium and tissue calcium content, when it was added either at 2CA- or histamine-induced steady states.

There are thus two possible interpretations of this data. Firstly, it could be that calcium is important for the initiation and early stages of the augmentation, but is not involved in the maintenance of the response. Alternatively, it could be that the intracellular machinery involved in the augmentation response becomes more sensitive to calcium as the response progresses, so that it becomes able to operate at a much lower intracellular calcium concentration. In this respect it is interesting that in both vascular and tracheal smooth muscle, there is a large transient rise in intracellular calcium following receptor activation (Reynolds & Dubyak, 1986; Takuwa *et al.*, 1987) which peaks at around 1–2 min and then falls to a plateau somewhat above basal, whilst the contractile response remains unchanged. The question remains as to how the response is maintained if it does not involve calcium, or how changes are brought about to enable the response to operate at a lower level of calcium. One possibility is that diacylglycerol, another product of inositol phospholipid hydrolysis, is involved. This hypothesis is supported by the observed augmentation of the cyclic AMP response to 2CA produced by phorbol esters (which mimic the actions of diacylglycerol) in the present study. As mentioned previously phorbol esters have been shown to augment the cyclic AMP response to 2CA in a synaptoneurosome preparation of guinea-pig cerebral cortex (Hollingsworth *et al.*, 1985). It should however be noted that the physiological significance of this phorbol ester response has recently been challenged (Danoff & Young, 1987) since higher concentrations are required in slice preparations to produce the same effect as in a purified preparation. However, in view of the additional diffusional barriers that the slice preparation possesses these findings are not surprising. Garbarg & Schwartz (1988) have recently shown that the concentrations of 4β-PMA and 4β-PDB as used in the present study were effective in augmenting the cyclic AMP response to H_2-receptor activation in guinea-pig hippocampus. The fact that the response to 4β-PMA was insensitive to EGTA provides further support for a role of diacylglycerol in the augmentation response at the later time points.

The hypothesis that the intracellular machinery becomes more sensitive to calcium as the response progresses, is attractive in view of data obtained in other tissues. Bruschi *et al.* (1988) have presented some evidence for the existence of factors in vascular smooth muscle which sensitize the contractile machinery to calcium. These authors also proposed

that this 'sensitization' may be brought about by a phosphorylation of the myosin light chain. Protein kinase C is a phosphorylating enzyme which, while being calcium-dependent, has been reported to lower the calcium requirements for cellular activation (Nishizuka, 1986). One could therefore envisage that in guinea-pig brain, the diacylglycerol produced upon H_1-receptor activation could activate protein kinase C, which would in turn phosphorylate adenylate cyclase and thus make it more sensitive to calcium. This theory is particularly appealing since it has been demonstrated *in vitro* that protein kinase C can phosphorylate the catalytic unit of adenylate cyclase purified from bovine brain (Yoshimasa *et al.*, 1987).

In conclusion, the calcium sensitivity of the histamine-induced cyclic AMP response changes with time. The earlier but not the later stages of the augmentation response are particularly sensitive to calcium removal. A role for diacylglycerol in maintaining the histamine response at later time points is suggested.

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The protein kinase C inhibitor 1-(5-isoquinolinesulphonyl)-2-methylpiperazine (H-7) disinhibits CA1 pyramidal cells in rat hippocampal slices

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1 The effects of the protein kinase C (PKC) inhibitor 1-(5-isoquinolinesulphonyl)-2-methylpiperazine (H-7) on evoked synaptic potentials were investigated in the CA1 region of rat hippocampal slices by use of extracellular and intracellular recording techniques.

2 Extracellular recordings showed that superfusion with H-7 (10–100 μM) increased the amplitude of the population spike and the initial slope of the dendritic field e.p.s.p. H-7 also produced the appearance of multiple population spikes in the somatic region and in the dendritic field e.p.s.p.

3 H-7 (30 μM) induced the disappearance of intracellularly recorded inhibitory potentials elicited by orthodromic stimulation of CA1 pyramidal cells. At this concentration H-7 had no effect on resting membrane potential, input membrane resistance, and spike threshold. In voltage-clamped neurones H-7 blocked the antidromically evoked inhibitory currents and the spontaneous miniature inhibitory currents.

4 The hyperpolarizing effect of bath applied γ-aminobutyric acid (GABA, 500 μM) or isoguvacine (30 μM) was not affected by 30 μM H-7.

5 Neither the PKC activity regulator sphingosine (10–40 μM) nor the H-7 analogue N-(2-guanidinoethyl)-5-isoquinolinesulphonamide (HA-1004, 20–50 μM) which is devoid of activity on PKC at these concentrations, affected the extracellularly recorded dendritic field e.p.s.p. or population spike.

6 It is concluded that the disinhibitory effect produced by H-7 is due to the block of a H-7-sensitive PKC which is involved in the spontaneous and evoked release of GABA.

Introduction

Protein kinase C (PKC) is believed to influence important processes of neuronal physiology from neuronal growth to the long-term potentiation of neurotransmission in the hippocampus (Nishizuka, 1988; Chiarugi *et al.*, 1989). Among its multiple effects, it has been suggested that PKC regulates the release of neurotransmitters (Shapira *et al.*, 1987; Malenka *et al.*, 1987; Dekker *et al.*, 1989). In the hippocampus, a brain area containing high concentrations of PKC (Worley *et al.*, 1986) phorbol esters, which directly activate PKC, induce an augmentation of the frequency of spontaneous miniature excitatory and inhibitory potentials (Malenka *et al.*, 1987) and an increase in the size of evoked excitatory potentials (Malenka *et al.*, 1986). These findings suggest an involvement of PKC in the release of excitatory and inhibitory neurotransmitters in the hippocampus. To investigate this hypothesis we studied the effects of the PKC inhibitor, 1-(5-isoquinolinesulphonyl)-2-methylpiperazine (H-7). H-7 has been shown to penetrate cell membranes and to inhibit PKC with an IC_{50} of 6 μM (Hidaka & Hagiwara, 1987). We compared the action of H-7 with that of its analogue, N-(2-guanidinoethyl)-5-isoquinolinesulphonamide (HA-1004), which inhibits the same protein kinases blocked by H-7 but is 7 times less potent in inhibiting PKC activity. For further comparison we used sphingosine, which has

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been shown to inhibit PKC in platelets, neutrophils and fibroblasts with an IC_{50} of less than 10 μM (Hannun *et al.*, 1986).

Methods

Preparation of slices and superfusion apparatus

Preparation of slices was carried out as previously described (Corradetti *et al.*, 1983). Male Sprague-Dawley rats (Charles River Italia; 150–200 g) were killed by decapitation and their hippocampi rapidly dissected in chilled (0°C) oxygenated (95% O_2/5% CO_2) artificial cerebrospinal fluid (aCSF) of the following composition (mM): NaCl 124, KCl 3.33, KH_2PO_4 1.25, $MgSO_4$ 2, $CaCl_2$ 2, $NaHCO_3$ 25, D-glucose 10. Slices (400 μm thick) were cut with a McIlwain tissue chopper and placed in oxygenated aCSF for at least 1 h at room temperature. A single slice was then placed on a nylon mesh, completely submerged in a small chamber and superfused with oxygenated aCSF (32–33°C or 22–25°C for voltage-clamp experiments) at a constant flow rate of 2–3 ml min^{-1}. Application of drugs was allowed by a three way tap and complete substitution of the fluid in the chamber was achieved within 3 min of the start of drug application.

Stimulation of slices and recording techniques

Test pulses (80 μs, 0.1 Hz) were delivered through bipolar nichrome electrodes positioned in the stratum radiatum or in the alveus. Evoked orthodromic potentials were recorded extracellularly with 3 M NaCl-filled microelectrodes (1–5 MΩ) placed in the CA1 region, either in the stratum radiatum or pyramidale, to record dendritic field or somatic potentials, respectively. Responses were amplified (Neurolog, Digitimer Ltd.), digitized, averaged (4–5 responses) and stored on floppy disks for later analysis (DATA 6000, Analogic). Conventional intracellular recordings, by current- or voltage-clamp techniques, were made in CA1 pyramidal cells with K-methylsulphate (3 M, 70–110 MΩ) or KCl-containing (3 M, 40–80 MΩ) microelectrodes. Signals were amplified (Axoclamp 2A, Axon Instruments) digitized, displayed on an oscilloscope and a chart recorder (Gould 2400S) and simultaneously recorded on videotape for further analysis.

Cells were considered viable when the resting membrane potential was -55 mV or more negative and the spike amplitude was greater than 75 mV. Before adding drugs, slices were allowed to equilibrate under stimulation for 30 min or longer, until the recorded potentials remained stable for at least 15 min.

Measurement of recorded potentials

The amplitude of the population spike was calculated by averaging the positive component of the response that preceded and followed the negativity, then measuring the size of the spike relative to this average. To avoid imprecise measurement of the amplitude of dendritic field e.p.s.ps due to contamination by positive-going population spikes, we measured the initial slope of the field e.p.s.p. In some experiments input-output curves were constructed by changing the stimulus strength; the initial slope of the field e.p.s.p. was plotted vs. the fibre volley amplitude. The slope of the linear part of the input-output curves was fitted by linear regression and the statistical significance of the changes in the slope of the input-output curves was assessed with Student's t test for paired data (Tallarida & Murray, 1981). Values quoted in the text are means $\pm$ s.e.mean and have been analyzed for their statistical significance by the paired Student's t test.

Sources of drugs and chemicals

1-(5-Isoquinolinesulphonyl)-2-methylpiperazine (H-7) and N-(2-guanidinoethyl)-5-isoquinolinesulphonamide (HA-1004) were obtained from Seikagaku Kogyo Co. (Tokyo); sphingosine, γ-aminobutyric acid (GABA), bicuculline methiodide, and tetrodotoxin were from Sigma; isoguvacine was from CRB (Cambridge). Sphingosine (40 μM), tested for its PKC inhibitory action on platelet 47 kD-protein phosphorylation induced by thrombin (0.4 u ml^{-1}), resulted in more than 90% inhibition.

Results

Extracellular recordings

Effects of H-7 In the CA1 pyramidal cell layer, bath application of H-7 (10–100 μM) produced an increase in amplitude of the population spike and the appearance of multiple population spikes. An example of this effect is shown in Figure 1a. The effect of H-7 appeared to be maximal at 50 μM ($n = 7$) as judged by the results of 3 experiments in which raising the concentration of H-7 from 50 μM to 100 μM did not produce any further increase of either the amplitude or the number of population spikes. The effect of H-7 developed within 10 min of application, persisted for as long as the drug was applied and disappeared after prolonged wash (Figure 1b).

A similar effect of H-7 was observed in recordings from the dendritic region in CA1. As shown in Figure 2a, H-7 increased the initial slope of the dendritic field e.p.s.p. and induced the appearance of

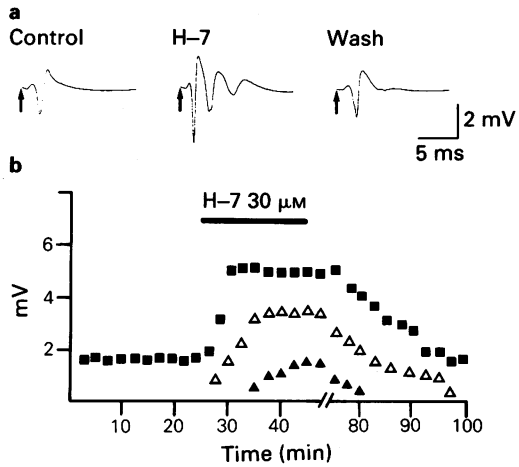

Figure 1 Extracellular recording of the effects of 1-(5-isoquinolinesulphonyl)-2-methylpiperazine (H-7) on evoked responses in CA1 pyramidal cell layer. (a) Stimulation (arrow) of the stratum radiatum evoked an e.p.s.p. with a superimposed population spike (p.s.) under control conditions (left trace). Superfusion of H-7 (30 μM, 15 min) increased the amplitude of the p.s. and elicited the appearance of multiple p.s.s (middle trace). The effect of H-7 was reversible upon 60 min wash (right trace). Traces are the average of 5 evoked potentials. (b) Time course of the effect of H-7 shown in (a). Addition of H-7 increased the amplitude of the p.s. (■) and produced the appearance of a second (△) and a third (▲) p.s. Each point represents the amplitude of the p.s. measured from the average of 5 evoked potentials.

positive going deflections, interpreted as population spikes. Input-output curves, constructed by plotting the fibre volley amplitude vs the initial slope of the field e.p.s.p., were significantly ($P < 0.05$, $n = 6$) steeper in the presence of 30 μM H-7 than in control conditions (Figure 2b).

The effect of H-7, which produced multiple population spikes, resembles that of bicuculline in the same preparation (Schwartzkroin & Prince, 1978). In fact, when the preparation was already disinhibited by superfusion with bicuculline (30 μM), the addition of 100 μM H-7 did not modify the initial slope of the field e.p.s.p. (102 ± 3% of values in bicuculline;

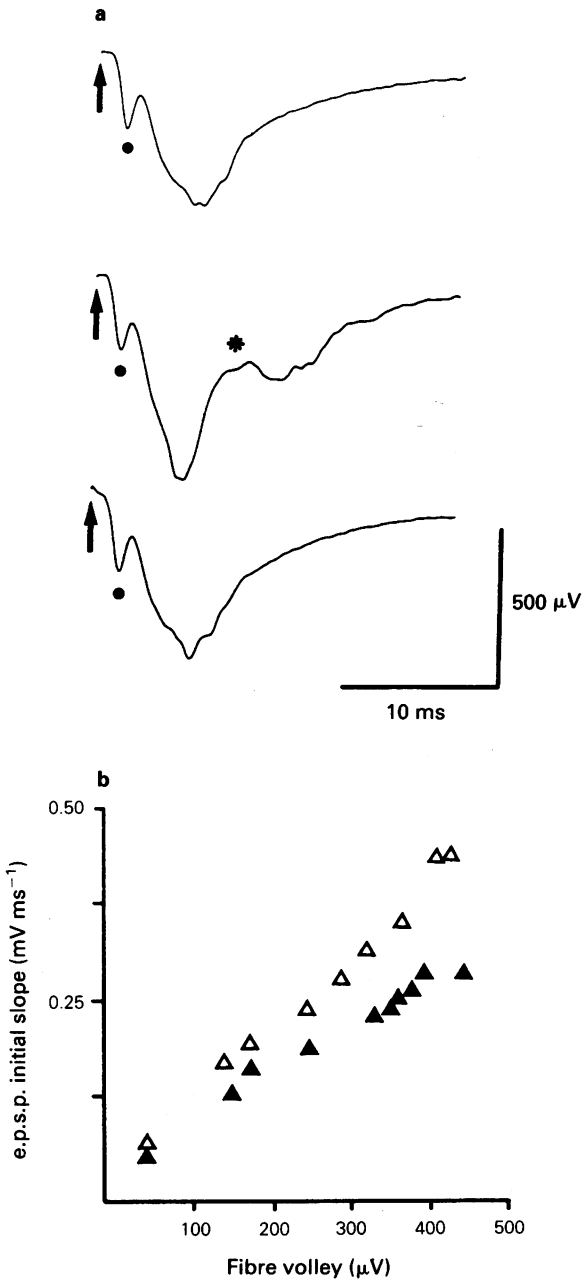

Figure 2 Extracellular recording of the effects of 1-(5-isoquinolinesulphonyl)-2-methylpiperazine (H-7) on the evoked responses in the stratum radiatum. (a) Stimulation (arrow) of the stratum radiatum evoked a field e.p.s.p. in control conditions (upper trace). Addition of H-7 (30 μM, 15 min) increased the initial slope and the amplitude of the field e.p.s.p. (middle trace). Note that the afferent volley (dot) was unchanged. Asterisk indicates the upward deflection due to the appearance of a population spike. The effect of H-7 was reversible upon 45 min wash (lower trace). Traces are the average of 5 evoked potentials. (b) Same experiment as in (a). Input/output curves constructed by changing the stimulus strength under control conditions (▲) and during superfusion with H-7 (△, 30 μM, 15 min). In the presence of H-7 the curve is significantly ($P < 0.05$) steeper than in control conditions.

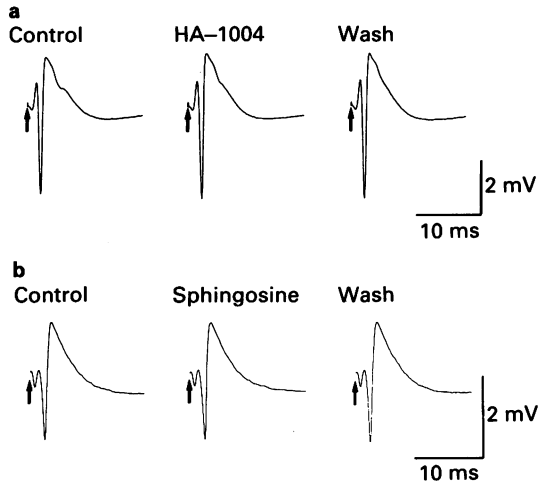

Figure 3 N-(2-guanidinoethyl)-5-isoquinolinesulphonamide (HA-1004) and sphingosine did not affect the evoked responses in CA1 pyramidal layer. (a) Extracellular recordings show somatic responses under control conditions (left trace), in the presence of 50 μM HA-1004 superfused for 30 min (middle trace) and after 60 min of wash (right trace). (b) Extracellular recordings show somatic responses under control conditions (left trace), in the presence of 40 μM sphingosine superfused for 30 min (middle trace), and after 60 min of wash. Traces in (a) and (b) are the average of 5 evoked responses. Arrows indicate the time of stimulation.

$n = 3$, $P > 0.1$) indicating that the action of H-7 was occluded.

Effects of HA-1004 and sphingosine Bath application of HA-1004 (50 μM; $n = 5$, Figure 3a), the H-7 analogue which is devoid of inhibitory action on PKC, slightly decreased the population spike amplitude ($-12 \pm 3\%$) and failed to produce multiple population spikes. HA-1004 (50 μM, $n = 5$), did not significantly ($P > 0.1$) affect the initial slope, the amplitude, and duration of the field e.p.s.p. ($101 \pm 2\%$, $98 \pm 2\%$, and $97 \pm 3\%$ respectively, expressed as % of control values).

Sphingosine applied at a concentration of 20 μM ($n = 5$) or 40 μM ($n = 5$, Figure 3b) did not change the population spike amplitude or produce multiple population spikes. Consistently, the field e.p.s.ps were unaffected by sphingosine 40 μM (initial slope $100 \pm 2\%$ of control values, $n = 7$, $P > 0.1$).

Intracellular recordings

Effects of H-7 on orthodromically evoked potentials and antidromically evoked inhibitory currents Intracellular recordings from CA1 pyramidal cells ($n = 11$, -66 ± 3 mV resting membrane potential, 45 ± 5 MΩ membrane input resistance, action potentials greater than 75 mV) showed that the inhibitory postsynaptic potential (i.p.s.p.) which follows the orthodromically evoked e.p.s.p. was blocked by H-7 (30 μM; Figure 4a). H-7 had no effect on the resting membrane potential, membrane input resistance or action potential threshold. Figure 4b illustrates the effect of H-7 (30 μM, $n = 3$) on antidromically evoked inhibitory currents in voltage-clamp conditions. The GABAergic nature of the currents was demonstrated by its sensitivity to bicuculline or picrotoxin in other neurones (Ropert & Korn, 1988). The block of the inhibitory currents was almost completely reversible ($80 \pm 5\%$) after 45 min wash.

Effect of H-7 on responses to GABA and isoguvacine To rule out the possibility that the block of the inhibition by H-7 was due to antagonism at the GABA-receptor level, we tested the effect of H-7 (30 μM) on responses produced by bath application of GABA (500 μM, $n = 3$) or isoguvacine the selective $GABA_A$-receptor agonist (30 μM, $n = 3$). Figure 5 shows that H-7 did not modify the response of intracellularly recorded CA1 pyramidal cells to application of either GABA or isoguvacine.

Effect of H-7 on the miniature inhibitory postsynaptic currents (i.p.s.cs)

The previous results suggest a presynaptic action of H-7 on GABA release. In order to test this possibility we examined the effect of H-7 on the frequency of miniature i.p.s.cs (Figure 6) and observed the suppression of i.p.s.cs in the presence of H-7 (30 μM). In control conditions CA1 pyramidal neurones exhibit continuous spontaneous activity sensitive to bicuculline (Collingridge *et al.*, 1984) and to tetrodotoxin (TTX, Figure 6). This is believed to reflect the spontaneous i.p.s.cs which occur in response to the GABAergic interneurone activity. In the presence of TTX most activity disappears but small currents are still visible. They are sensitive to bicuculline (Ropert & Korn, 1988) and are presumably due to the activity-independent release of GABA. The occurrence of these miniature postsynaptic currents could be blocked by H-7 (30 μM, Figure 6c).

Discussion

Our results show that the PKC inhibitor H-7 produced an increase in the size of excitatory responses to electrical stimulation in the CA1 region of the rat hippocampus. This is indicated by the increase in

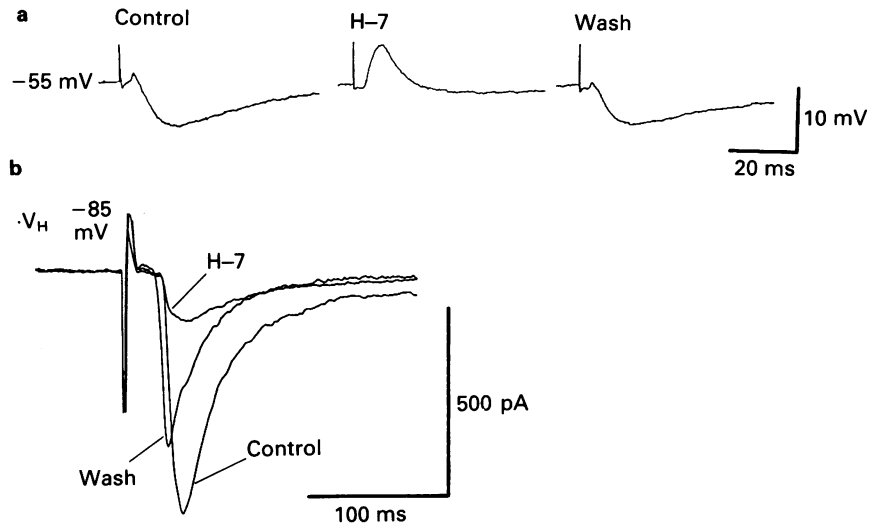

Figure 4 Intracellular recordings of evoked responses from CA1 pyramidal cells. (a) Stimulation (rapid upward deflections) of the stratum radiatum evoked an e.p.s.p.-i.p.s.p. sequence in a neurone recorded with a 3 M K-methylsulphate-filled electrode under control conditions (left trace). Superfusion with 1-(5-isoquinolinesulphonyl)-2-methylpiperazine (H-7, 30 μM, 15 min) induced the disappearance of the i.p.s.p. and unmasked a large e.p.s.p. (middle trace). This effect was reversible upon 45 min wash (right trace). To keep the membrane potential at −55 mV a positive (+220 pA) steady current was injected through the recording electrode. Resting membrane potential −67 mV, membrane input resistance 43 MΩ. (b) In a pyramidal neurone, recorded with a 3 M KCl-filled electrode and voltage clamped at −85 mV, stimulation of the alveus (rapid, biphasic deflection) evoked an inward current due to the GABA-mediated recurrent inhibition. This current was greatly reduced by bath application of H-7 (30 μM, 15 min). Prolonged wash (45 min) allowed a substantial recovery. Each trace is the average of 20 responses. Resting membrane potential −57 mV, input membrane resistance 35 MΩ.

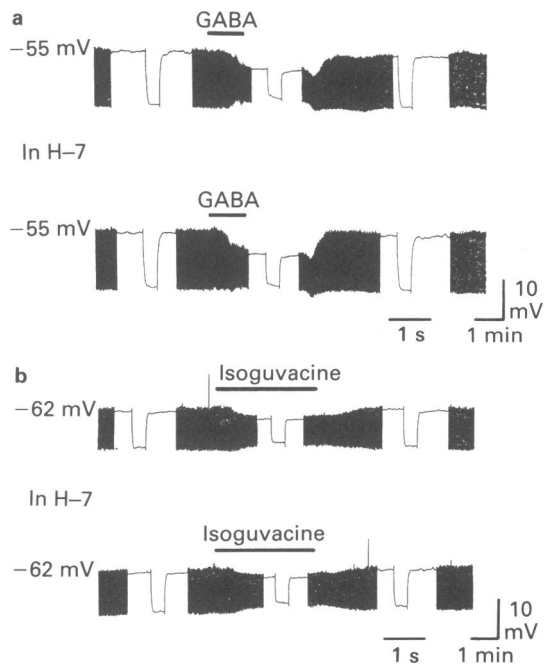

Figure 5 1-(5-Isoquinolinesulphonyl)-2-methylpiperazine (H-7) did not affect the response to γ-aminobutyric acid (GABA) and isoguvacine. (a) Intracellular recordings from a CA1 pyramidal cell in the presence of tetrodotoxin 1 μM. A current pulse (400 ms, −0.3 nA, 0.2 Hz) was injected throughout the experiment to measure the membrane input resistance. Upper trace: application of GABA (500 μM, 1 min) induced a hyperpolarization and a decrease in membrane input resistance. Both effects washed within 5 min. Lower trace: in the presence of H-7 (30 μM, 30 min) the response to GABA application was unaffected. Note that H-7 did not change the membrane input resistance. The membrane potential was kept depolarized by injecting a positive (+290 pA) steady current through the recording electrode. Resting membrane potential −65 mV, 3 M K-methylsulphate-containing electrode. (b) Intracellular recordings from a CA1 pyramidal cell. Upper trace: at resting membrane potential the $GABA_A$-receptor agonist isoguvacine (30 μM, 3 min) hyperpolarized the cell and produced a decrease in the membrane input resistance (current pulses 400 ms, −0.3 nA, 0.2 Hz). Lower trace: The presence of H-7 (30 μM, 30 min) did not affect the response to isoguvacine. Note that H-7 neither changed the resting membrane potential nor the membrane input resistance. Rapid upward deflections are truncated action potentials. 3 M K-methylsulphate-containing electrode.

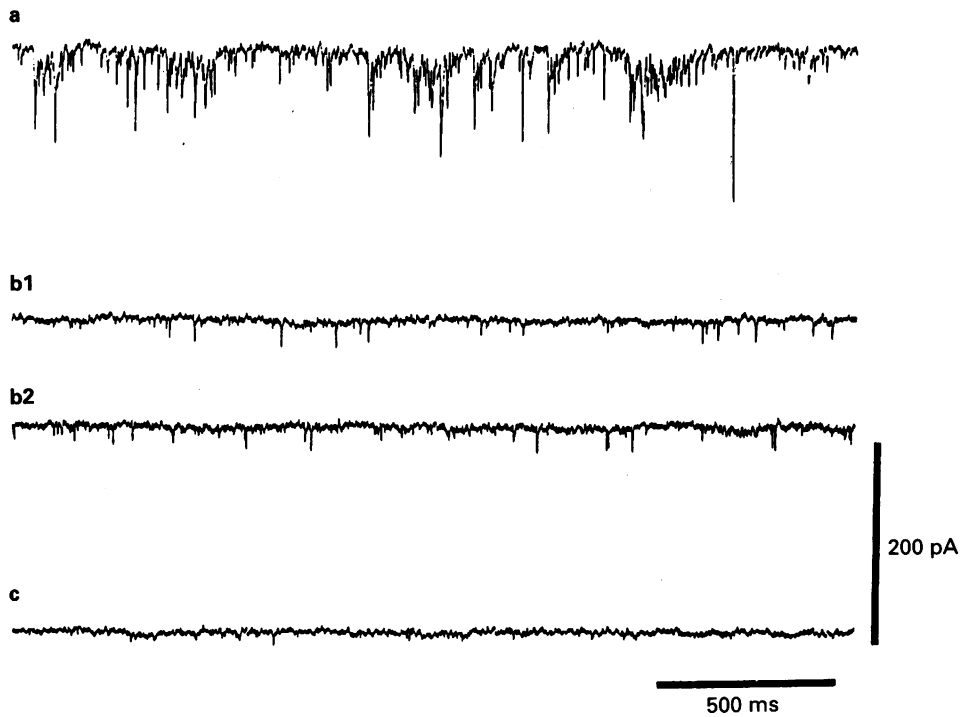

Figure 6 1-(5-Isoquinolinesulphonyl)-2-methylpiperazine (H-7) reduced the frequency of miniature inhibitory post synaptic currents. Spontaneous i.p.s.cs in a CA1 pyramidal cell recorded under single electrode voltage-clamp with a KCl-containing electrode (a). In the presence of 0.5 μM tetrodotoxin (b1, b2) most activity disappeared, but small currents were still present. These miniature postsynaptic currents were blocked by 30 μM H-7 (c). In (a) and (b1) holding potential −86 mV; in (b2) and (c) holding potential −96 mV.

population spike amplitude and in field e.p.s.p. initial slope, and by the apparance of multiple population spikes. Our results also demonstrate that the effects induced by H-7 are due to the blockade of the inhibitory synaptic potentials in the CA1 region of the rat hippocampus. This disinhibitory effect is probably due to block of PKC activity, since H-7, at a concentration of 30 μM, abolished the intracellularly recorded inhibitory responses and produced multiple population spikes in the field, whereas its analogue HA-1004, which does not block PKC at this concentration, did not show any disinhibitory action. Furthermore, in a recent communication, Agopyan & Krnjević (1988b) described a decrease of the size of evoked i.p.s.ps, produced by iontophoretically applied H-7. This effect, which is consistent with our data, appeared to be opposite to those of phorbol esters on the evoked i.p.s.ps. In addition, in experiments investigating the involvement of PKC in long-term potentiation in the CA1 region, Muller *et al.* (1988) noted that H-7 greatly increased the neuronal excitability and produced a reduction of the paired pulse inhibition, an effect opposite to that of phorbol esters.

The effect of H-7 appears to be due to a presynaptic action on GABA containing terminals since H-7 blocked the spontaneous miniature inhibitory synaptic currents. On the other hand the block of the evoked inhibitory synaptic responses, cannot be easily ascribed to a decreased excitatory input to the GABAergic interneurones via the recurrent axon collaterals. In fact, the threshold for action potential discharge and the input membrane resistance of CA1 pyramidal cells were unaffected by H-7 at concentrations that fully blocked the inhibitory potentials and the cells were shown to fire repeatedly following adequate stimulation (Figure 1a). Thus, unless a peculiar sensitivity of axon collaterals to H-7 is assumed, the input to the interneurones should be preserved.

On the other hand, in agreement with *in vivo* observations by Agopyan & Krnjević (1988a), H-7 did not block the postsynaptic action of GABA or isoguvacine, thus ruling out a possible antagonism by H-7 at the GABA receptor level.

Thus the disinhibitory effect of H-7 suggests an important regulatory role of an H-7 sensitive PKC, both on spontaneous and on evoked GABA release.

It is worth noting that the excitatory transmission appears not to be greatly affected by H-7: prolonged (30 min) superfusion with the drug did not decrease the excitatory response. On the other hand, sphingosine failed to modify the evoked potentials thus indicating that a sphingosine-sensitive PKC is not involved in CA1 hippocampal neurotransmission under control conditions. In keeping with the present results it has recently been reported (Kauer *et al.*, 1988; Malinow *et al.*, 1988) that sphingosine did not change the field e.p.s.p., but was able to block the electrically induced long-term potentiation in the rat CA1 region. Since multiple forms of PKC have been described in the rat brain (Housey *et al.*, 1987; Nishizuka, 1988) it is conceivable that they differ in sensitivity to inhibitors. A similar suggestion has recently been made by Malinow *et al.* (1988) to explain different actions of H-7 and sphingosine on the suppression of long-term potentiation.

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Evidence for the involvement of a plasma kallikrein-kinin system in the immediate hypotension produced by endotoxin in anaesthetized rats

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1 *In vitro* incubation of normal rat plasma with endotoxin from *E. coli* (3–10 mg ml^{-1} in the incubation mixture) caused a dose-dependent increase in levels of free kinin and plasma kallikrein in the presence of *o*-phenanthroline, together with a mirror-image, dose-dependent decrease in the residual levels of the precursors, plasma prekallikrein and high-molecular-weight kininogen. Low-molecular-weight kininogen levels were not modified.

2 Intravenous injection of endotoxin (3–30 mg kg^{-1}) into the femoral vein of anaesthetized rats resulted in dose-dependent hypotension. In blood collected up to 15 min after injection, the levels of prekallikrein and high-molecular-weight kininogen in plasma were decreased while levels of the active forms, plasma kallikrein and free kinin, showed a transient increase in the blood 1 min after administration of endotoxin.

3 A degradation product of bradykinin, des-Phe^8-Arg^9-bradykinin, as measured by a newly developed enzyme immunoassay, was detectable up to 5 min after administration of endotoxin.

4 Intravenous infusion of soybean trypsin inhibitor inhibited both the formation of bradykinin and des-Phe^8-Arg^9-bradykinin and the initial hypotension.

5 It can be concluded from our results that plasma prekallikrein is activated in the blood immediately after administration of endotoxin to rats and that bradykinin is a major cause of the immediate hypotension.

Introduction

Circulating bacterial endotoxins are known to induce shock (Lillehei *et al.*, 1967; Cardis *et al.*, 1972). Because of the seriousness of the clinical symptoms associated with gram-negative septicaemia, several studies have been conducted to clarify the mechanisms responsible for the development of metabolic and haemodynamic abnormalities observed during endotoxin shock (Baue, 1968; Neuhof *et al.*, 1970; Sardesai & Walt, 1971). Among the vasoactive substances released by endotoxin, such as histamine (Hinshaw *et al.*, 1961; Vick *et al.*, 1971), 5-hydroxytryptamine, catecholamines (Rosenberg *et al.*, 1961) and kinins, bradykinin has been suspected of playing a major role in the widespread vascular disturbances seen during endotoxaemia. The involvement of the plasma kallikrein-kinin system in endotoxin shock has been examined and predicted by many authors: Robinson *et al.* (1975) measured kallikrein activity in blood of patients in endotoxin shock, using TAME (*p*-tosyl-arginine methyl ester hydrochloride), a non-specific synthetic substrate of esterase. Sardesai & Rosenberg (1974) also measured the increase in the TAME esterase activity, with an increase in levels of free bradykinin and a decrease in levels of total bradykininogen after injection of rats and dogs with endotoxin. They did not identify the types of kininogen involved. Gillimore *et al.* (1978) reported a reduction in the levels of prekallikrein and of high-molecular-weight kininogen in plasma during endotoxin shock, but free kinin was not detected. Similar results were obtained by Nies *et al.* (1968), who showed only an increase in levels of free kinin and a reduction in levels of total kininogen during endotoxin shock in monkeys. These workers presented only partial evidence of activation of plasma kallikrein-kinin

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systems. Conclusive evidence for the activation of plasma prekallikrein and in particular, the time course of the activation still remain to be demonstrated.

We have established, in our laboratory, systems for the measurement of the levels of plasma prekallikrein (Oh-ishi & Katori, 1979), high- and low-molecular-weight kininogens (Uchida & Katori, 1979; Uchida *et al.*, 1986) and bradykinin itself (Ueno *et al.*, 1981). A novel assay system for measurement of levels of a degradation product of bradykinin, des-Phe^8-Arg^9-bradykinin, was recently developed and has been used as a marker of the release of kinin at sites of inflammation (Katori *et al.*, 1988; Majima *et al.*, 1988a). Using these assays, we have attempted to obtain the conclusive evidence for the involvement of the plasma kallikrein-kinin system in the immediate phase of endotoxin shock.

Methods

Activation of the plasma kallikrein-kinin system in rat plasma by endotoxin in vitro

Blood was collected under light anaesthesia with ether from the carotid artery of rats (Sprague-Dawley, male rats, specific pathogen free, 8–10 weeks old, from the Shizuoka Laboratory Animal Center, Hamamatsu) into tubes that contained 1/10 volume of 3.8% sodium citrate and the mixture was centrifuged at 1500 *g*. The citrated rat plasma was mixed with an equal volume of a solution of endotoxin (6–20 mg ml^{-1} saline). After a 30 min incubation at 37°C, the residual levels of prekallikrein and kininogens in the incubation mixture were determined. Plasma kallikrein activity in the incubation mixtures was also assayed using a selective, synthetic substrate, Z-Phe-Arg-MCA for plasma kallikrein, according to the method for the measurement of levels of prekallikrein described below. The amount of free kinin released in the incubation mixture was measured in the presence of *o*-phenanthroline (0.2 mg ml^{-1} plasma), an inhibitor of kininase I and II, by use of an enzyme immunoassay (Markit-A, Dainippon Pharmaceutical Co., Osaka).

Assay of prekallikrein

The residual level of prekallikrein was measured by the method described by Oh-ishi & Katori (1979). Briefly, plasma prekallikrein was activated by kaolin in the presence of acetone, and plasma kallikrein activity was assayed using a peptidyl fluorogenic substrate, Z-Phe-Arg-MCA, whch is a selective, synthetic substrate for plasma kallikrein (Morita *et al.*, 1977). One unit was arbitrarily defined as the amount of prekallikrein that released 1×10^{-7} M of AMC during a 10 min period under our assay conditions. The difference between the amidolytic activities in the presence of soybean trypsin inhibitor and limabean trypsin inhibitor was considered to represent the plasma kallikrein activity, since the synthetic substrate is also hydrolyzed by other serine proteases, such as plasmin. The residual levels of plasma prekallikrein were expressed as arbitrary units per mg protein after activation. Levels of plasma proteins were determined by the method of Lowry *et al.*, (1951).

Assay of high-molecular-weight and low-molecular-weight kininogens

The residual levels of kininogens were determined by the method reported previously (Uchida & Katori, 1979; Uchida *et al.*, 1986). For high-molecular-weight kininogen, plasma was incubated with glass powder (Ballotini, Jencons, England) in the presence of *o*-phenanthroline. Bradykinin released from high-molecular-weight kininogen was measured by the enzyme immunoassay (Markit-A, Dainippon Pharmaceutical Co, Osaka). For low-molecular-weight kininogen, plasma was incubated with glass powder in the absence of *o*-phenanthroline. The plasma, in which the high-molecular-weight kininogen was consumed, was acidified at pH 2.0 and was incubated with trypsin after neutralization. The bradykinin released was measured by the enzyme immunoassay. The levels of high-molecular-weight and low-molecular-weight kininogens were expressed as the amount, in ng, of bradykinin released per mg of protein.

Endotoxin shock model

Male Sprague-Dawley rats (specific pathogen-free, weighing 270–390 g, obtained from Shizuoka Laboratory Animal Center Hamamatsu), were anaesthetized with sodium pentobarbitone (50 mg kg^{-1}, i.p.) before experimental procedures were started. Anaesthesia was maintained by occasional intraperitoneal administration of sodium pentobarbitone (25 mg kg^{-1}). The right femoral vein was cannulated with a polyethylene cannula (PE-50, Clay Adams, NJ, U.S.A.). Injection of endotoxin and infusion of soybean trypsin inhibitor were given through this cannula. The mean systemic blood pressure was measured by a pressure transducer (MPU-0.5, Nihon Kohden, Tokyo, Japan) attached to a cannula inserted into the right femoral artery and this parameter was recorded on a polygraph (RM-85, Nihon Kohden, Tokyo, Japan). Respiratory movements were recorded by a force-displacement transducer (SB-1T, Nihon Kohden, Tokyo, Japan). The

heart rate was measured from the ECG using a tachometer (RT-5A, Nihon Kohden, Tokyo, Japan). The blood samples were collected from a polyethylene cannula (PE-60, Clay Adams, New Jersey, U.S.A.) inserted into the left carotid artery. Blood (5 ml) was collected only once from each rat.

After completion of the experimental setup, endotoxin (3–30 mg ml^{-1} saline kg^{-1}), was injected as a single bolus into the right femoral vein. For control studies, only saline (1 ml kg^{-1}) was injected. For inhibition of release of kinins, soybean trypsin inhibitor (5 mg 0.5 ml^{-1} saline 10 min^{-1}) was infused for 20 min into the right femoral vein. Endotoxin was injected 15 min after the start of the infusion of soybean trypsin inhibitor.

Haematocrits of the samples of blood from the carotid artery were determined by a micro-method (Chien *et al.*, 1965).

Measurements of plasma kallikrein activity in blood

One ml of blood from the carotid artery was collected directly into a plastic tube (Falcon 2029) that contained 2.0 ml of the solution of MCA synthetic substrate (Z-Phe-Arg-MCA, 1.0×10^{-4} M in 150 mM phosphate buffer, pH 7.4) and incubated at 37°C for 15 min in the presence of soybean trypsin inhibitor or limabean trypsin inhibitor (Oh-ishi & Katori, 1979). The reaction was terminated by addition of 750 μl of a 20% solution of trichloracetic acid (TCA). After centrifugation (1500 *g*, for 15 min, at 4°C), the supernatant was diluted 10 fold with a 17% solution of acetic acid in order to prevent quenching. The amounts of AMC released were measured fluorometrically (excitation, 380 nm; emission, 436 nm). The difference between the amidolytic activities in the presence of soybean trypsin inhibitor and in the presence of limabean trypsin inhibitor was calculated and taken as the plasma kallikrein activity.

Detection of bradykinin and des-Phe^8-Arg^9-bradykinin in blood by enzyme immunoassay

Five ml of blood from the carotid artery was collected directly into the plastic tubes (Falcon 2099) which contained absolute ethanol in which 10 μM *o*-phenanthroline was dissolved. The mixture was heated at 70°C for 10 min. After centrifugation (1500 *g*, at 4°C, for 15 min), the supernatant was evaporated under reduced pressure. The residue was washed three times with 10 ml of diethylether in order to remove lipids, and it was then dissolved in the assay buffer for the enzyme immunoassay of bradykinin or des-Phe^8-Arg^9-bradykinin. The amounts of bradykinin were determined by a previously developed enzyme immunoassay (Markit-A, Dainippon Pharmaceutical Co., Osaka, Japan) (Uchida *et al.*, 1986), whereas amounts of des-Phe^8-Arg^9-bradykinin were measured by a recently established enzyme immunoassay (Katori *et al.*, 1988; Majima *et al.*, 1988a). Limits of detection for either peptide were 80 pg ml^{-1} blood. The recovery rate was greater than 90% for each peptide, as evaluated from measurements of authentic bradykinin or des-Phe^8-Arg^9-bradykinin added to the blood of normal, healthy rats.

Drugs

Drugs used were: *o*-phenanthroline (Wako Pure Chemical Co., Osaka, Japan); peptidyl fluorogenic substrate, Z-Phe-Arg-MCA (Peptide Institute Inc., Osaka, Japan); soybean trypsin inhibitor and limabean trypsin inhibitor (Worthington Biochemical Co., Freehold, NJ, U.S.A.); trypsin (twice-crystallized, salt-free, from bovine pancreas, Nutritional Biochemical Co., Cleveland, OH, U.S.A.); sodium pentobarbitone (Nembutal, Abbot Labs., North Chicago, IL, U.S.A.); endotoxin (lipopolysaccharide W, from *E. coli*, 0127 : B5, Difco Laboratories, Detroit, Michigan, U.S.A.). Other reagents were all of analytical grade and were obtained from commercial sources.

Statistical analysis

When variances were not heterogeneous, Student's *t*-test was used to evaluate the significance of differences. When variances were heterogeneous, statistical analysis was performed by the Aspin-Welch method or the Wilcoxon's rank sum test.

Results

Activation in vitro *of the plasma kallikrein-kinin system in rat plasma by endotoxin*

As shown in Figure 1b, the incubation of rat plasma with a solution of endotoxin for 30 min generated plasma kallikrein activity in a dose-dependent manner (to levels 2, 15 and 27 times the control value for 3, 5 and 10 mg ml^{-1} endotoxin, respectively). Concomitantly, the residual level of plasma prekallikrein was reduced in a dose-dependent manner (by 23%, 32% and 38% for 3, 5 and 10 mg ml^{-1} endotoxin, respectively) (Figure 1a).

Levels of free bradykinin in the incubation mixture also increased in a dose-dependent manner (to 16 and 23 times the control value for 3 and 10 mg ml^{-1} endotoxin, respectively) in the presence of *o*-phenanthroline (Figure 1d). Concomitantly, the residual level of high-molecular-weight kininogen

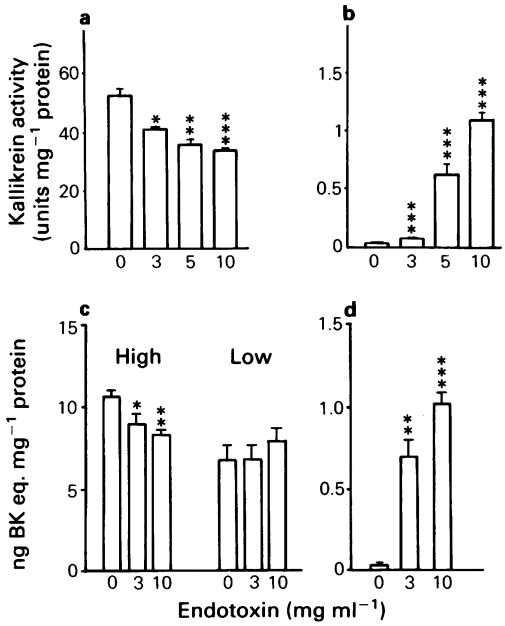

Figure 1 Changes of the levels of the components of the plasma kallikrein-kinin system after incubation of rat plasma with endotoxin *in vitro*. This figure illustrates residual levels of plasma prekallikrein (a), high-molecular-weight (High) and low-molecular-weight (Low) kininogens (c), plasma kallikrein activity (b), and free bradykinin (d). The ordinates in (a) and (b) are arbitrary units mg^{-1} of plasma protein and those in (c) and (d) are ng of bradykinin (BK) mg^{-1} of plasma protein. Levels of endotoxin are final concentrations in the incubation mixture. Each column represents the mean of results of four experiments (vertical bars show s.e. mean) and is compared with an appropriate saline control (no endotoxin). $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

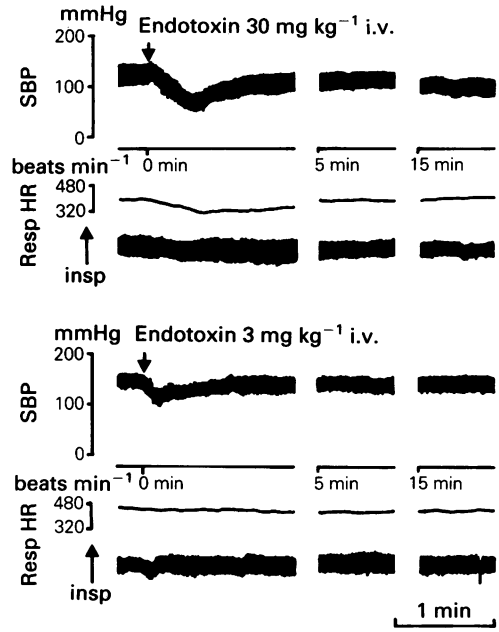

Figure 2 Typical tracings showing the changes in systemic blood pressure (SBP), heart rate (HR) and respiratory movement (Resp) after injection of endotoxin. Insp: direction of record of inspiration. The bar below the trace indicates 1 min.

decreased significantly (by 15% and 22% for 3 and 10 mg ml^{-1} endotoxin, respectively) (Figure 1c). However, the level of low-molecular-weight kininogen was not changed.

These results indicate that endotoxin from *E. coli* caused the release of bradykinin in rat plasma from high-molecular-weight kininogen by the conversion of plasma prekallikrein to the active plasma kallikrein.

Changes in systemic blood pressure induced by the intravenous injection of endotoxin

Figure 2 shows typical tracings of the systemic blood pressure, heart rates and respiratory movements induced by a bolus injection of endotoxin (30 or 3 mg kg^{-1}). When a solution of endotoxin was injected into the femoral vein of rats, the systemic blood pressure fell gradually. The drop in systemic blood pressure caused by injection of 3 or 30 mg kg^{-1} of endotoxin was completely reversed and blood pressure returned to the preinjection level 5 min after the injection of endotoxin.

Figure 3a shows the initial maximum decrease in the mean systemic blood pressure (−26.3, −44.3 and −46.9 mmHg for 3, 10 and 30 mg kg^{-1} endotoxin, respectively). As shown in Figure 3b, the haematocrits of the blood samples, which were collected 15 min after the injection of endotoxin increased in a dose-dependent manner to 104%, 109% and 123% of control values for 3, 10 and 30 mg kg^{-1} endotoxin, respectively. These values suggest that haemoconcentration due to plasma leakage, probably as a result of the formation of bradykinin, was induced in parallel with the decrease in blood pressure.

Changes in the levels of the components of the plasma kallikrein-kinin system after the intravenous injection of endotoxin

As shown in Figure 4b, plasma kallikrein activity was increased to 450% of the saline injected control 1 min after the injection of endotoxin (10 mg kg^{-1}).

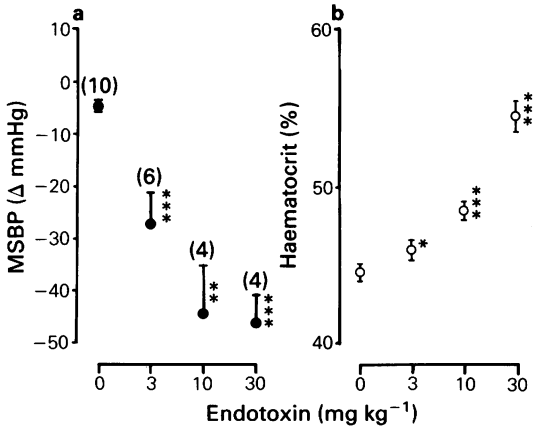

Figure 3 Changes of mean systemic blood pressure (MSBP, a) and haematocrit (%, b) after injection of endotoxin. The ordinate scale in (a) indicates the maximal decrease in mean systemic blood pressure. The numbers in parentheses indicate the numbers of experiments. Each point represents the mean value (s.e.mean shown by vertical bar) and is compared with the value of the saline control (no endotoxin). $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

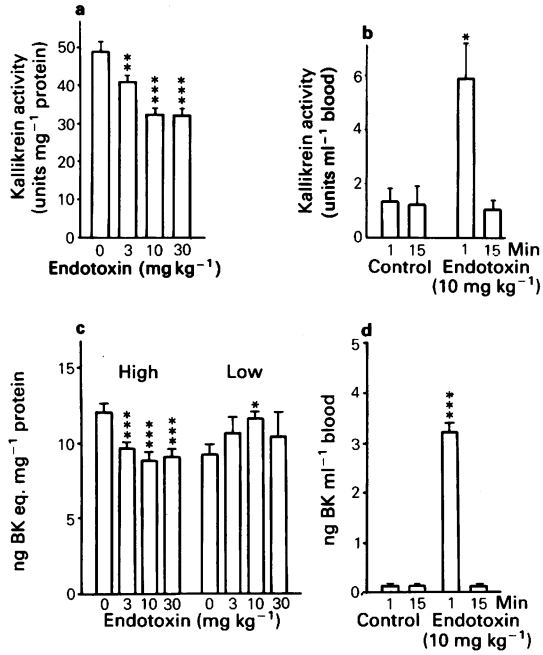

Figure 4 Changes in the levels of the components of the plasma kallikrein-kinin system in the blood collected after injection of endotoxin. This figure illustrates residual levels of plasma prekallikrein at 15 min (a), high-molecular-weight (High) and low-molecular-weight (Low) kininogen at 15 min (c), plasma kallikrein activity (b), and free bradykinin (d). The ordinate scale in (a) shows the arbitrary units mg^{-1} of plasma protein and that in (b) shows arbitrary units ml^{-1} of blood. The ordinate scale in (c) shows ng of bradykinin (BK) mg^{-1} of plasma protein and that in (d) shows ng of bradykinin ml^{-1} of blood. Control indicates the saline-injected group. Each column represents the mean of results from 4–8 experiments (s.e.mean shown by vertical bars) and is compared with the saline control (no endotoxin). $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

However, 15 min after the injection of endotoxin, the level of plasma kallikrein returned to that in controls. However, the residual level of plasma prekallikrein in rat plasma remained significantly lower even 15 min after the injection of endotoxin and the extent of the reduction was increased by 17%, 34% and 34% for 3, 10 and 30 mg kg^{-1} endotoxin, as compared to values for controls (no endotoxin) (Figure 4a).

At the same time, free bradykinin was detectable (3.2 ± 0.2 ng ml^{-1} blood) only 1 min after the injection of endotoxin, when there was evidence of activated plasma kallikrein. Fifteen min after the injection of endotoxin, the levels of free bradykinin in the blood had returned to the preinjection level (Figure 4d). The residual level of high-molecular-weight kininogen was significantly reduced by 21%, 25% and 24% with 3, 10 and 30 mg kg^{-1} endotoxin, respectively, and these low levels persisted for at least 15 min after the injection. The residual level of low-molecular-weight kininogen was not reduced (Figure 4c).

These results indicate that, even in this model *in vivo*, the intravenous injection of endotoxin resulted in the activation of the plasma prekallikrein, so that free bradykinin could be detected together with active plasma kallikrein in the blood, but the active forms were found only immediately after the injection of endotoxin.

Detection of des-Phe^8-Arg^9-bradykinin after injection of endotoxin in vivo.

The results on the activation of plasma prekallikrein, with the generation of kinin, were further validated by the detection of des-Phe^8-Arg^9-bradykinin in the blood after intravenous injection of endotoxin.

As shown in Figure 5a, free bradykinin was detected only 1 min after injection of endotoxin (960 ± 450 pg ml^{-1}, $n = 5$). Three minutes later, the level of free kinin had returned to the control level (below 80 pg ml^{-1} blood). In contrast, des-Phe^8-Arg^9-bradykinin could be detected for at least 5 min (Figure 5b). Fifteen minutes later, when blood pressure was restored to normal levels, the level of this

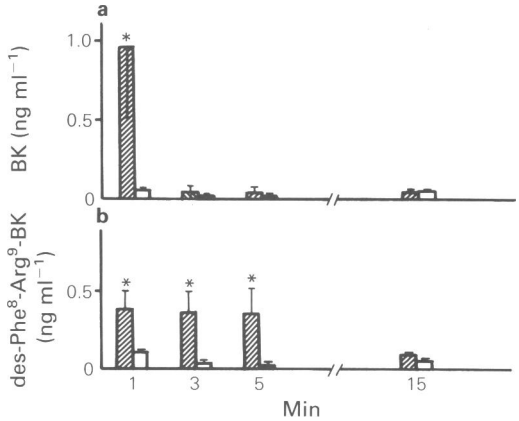

Figure 5 Time course of the increase in the levels of bradykinin (BK) and des-Phe^8-Arg^9-bradykinin (des-Phe^8-Arg^9-BK) in blood after injection of endotoxin. Open columns indicate results from saline-injected (1 ml kg^{-1}) rats and hatched columns indicate those from endotoxin-injected (10 mg kg^{-1}, i.v.) rats. Each column represents the mean of results from 3–6 experiments (s.e.mean shown by vertical bars) and is compared with the results from the saline-injected rats at each time point. $*P < 0.05$.

metabolite did not differ from the control value measured after injection of saline.

This result indicates that des-Phe^8-Arg^9-bradykinin is a long-lasting marker of the release of bradykinin in blood.

Inhibition of the immediate drop in blood pressure by soybean trypsin inhibitor

Intravenous infusion of soybean trypsin inhibitor (10 mg 20 min^{-1} per rat) clearly prevented the immediate hypotension caused by endotoxin, as shown in Figure 6a. The extent of hypotension was significantly reduced from 44.3 mmHg to 4.7 mmHg (mean systemic blood pressure) (Figure 6b). The infusion of this inhibitor also prevented release of bradykinin, as well as of des-Phe^8-Arg^9-bradykinin, in arterial blood as shown from measurements made 1 min and 3 min after the injection of endotoxin (Figure 7).

These results clearly indicate that bradykinin was formed as a result of the intravenous injection of endotoxin and that the immediate hypotension induced by endotoxin may be caused mainly by bradykinin formed.

Discussion

Bradykinin has been reported to have very potent hypotensive effects, to increase vascular permeability, and to heighten pain sensation (Johnson, 1979).

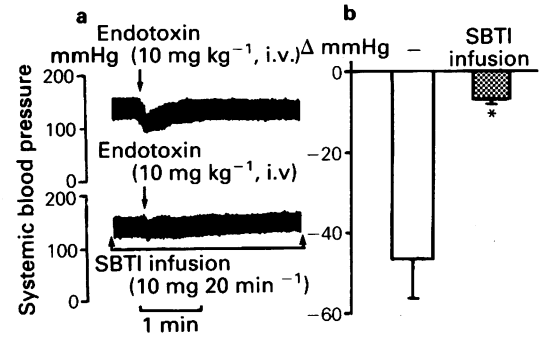

Figure 6 Inhibition of the endotoxin-induced immediate drop in systemic blood pressure by soybean trypsin inhibitor (SBTI): (a) shows typical tracings of changes in the systemic blood pressure. In (b), the immediate drop in the mean systemic pressure is seen to be significantly inhibited ($*P < 0.05$) during infusion of SBTI (stippled column), compared with the systemic pressure during infusion of saline (open column).

Thus, bradykinin has been claimed to be a major cause of hypotension in endotoxin shock (Nies *et al.*, 1968; Sardesai & Rosenberg, 1974; Robinson *et al.*, 1975; Gillimore *et al.*, 1978; Assen *et al.*, 1983; McConn *et al.*, 1983; Högström *et al.*, 1987). However, the evidence presented in previous papers in somewhat incomplete and fragmentary, and, thus, the involvement of bradykinin in endotoxin shock

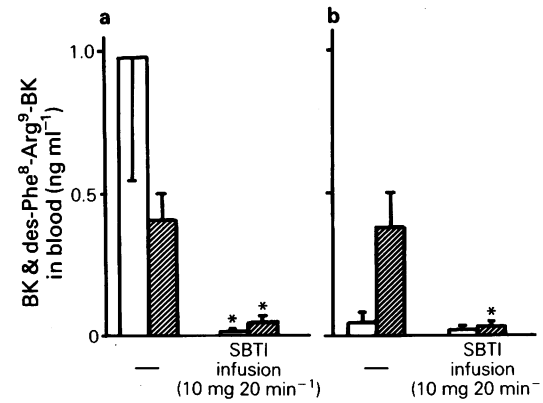

Figure 7 Inhibition of the generation of bradykinin (BK, open column) and des-Phe^8-Arg^9-bradykinin (des-Phe^8-Arg^9-BK, hatched column) in blood after injection of endotoxin (10 mg kg^{-1}) during infusion of soybean trypsin inhibitor (SBTI). The ordinate scale indicates ng of BK and des-Phe^8-Arg^9-BK ml^{-1} blood at 1 (a) and 3 min (b) after injection of endotoxin. Each column represents the mean of results from 3–6 experiments, vertical bars show s.e.mean. Values during infusion of SBTI were compared with those during infusion of saline. $*P < 0.05$.

has been a matter of debate. The controversy can be, in part, attributed to the rapid destruction of and the difficulties in the detection of bradykinin in pathological states. Plasma kallikrein is also readily inactivated by various types of inhibitor in plasma. Our experiments were designed to provide comprehensive and conclusive results. In the present study, free bradykinin and active plasma kallikrein could only be detected 1 min after the injection of endotoxin; 15 min later neither bradykinin nor plasma kallikrein were elevated (Figure 4). In the present experiments, the respective decreases in levels of precursors and products were clearly seen to be mirror images. In our experiment *in vitro*, the incubation of plasma with endotoxin resulted in the consumption of residual prekallikrein and high-molecular-weight kininogen, together with increases in levels of active plasma kallikrein and bradykinin (Figure 1). These mirror-image processes were corroborated *in vivo* after a bolus injection of endotoxin in rats. In this case, low-molecular-weight kininogen was not consumed since plasma kallikrein acts only on high-molecular-weight kininogen *in vivo*. In inflammatory models, a reduction in the residual levels of plasma prekallikrein and high-molecular-weight kininogen, and not of low-molecular-weight kininogen, has been taken as indirect evidence of involvement of bradykinin and interpreted as the activation of plasma kallikrein-kinin (Uchida *et al.*, 1983).

Endotoxin from *E. coli* is a sulphated polysaccharide (Morrison & Cochrane, 1974) and is analogous to carrageenin, which has been reported, by groups including ours, to activate factor XII (Erdös & Miwa, 1968; Morrison & Cochrane, 1974; Uchida *et al.*, 1983). It has been assumed, therefore, that endotoxin from *E. coli* can activate factor XII (Hageman factor) in plasma, resulting in plasma prekallikrein being converted to active plasma kallikrein.

The increase in the level of bradykinin in blood after injection of endotoxin was only transient but this brief generation of bradykinin was detectable via a long-lasting metabolite of bradykinin, des-Phe^8-Arg^9-bradykinin, present in blood for at least 5 min after the injection. We have developed an enzyme immunoassay for this metabolite in order to overcome the difficulties involved in the detection of bradykinin in body fluid (Katori *et al.*, 1988; Majima *et al.*, 1988a). Bradykinin is degraded to des-Arg^9-bradykinin by kininase I and to des-Phe^8-Arg^9-bradykinin by kininase II in rats and man (Majima *et al.*, 1988a). Since des-Phe^8-Arg^9-bradykinin is the first major degradation product that can be detected in rat plasma, it can be used as a marker for the previous formation of the kinin in pathological states; large amounts of this metabolite can be detected in pleural exudates in carrageenin-induced and kaolin-induced pleurisy in rats (Katori *et al.*, 1988; Majima *et al.*, 1988a, b; Majima *et al.*, 1989). Thus, the detection of des-Phe^8-Arg^9-bradykinin provides a useful tool for verification of the involvement of bradykinin in pathological states, even in cases of endotoxin shock, when there is rapid destruction of bradykinin. The detection of des-Phe^8-Arg^9-bradykinin in the blood for up to 5 min after injection of endotoxin provides further support for the involvement of bradykinin in hypotension during endotoxin shock and becomes a good marker for the formation of kinin (Figure 5).

Soybean trypsin inhibitor is a potent inhibitor of plasma kallikrein, but not of glandular kallikrein (Imanari *et al.*, 1974–76). The reduction in amounts of bradykinin as well as of des-Phe^8-Arg^9-bradykinin in the blood to undetectable levels after infusion of soybean trypsin inhibitor provides still further evidence for the generation of bradykinin by endotoxin from *E. coli*. Furthermore, abolition of the immediate endotoxin-induced hypotension by soybean trypsin inhibitor is strong evidence that the newly generated bradykinin plays a major role in this immediate hypotension. Increases in haematocrit after the injection of endotoxin, provide further, if indirect, support for the role of bradykinin in this immediate hypotension, since bradykinin is a potent effector of plasma leakage. Attenuation of the immediate drop in the blood pressure by an antagonist of bradykinin, namely, Lys-Lys-[Hyp^2, Thi^{5,8}, D-Phe^7]-bradykinin (B4148), which was reported by others (Weipert *et al.*, 1988) is further evidence in favour of this conclusion.

The protective effects of indomethacin (Anderson *et al.*, 1975; Katori, 1978) and of a specific antagonist of platelet-activating factor (Terashita *et al.*, 1985; Adnot *et al.*, 1986; Casala-Stenzel, 1987) suggest the possible involvement of prostaglandins and platelet-activating factor in endotoxin shock. However, the protective effects of these agents were implicated by survival ratios (Casala-Stenzel, 1987) or the late phase of a decrease in blood pressure (Anderson *et al.*, 1975; Katori, 1978; Terashita *et al.*, 1985), rather than the immediate decrease examined in our experiments. From our results, we can conclude that bradykinin is, without doubt, released during the very early stages and plays a major role in the immediate development of hypotension after the injection of endotoxin.

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Synergistic effects of calcium-mobilizing agents and adenosine on histamine release from rat peritoneal mast cells

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1 Adenosine and its metabolically stable analogue N-ethyl-carboxamidoadenosine (NECA) enhance histamine release from rat peritoneal mast cells when these are stimulated by calcium-mobilizing agents. NECA and adenosine shift the concentration-response curve of the calcium ionophore A23187 to lower concentrations.

2 The potencies of NECA or adenosine in enhancing A23187-induced histamine release are dependent on the level of stimulated release in the absence of adenosine analogues. At high levels of release their potencies are up to 20 times higher than at low levels. Consequently, averaged concentration-response curves of adenosine and NECA for enhancing histamine release are shallow.

3 The adenosine transport blocker S-(*p*-nitrobenzyl)-6-thioinosine (NBTI) has no effect by itself at low levels of stimulated histamine release, but abolishes the enhancing effect of adenosine. At high levels of release, however, NBTI alone enhances the release of histamine.

4 It is concluded that adenosine and calcium reciprocally enhance the sensitivity of the secretory processes to the effects of the other agent. The levels of intracellular adenosine obtained by trapping adenosine inside stimulated mast cells are sufficient to enhance histamine release substantially, suggesting that this effect may play a physiological and pathophysiological role.

Introduction

Mast cells release histamine and other mediators of allergy in response to a variety of IgE-directed stimuli. The release is generally thought to be intimately related to an elevation of free cytosolic calcium (Foreman, 1980) which increases upon IgE-receptor stimulation (White *et al.*, 1984). This increase of free intracellular calcium levels appears to be mediated by generation of inositol-trisphosphate (Beaven *et al.*, 1984; Nakamura & Ui, 1985). *In vitro*, release can also be initiated by the calcium-ionophore A23187, which causes a direct increase of cytosolic calcium (Foreman, 1980).

However, there is increasing evidence that an elevation of intracellular calcium within the physiological range is not sufficient to trigger mast cell mediator release (Neher & Almers, 1986). Consequently, additional signalling pathways have been postulated that act in concert with intracellular calcium (Beaven *et al.*, 1987; Penner & Neher, 1988; Penner, 1988). In a recent study we identified adenosine as a candidate for such a pathway which acts by increasing the calcium-sensitivity of the release process (Lohse *et al.*, 1988).

Adenosine has been known for several years to enhance mediator release from different types of mast cells (Marquardt *et al.*, 1978; Welton & Simko, 1980). Whereas earlier studies showed that the stimulation of mast cell mediator release by adenosine is antagonized by the adenosine receptor antagonist theophylline (Marquardt *et al.*, 1978; Church *et al.*, 1983; Hughes *et al.*, 1984), more recent studies have failed to confirm this observation (Vardey & Skidmore, 1985; Hughes & Church, 1986; Leoutsakos & Pearce, 1986; Lohse *et al.*, 1987). The latter data suggest that the effects of adenosine on mast cell release are not mediated via typical adenosine receptors. Since adenosine uptake blockers prevent the stimulation of mast cell histamine release by adenosine (Lohse *et al.*, 1987) it is reasonable to assume that these effects are mediated via an intracellular site. We have suggested that the action of adenosine at this site results in an enhanced sensitivity of the release process to calcium (Lohse *et al.*, 1988).

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In the present study we have investigated the relationship between the effects of calcium-mobilizing agents and adenosine in order to characterize further the site at which adenosine exerts its stimulatory effects on histamine release from rat peritoneal mast cells.

Methods

Preparation of mast cells

Rat peritoneal mast cells from male Wistar rats were obtained by peritoneal lavage with HEPES-buffered saline (washing buffer) containing (mM): NaCl 137, KCl 2.7, NaH_2PO_4 0.4, HEPES 10, glucose 5.5 and 0.05% bovine serum albumin, pH 7.4 (adjusted with NaOH). All subsequent steps were carried out at 4°C. The cell suspension was filtered through a 250 μm nylon mesh, and the filtrate centrifuged for 5 min at 120 g. The pellet was resuspended in the original volume washing buffer and centrifuged as above. This washing step was repeated once and the cells resuspended in incubation buffer (= washing buffer supplemented with 1 μM $CaCl_2$ and 0.5 mM $MgCl_2$), giving a suspension of about 2×10^5 mast cells per ml. It has been shown previously that the effects of adenosine on histamine release are indistinguishable in crude mast cell preparations and in cells purified to >95% homogeneity (Lohse *et al.*, 1987; 1988).

Determination of histamine release

Approximately 80,000 cells were preincubated at 37°C for 10 min in 0.95 ml of incubation buffer together with the compounds to be investigated. This preincubation time was found to result in an optimal stimulation of release by adenosine analogues. The release was initiated by addition of 50 μl of incubation buffer containing either the calcium ionophore A23187 (0.1–0.4 μM final concentration) or the lectin concanavalin A (100 $\mu g\,ml^{-1}$).

The reaction was stopped after 10 min by placing a 900 μl aliquot on ice, followed by centrifugation at 200 g for 10 min at 0°C. The histamine content of both the supernatant and the pellet containing the cells were determined by the fluorometric method of Shore *et al.* (1959), as modified by Anton & Sayre (1969) and May *et al.* (1971). Histamine release was expressed as a percentage of the total histamine content (cells + medium).

Data analysis

Concentration-response data were analyzed by non-linear nonweighted regression using a general form of the Hill-equation (Lohse *et al.*, 1986):

$$E = E_b + E_{max} \frac{[L]^n}{EC_{50}^n + [L]^n} \quad (I)$$

providing parameter estimates for E, effect; E_b, basal effect; E_{max}, maximal effect of agent; n, Hill coefficient; and [L] ligand concentration. Results of individual concentrations are presented as means, or means ± s.e.means.

Materials

The ionophore A23187, the adenosine uptake blocker S-(*p*-nitrobenzyl)-6-thioinosine (NBTI), adenosine and histamine were purchased from Sigma, München, Concanavalin A and HAM-F10 medium were obtained from Serva, Heidelberg, and orthophthaldialdehyde from Merck, Darmstadt. 5'-N-ethylcarboxamido-adenosine (NECA) was a gift from Byk Gulden, Konstanz. All other drugs were of analytical grade.

Results

We have previously suggested that adenosine and its metabolically stable analogues increase histamine

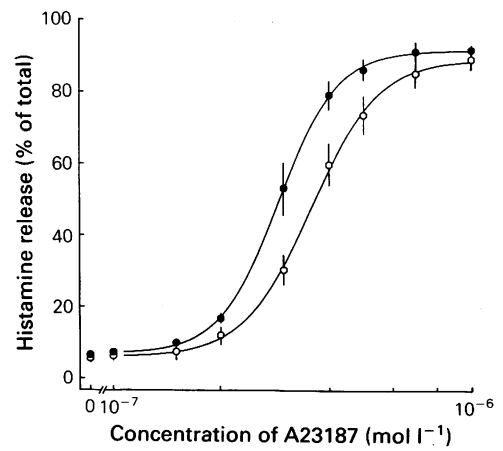

Figure 1 Effect of N-ethylcarboxamidoadenosine (NECA) on the histamine release from mast cells induced by various concentrations of A23187. Histamine release was induced by the indicated concentrations of A23187 in the absence (○) or presence (●) of 10 μM NECA and in the presence of 1.0 mM free extracellular calcium. EC_{50} values (and 95% confidence limits) were: 0.34 μM (0.32–0.37) under control conditions and 0.26 μM (0.24–0.28) in the presence of NECA. Points show means of four experiments with duplicate samples; s.e.means shown by vertical lines.

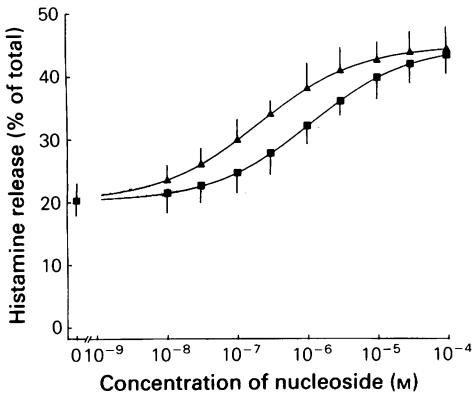

Figure 2 Enhancement of the A23187-induced histamine release by various concentrations of adenosine and N-ethylcarboxamidoadenosine (NECA). Histamine release was induced by 0.35 μM A23187 in the presence of the indicated concentrations of NECA (▲) or adenosine (■). EC_{50} values calculated from the averaged curves were 0.2 μM for NECA and 1.1 μM for adenosine. Slope factors were 0.6 for both compounds. Points are means of eight experiments with duplicate samples, s.e.means shown by vertical lines.

release from rat peritoneal mast cells initiated by calcium-mobilizing agents by sensitizing the release process to calcium (Lohse *et al.*, 1988). Figure 1 demonstrates this for the calcium ionophore A23187. The presence of a maximally effective concentration of the adenosine analogue NECA (10 μM) shifted the concentration-response curve of A23187 to lower concentrations, changing the EC_{50} value from 0.34 μM to 0.26 μM. In the presence and absence of NECA the concentration-response curves were very steep with slope factors of 4.6 (control) and 5.3 (10 μM NECA). NECA had no significant effect on the release in the absence of A23187.

Figure 2 shows the concentration-response curves of adenosine and NECA in enhancing A23187-induced histamine release. Both compounds have shallow curves with slopes significantly less than 1, when a large number of experiments is averaged. One possible explanation for such shallow concentration-response curves is that they result from the averaging of a heterogeneous population of curves. Such heterogeneity of the concentration-response curves has been observed for agents that inhibit histamine release from basophils by increasing adenosine 3':5'-cyclic monophosphate (cyclic AMP). The inhibitory effects of these agents are strongly dependent on the level of stimulated release: the higher the release, the lower the inhibition obtained by these agents (Tung & Lichtenstein, 1981). In order to examine whether a similar dependence exists for the enhancing effects of adenosine analogues we investigated their effects at different levels of A23187-stimulated histamine release.

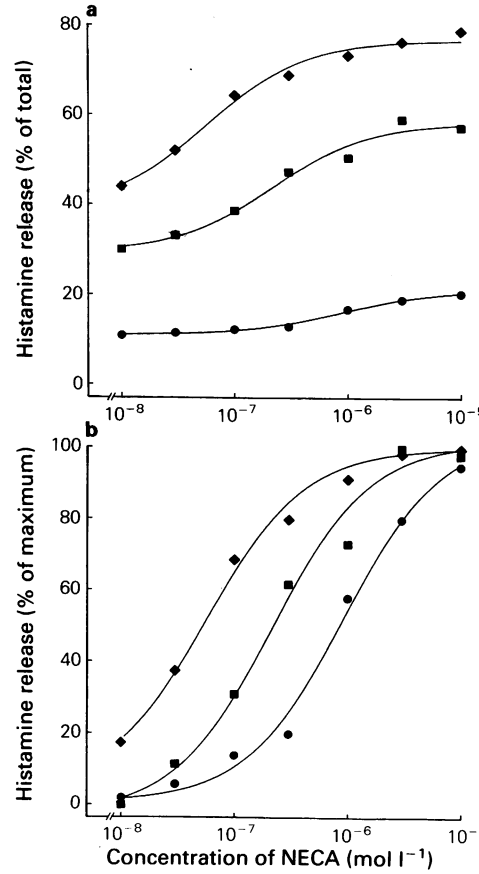

Figure 3 Effects of N-ethylcarboxamidoadenosine (NECA) on the histamine release from mast cells at different degrees of stimulation by A23187. Histamine release was measured in the presence of 0.3 (●), 0.35 (■) or 0.4 (◆) μM A23187. Each curve is the mean of two experiments with duplicate samples. (a) Release expressed in % of total histamine content. (b) Release normalized to the E_{max} of the individual curve. The calculated values of prestimulated release (E_b) and EC_{50} were: 0.3 μM A23187: E_b = 11.1%, EC_{50} = 930 nM; 0.35 μM A23187: E_b = 29.5%, EC_{50} = 215 nM; 0.4 μM A23187: E_b = 38.5%, EC_{50} = 57 nM.

Figure 3a shows the concentration-response curves of NECA in enhancing the histamine release induced by 0.3, 0.35 and 0.4 μM A23187. A higher level of stimulation markedly reduced the EC_{50} value of NECA, which is most clearly seen when the curves are normalized to their respective E_{max} values (Figure 3b). Increasing the level of A23187-induced

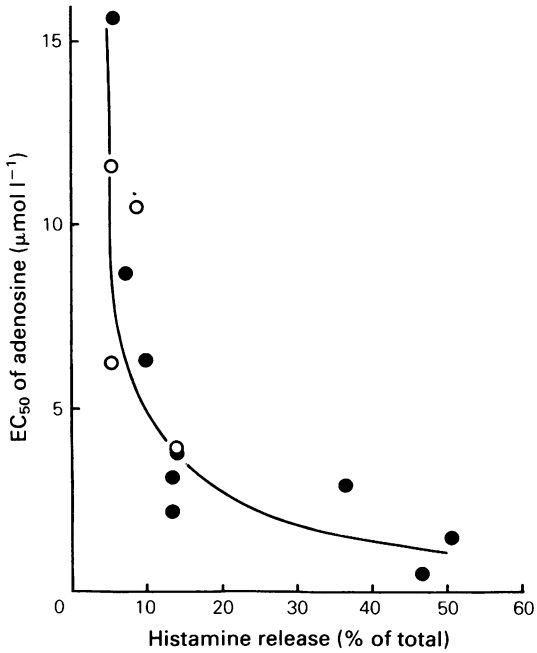

Figure 4 Dependence of the EC_{50} value of adenosine in enhancing mast cell histamine release on the degree of stimulation in the absence of adenosine. Histamine release was induced by different concentrations of A23187 (●) or Concanavalin A (○) in the presence of various concentrations of adenosine. Concentration-response curves of the adenosine-induced enhancement of histamine release were analyzed as in Figure 3, giving EC_{50} values and E_b values (= the release induced by A23187 or Concanavalin A alone). Shown is a plot of the EC_{50} values vs. the E_b values (% stimulation). Each point is the EC_{50} value of one experiment with duplicate samples.

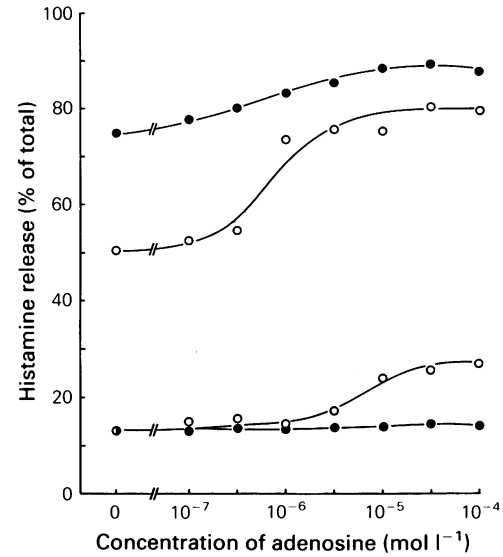

Figure 5 Effects of S-(*p*-nitrobenzyl)-6-thioinosine (NBTI) on adenosine-stimulation of mast cell histamine release. Histamine release was measured in the absence (○) or presence (●) of 1 μM NBTI and the presence of 0.3 μM (bottom curves) or 0.4 μM (top curves) of A23187. Data are the means of two experiments with duplicate samples for each concentration of A23187.

release from 10% to 35% of the total histamine content resulted in a 15 fold lower EC_{50} value of NECA in enhancing this release. Thus, it appears that stronger stimulation increases the sensitivity to NECA.

This effect was also seen with adenosine itself: Figure 4 shows a plot of the EC_{50} values of adenosine in enhancing histamine release versus the level of release induced by either A23187 or Concanavalin A. The EC_{50} values were derived from experiments analogous to those shown in Figure 3 for NECA. Again, the potency of adenosine increases when the release induced by A23187 alone increases. There is a concave relationship, with EC_{50} values ranging from 0.5 μM to 16 μM. An apparently identical relationship was found for Concanavalin A-induced release, although higher levels of release could only be achieved with A23187.

We have previously shown that exogenous adenosine has to be taken up via the nucleoside transporter in order to enhance mast cell mediator release (Lohse *et al.*, 1987). Because the nucleoside transport is a facilitated bidirectional process, the presence of nucleoside transport inhibitors traps intracellular adenosine (Paterson *et al.*, 1983). Therefore, if high levels of stimulation sensitize mast cell histamine release to adenosine, the effects of the nucleoside transport inhibitor NBTI might be different at higher levels of release. Indeed, Figure 5 shows that in the absence of exogenous adenosine, NBTI 1 μM alone had no effect at low levels of histamine release. However, NBTI markedly enhanced the release at a high level of A23187-induced release. NBTI almost completely abolished the enhancing effect of exogenous adenosine at either level of prestimulation.

Figure 6 shows the dependence of the enhancing effect of NBTI on the degree of stimulation of histamine release induced by various concentrations of A23187. There was little enhancement by NBTI when the histamine release induced by A23187 alone was below 10%, but a marked effect was observed at higher levels of release, reaching a maximum when the A23187-induced release exceeded 20%. This relationship is very similar to that seen for the EC_{50}

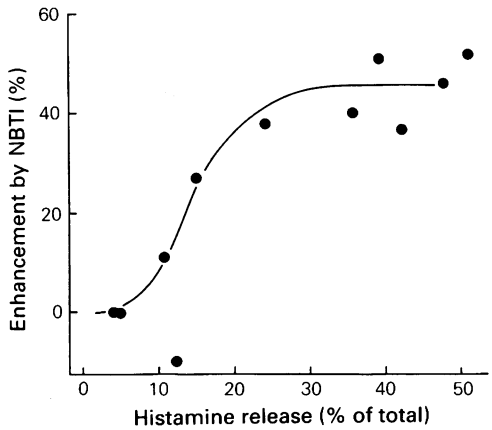

Figure 6 Dependence of the effect of S-(*p*-nitrobenzyl)-6-thioinosine (NBTI) on mast cell histamine release on the stimulated release. The release was stimulated in the absence or presence of 1 μM NBTI with different concentrations of A23187 as in Figure 5. Plotted is the enhancement induced by NBTI as a function of the release in the presence of A23187 alone. Each point represents one experiment with duplicate samples.

value of adenosine (Figure 4), suggesting that it reflects the same underlying mechanism.

Discussion

Adenosine and several of its metabolically stable analogues, such as NECA, appear to stimulate histamine secretion from rat peritoneal mast cells by a mechanism distinct from stimulation of A_2 receptors and elevation of cyclic AMP. The inhibition of this effect by the nucleoside transport inhibitor NBTI suggests that elevation of intracellular adenosine is required (Lohse *et al.*, 1987). The enhancement of histamine release appears to be related to an enhanced sensitivity of the release process to free intracellular calcium (Lohse *et al.*, 1988). This enhanced sensitivity was seen in the present study as a shift of the concentration-response curve of the calcium ionophore A23187 to lower concentrations in the presence of NECA.

Tung & Lichtenstein (1981) have demonstrated that basophils are more sensitive to inhibitory stimuli at low levels of release. Our data show that the reverse is true for the enhancing effects of adenosine: the potencies of adenosine and its metabolically stable analogue NECA are markedly increased at higher levels of release. Thus, 100 nM NECA has almost no effect when the release is below 10% of the total histamine content, but the same concentration causes a more than half maximal enhancement when the release exceeds 40%. This dependence of the response to adenosine and its analogues on the level of stimulation is a likely explanation for the large variations of its EC_{50} value and the flat concentration-response curves when a large number of experiments are averaged, which has been found by various workers (Marquardt *et al.*, 1978; Holgate *et al.*, 1980; Welton & Simko 1980; Church *et al.*, 1986).

There are several reports in the literature that calcium may not alone be responsible for the mediation of secretory responses (see Gomperts, 1986, and Penner, 1988, for reviews). Our data presented here and in previous papers (Lohse *et al.*, 1987; 1988) suggest that intracellular adenosine might represent a factor which is required in addition to an elevation of intracellular calcium for release to occur. Although more complex models can be proposed, the observation of a reciprocal enhancement of the effects of adenosine and calcium is compatible with their action at the same or closely related sites. This supports our assumption that the effects of adenosine are mediated via an intracellular site. However, other authors assume that adenosine acts on an atypical cell surface receptor on mast cells (Church *et al.*, 1986; Marquardt *et al.*, 1988). Our hypothesis was based mainly on the observations that the potent nucleoside transport inhibitors NBTI and S-(*p*-nitrobenzyl)-6-thioguanosine (NBTG) virtually abolish the enhancement of histamine release by adenosine. Although other nucleoside transport inhibitors such as dipyridamole have been found to be inactive in this respect, this may be explained by their well-known low potency in the rat (Lohse *et al.*, 1987). It might be speculated that NBTI and NBTG act as antagonists at an atypical adenosine receptor on mast cells. Such a hypothesis is, however, incompatible with the observation that at high levels of release NBTI, while still blocking the effects of exogenous adenosine, has an effect comparable to the maximal enhancement of adenosine, i.e. would appear to be a full agonist (see Figure 5). Consequently, we feel that an intracellular site of action of adenosine is the most sensible interpretation of our results.

This intracellular site may be related to the G_E-protein postulated by Gomperts and coworkers to link elevation of cytosolic free calcium and additional signals to exocytosis (Gomperts, 1986; Howell *et al.*, 1987; Cockcroft *et al.*, 1987). This hypothesis is also compatible with the observation that pretreatment of bone marrow-derived mast cells with pertussis toxin inhibits the enhancement of mediator release by adenosine (Marquardt & Walker, 1988), since pertussis toxin appears to affect the putative G_E-protein (Penner, 1988). Finally, it has also been suggested that cyclic AMP exerts its inhibitory

effects on mediator release at the level of G_E (Penner, 1988). According to this hypothesis there may be an integration of several stimulatory and inhibitory intracellular signals at the level of G_E. Whereas the inhibitory effects of cyclic AMP are reduced in the face of strong stimuli (Tung & Lichtenstein, 1981), this paper shows that stimulatory signals may synergistically enhance each other.

Thus, intracellular adenosine and calcium may have synergistic effects on histamine release, by reciprocally increasing the sensitivity of the secretory process to the other agent. The data presented here show that physiological intracellular concentrations of adenosine are sufficient to produce these effects: first, trapping adenosine inside stimulated mast cells with NBTI can cause a considerable enhancement of histamine release, which corresponds to the effect produced by 1–3 μM exogenous adenosine in the absence of NBTI. Second, micromolar concentrations of adenosine are found in the extracellular space of many tissues and species (see Gerlach & Becker, 1987, for a review).

Adenosine is released upon stimulation of mast cells (Marquardt *et al.*, 1984). This may reflect either true release, or may be due to increased intracellular adenosine concentrations, e.g. due to ATP consumption. In any case, the increased concentrations of adenosine found in the extracellular space should coincide with elevated concentrations inside the cells as adenosine rapidly equilibrates across the nucleoside transporter (Paterson *et al.*, 1983). By increasing the sensitivity of the secretory process to the prevailing concentrations of calcium, adenosine might thus constitute a positive feed-back signal resulting in accelerated degranulation of mast cells.

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The role of microsomal phospholipids and their fatty acid composition in the control of hepatic metabolism of lignocaine

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1 Sex differences exist in the metabolism of lignocaine by the rat liver. Microsomal phospholipids have been implicated in the control of these sex differences. Induction of diabetes in the male rat abolishes these sex differences. The difference in drug metabolism between the male and female rat is, thus, the same as that between the control and diabetic male rat.

2 By using reconstitution of delipidated male microsomal proteins with male-, female- and diabetic-derived phospholipids as well as synthetic phospholipids, it should be possible to delineate the role of phospholipids in the control of drug metabolism.

3 Female- and diabetes-derived phospholipids decrease the activity of the male-specific lignocaine N-deethylase specifically by between 35 and 52%.

4 Analysis of the phospholipid classes and fatty acid content of the various fractions indicated that stearic acid content was increased and arachidonic acid content decreased in both female- and diabetic-derived lipids as compared to control males. Linoleic acid content was decreased in female- but increased in diabetic-derived lipids as compared to control males. Subsequent correlation to N-deethylase activity, however, rules out all but the arachidonic acid content of the phospholipids as a controlling factor of lignocaine metabolism.

5 Use of synthetic phosphatidylcholine (PC) species for reconstitution indicates that diarachidonyl-PC is the most efficient at activating the N-deethylase and indicates that the degree of unsaturation of the fatty acyl side-chains of PC is of major importance in the regulation of this enzyme activity.

6 The presence of unsaturated fatty acids, and especially arachidonic acid, in the phospholipids is, thus, a major controlling influence on the specific activation of lignocaine N-deethylase in the rat liver.

Introduction

There are three essential components of the cytochrome P-450-dependent hepatic mixed function oxidase enzyme system; cytochrome P-450, NADHP-cytochrome P-450 reductase and phospholipid (Lu & Coon, 1968) and of these, the amount and type of cytochrome P-450 has long been considered the major controlling influence.

Indeed, if sex differences in drug metabolism in the rat are examined, it is seen that there are sex-dependent isozymes of cytochrome P-450 which could explain most of the sexual differentiation seen. For instance, a female-specific cytochrome P-450 (isozyme 15β-hydroxylase, MacGoech *et al.*, 1984), P-450-female (Kato & Kamataki, 1982) or UT-I/2d (Waxman, 1984; now designated cytochrome P-450IIC12) has been purified as has a male-specific isoform (variously termed cytochrome P-450 2c/UT-A or RLM5 (Waxman, 1984; Morgan *et al.*, 1985); now designated cytochrome P-450IIC11).

However, Barr & Skett (1984) provided evidence that, for at least one substrate, sex-dependent cytochrome P-450 was not the whole answer. In this study it was seen that the activity of the male-specific lignocaine N-deethylase could be controlled by the microsomal lipid. This finding was confirmed and extended by Meftah (Meftah & Skett, 1987a,b; Meftah, 1988) to show that the isoenzyme of cytochrome P-450 performing the N-deethylation was

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the male-specific isoform previously isolated (2c above) and that this isoenzyme specifically interacted with microsomal phospholipid to modify the activity of the enzyme.

The sex-dependent enzyme activities in the male rat are also susceptible to modulation by the diabetic state as previously described (Skett *et al.*, 1984; Skett & Joels, 1985; Skett, 1986), with the male-specific enzyme activities moving towards the female level (a feminisation). The N-deethylation of lignocaine was significantly and markedly reduced in the diabetic state without any effect on the non-sex-dependent 3-hydroxylation of the same substrate (Skett & Joels, 1985).

It was, thus, of interest to examine how the phospholipid fraction of the hepatic endoplasmic reticulum could modulate the male-specific N-deethylation of lignocaine by comparing the effects of female- and diabetic-derived phospholipids and the analysis of the type of phospholipid present and its fatty acid content.

Methods

Experimental animals

Mature rats of Wistar stock, bred in the Department, were used throughout the study. Males weighed 300–350 g and females 225–250 g at the start of the experiment. The animals were kept in a light- and temperature-controlled environment (lights on 07 h 00 min–19 h 00 min; temperature 19 ± 1°C) and were allowed free access to food (CRM Nuts, Labsure, Croydon, U.K.) and tap water. A single intravenous dose of streptozotocin (90 mg kg^{-1} in distilled water) was used to induce diabetes mellitus as described previously (Skett, 1986).

Preparation of the delipidated microsomal protein

The animals were killed by CO_2-asphyxiation followed by cervical dislocation and the livers quickly excised and rinsed in ice-cold 0.25 M sucrose. The microsomal fraction of the tissue was prepared by the ultracentrifugation method of Berg & Gustafsson (1973) and haemoglobin was removed from this fraction as described by Mihara & Sato (1978). The cleaned microsomal preparation was subsequently dissolved by the addition of sodium cholate and delipidated by elution from a Bio-Gel P-30 column as described previously (Meftah & Skett, 1987a). The protein fraction from this column is the delipidated microsomal protein and has been shown to contain no measurable phospholipid (Meftah & Skett, 1987a).

Reconstitution of an active enzyme system

The delipidated proteins were reconstituted with lipids of known composition by the method of Ingelman-Sundberg & Glaumann (1980) using extracted microsomal lipids, prepared as below, or synthetic phospholipids. The optimum ratio of lipid to protein, previously tested (Meftah & Skett, 1987a), was used in all cases. Assay of drug-metabolizing capacity was assessed by use of lignocaine as substrate, by a modification of the method of Skett *et al.* (1980), as described by Meftah & Skett (1987a). The cytochrome P-450 content of the reconstituted preparation was assayed by the method of Omura & Sato (1964) and the results are expressed as nmol product formed min^{-1} $nmol^{-1}$ cytochrome P-450.

Separation and assays of phospholipids

Microsomal lipids were extracted from the cleaned microsomal fraction by the organic solvent extraction method of Overturf & Dryer (1969) and stored at −20°C in the dark under a nitrogen atmosphere until used. The phospholipid fraction was prepared by silica gel column chromatography as described by Rouser *et al.* (1970) and the phospholipids further separated into their respective groups (based on head group analysis) by two-dimensional thin-layer chromatography on silica gel as previously described (Yavin & Zutra, 1977). This method can separate distinctly phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol from each other and from phosphatidic acid. The amount of phospholipid in each spot was assayed by determining the phosphorus content by the method of Rouser *et al.* (1970).

Preparation and assay of fatty acid content of microsomal phospholipids

The fatty acid composition of the microsomal phospholipids was assayed by purifying the phospholipid fraction as outlined above and converting the mixture to fatty acid methyl esters as described by Kates (1964). This method involved refluxing the lipids in the presence of methanolic sodium hydroxide solution and subsequent extraction of the methyl esters formed. Fatty acid methyl esters were stored at −20°C in the dark under a nitrogen atmosphere until assayed. The fatty acid methyl esters were analysed by capillary gas chromatography on a 1% OV-1 column (film thickness 250 nm, length 12 m) in a Perkin-Elmer 8420 Gas Chromatograph at 190°C (isothermal) by use of an FID detector. Authentic standard fatty acid methyl esters were also run through the column for positive identification of the

peaks obtained. Quantitation of the peaks was by triangulation.

Statistics

Results are calculated as the mean ± standard deviation (s.d.) of at least six independent samples from different microsomal preparations (actual number of samples given in the tables). Statistical analysis was by the use of Student's t test for unpaired samples (for comparing two groups) or Duncan's multiple range test (for three or more groups). The level of significance was set at $P < 0.05$.

Chemicals

Bio-Gel P-30 was purchased from Bio-Rad Laboratories, Watford, U.K., Sephadex G-50 from Pharmacia Fine Chemicals, Milton Keynes, U.K. and [carbonyl-^{14}C]-lignocaine from Amersham Intl. plc, Aylesbury, U.K. Unlabelled lignocaine was the kind gift of Astra Läkemedel AB, Södertälje, Sweden. NADPH, ammonium molybdate, streptozotocin, the free fatty acids and the phosphatidylcholine species were all obtained from Sigma Chemical Co. Ltd., Poole, U.K. All other reagents were of the highest purity available commercially.

Results

Effect of different microsomal phospholipids on metabolism of lignocaine

As can be seen in Table 1, reconstruction of microsomal proteins derived from the male rat liver with microsomal phospholipids derived from the same source gave a pattern of metabolism with a greater preponderance of monoethylglycylxylidide (MEGX). This is similar to the metabolism seen for the untreated microsomal preparation from the male rat liver (Skett *et al.*, 1980). Reconstitution with female phospholipids or phospholipids derived from the diabetic male animals gave a significantly lower N-deethylase activity without any effect on the 3-hydroxylase activity. Indeed, statistical analysis of the results indicated that the phospholipids derived from the chronic (21 days) diabetic animals were poorer than the female-derived lipids in reconstituting N-deethylase activity ($P < 0.05$).

Table 1 The effect of reconstitution with lipids from different sources on the metabolism of lignocaine by male delipidated microsomal proteins

Lipid source		*3-Hydroxylase*	*N-deethylase*
Male	(A)	0.50 ± 0.03	1.86 ± 0.02
Female	(B)	0.49 ± 0.02	1.04 ± 0.02
3 d diabetic male	(C)	0.48 ± 0.02	1.20 ± 0.15
21 d diabetic male	(D)	0.50 ± 0.03	0.90 ± 0.06
		C B A D	D B C A

Results are expressed as nmol product formed min^{-1} nmol^{-1} P-450 and are means ± s.d.
$n = 12$ for control animals and 6 for diabetic animals.
Data analysed by Duncan's multiple range test — letters refer to groups and those underlined are not significantly different ($P > 0.05$).

Analysis of phospholipids in microsomal fraction

As the source of the phospholipid had such a marked effect on the N-deethylation of lignocaine, it was of interest to analyse the composition of the added phospholipid fraction. Table 2 shows the results of this analysis (based on phosphorus content of the separated lipids). It is seen that there is a significant ($P < 0.05$) decrease in the total lipid content (expressed as g^{-1} liver) of all groups compared to the control male. This lowering is reflected in the decreased amount of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) in both the female- and chronic diabetic-derived lipids. The lipids

Table 2 Phospholipid content of microsomal lipids derived from male, female and diabetic male rats

Phospholipid class	*Male*	*Female*	*3 d STZ*	*21 d STZ*
		(μmol g^{-1} wet weight liver)		
Total	11.5 ± 0.5	9.0 ± 0.6*	10.5 ± 0.3*	7.8 ± 0.5***
PC	6.5 ± 0.2	5.5 ± 0.2*	6.0 ± 0.4	4.5 ± 0.3**
PE	3.9 ± 0.2	2.5 ± 0.4**	3.5 ± 0.2	2.5 ± 0.2**
PS	0.3 ± 0.2	0.3 ± 0.2	0.3 ± 0.1	0.3 ± 0.2
PI	0.8 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	0.5 ± 0.1
PC/PE	1.67	2.20	1.71	1.80

Results are expressed as mean ± s.d. of 6 independent samples for each group.
Data analysed by Student's unpaired t test; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ as compared to male control.
PC = phosphatidylcholine, PE = phosphatidylethanolamine, PS = phosphatidylserine, PI = phosphatidylinositol.
STZ = streptozotocin and denotes a diabetic animal. 3 d = 3 days after STZ; 21 d = 21 days after STZ.

Table 3 Analysis of fatty acyl side-chain content in phospholipids from male, female and diabetic male hepatic microsomes

Fatty acid	*Male*	*Female*	*3 d STZ*	*21 d STZ*
Palmitic	30 ± 3	29 ± 2	30 ± 3	30 ± 2
Palmitoleic	14 ± 2	14 ± 2	15 ± 1	11 ± 1*
Stearic	10 ± 1	25 ± 1***	10 ± 1	14 ± 1*
Oleic	10 ± 2	10 ± 1	10 ± 1	15 ± 2*
Linoleic	12 ± 1	8 ± 1**	11 ± 1	14 ± 1*
Arachidonic	12 ± 1	5 ± 1**	6 ± 1**	3 ± 1***
Others	12 ± 1	9 ± 1	18 ± 2	13 ± 1
% unsaturated	48	37	42	42

Results are expressed as mean ± s.d. of 6 independent samples.
Statistical analysis was by means of Student's *t* test for unpaired samples; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to the control male.
For key to abbreviations used see Table 2.

derived from the acute (3 day) diabetic rats showed no significant decrease in PC or PE as compared to the intact males. There were no significant differences in phosphatidylserine (PS) or phosphatidylinositol (PI) between any of the groups. There appeared to be a graded difference from the male-derived lipids with the highest content of each phospholipid through the acute diabetic- and female-derived lipids to the lipids derived from the chronically diabetic animals, which showed the lowest content of each phospholipid class on each occasion. The PC/PE ratio was markedly higher in female-derived microsomes than any of the other groups (2.2 compared to 1.67–1.8).

Analysis of fatty acid content of phospholipid fraction

A further analysis of the total phospholipid fraction to determine its fatty acid content resulted in the data shown in Table 3. It is seen that there were some marked differences between male- and female-derived phospholipids, with the male-derived lipids exhibiting a higher proportion of polyunsaturated fatty acids (linoleic (18:2) and arachidonic acid (20:4) ($P < 0.01$) and a lower proportion of stearic acid (18:0) ($P < 0.001$)). Analysis of the fatty acid content of microsomal phospholipids derived from diabetic male rats also gave changes from the control male. The acute diabetic gave a reduced content of arachidonate (20:4) ($P < 0.01$), whereas the chronic diabetic gave a reduced content of palmitoleate (16:1) ($P < 0.05$) and an increased content of stearate (18:0), oleate (18:1) and linoleate (18:2) (all $P < 0.01$), as well as a very marked reduction in arachidonate (20:4) ($P < 0.001$). It should be noted that the differences between the male- and female-derived phospholipids were not the same as those caused by diabetes (either acute or chronic). The percentage of unsaturated fatty acids was somewhat lower in the female- than male-derived phospholipids (37 compared to 48%) and the value for the diabetic state was intermediate between the two control values, and did not differ between acute and chronic diabetes (42%).

Effect of reconstitution with phosphatidylcholine species with differing fatty acyl chains on lignocaine metabolism

Delipidated microsomal proteins from male rats were reconstituted by the addition of synthetic phosphatidylcholine with known and differing fatty acyl side chains (ranging from dicaproyl (6:0) to diarachidonyl (20:4)). The results obtained are shown in Table 4. It is seen that the N-deethylation of lignocaine by these preparations was markedly affected by the species of PC used; diarachidonyl-PC (20:4) was the most effective in the reconstitution system

Table 4 The effect of reconstitution with phosphatidycholines (PCs) of differing acyl chains on the metabolism of lignocaine by a delipidated male microsomal protein preparation

Lipid used	*3-Hydroxylation*	*N-deethylation*
DAPC (A)	0.43 ± 0.03	1.49 ± 0.06
DOPC (B)	0.43 ± 0.02	1.24 ± 0.04
DBPC (C)	0.44 ± 0.01	1.03 ± 0.03
DSPC (D)	0.43 ± 0.02	0.97 ± 0.03
DLPC (E)	0.45 ± 0.01	0.97 ± 0.03
DCPC (F)	0.35 ± 0.01	0.79 ± 0.06
	A B C D E F	A B C D E F

Results are expressed as nmol product formed min^{-1} $nmol^{-1}$ P-450 and are means ± s.d. of 6 independent samples. Statistical analysis was by Duncan's multiple range test — those group letters underlined indicate that the groups were not significantly different ($P > 0.05$). DAPC = diarachidonyl-PC; DOPC = dioleoyl-PC; DBPC = dibehenoyl-PC; DSPC = distearoyl-PC; DLPC = dilauroyl-PC; DCPC = dicaproyl-PC.

whereas dilauryl- (12:0), disteoryl- (18:0) and dibehenoyl-PC (22:0) all gave a lower but constant activity. Dioleoyl-PC (18:1) gave a significantly higher activity than disteoryl-PC (18:0) ($P < 0.05$) and dicaproyl-PC (6:0) gave the lowest activity. There were no differences in the effects of the PC species on 3-hydroxylation, except for dicapropyl-PC which resulted in a significantly lower activity than any of the other PCs.

Discussion

Lignocaine is a clinically important drug whose metabolism has been shown to be sex differentiated in the rat liver (Nyberg *et al.*, 1977; Skett *et al.*, 1980) with the N-deethylation (thought to be catalysed by cytochrome P-450IIC11) being markedly greater in male than female rats, whereas the 3-hydroxylation (thought to be catalysed by cytochrome P-450IIA1) shows no sex difference. Although there is no reported sex difference in the metabolism of lignocaine in man, there are sex differences in the metabolism of other drugs (for review, see Skett, 1988), and any effects seen on the metabolism of lignocaine in the rat may be relevant to the metabolism of these drugs in man.

There is evidence to suggest that this sex difference may be related to the lipids in which the enzymes are embedded in the endoplasmic reticulum of the liver cell. Early work in this laboratory (Barr & Skett, 1984) indicated that male- and female-derived microsomal lipids had different effects on lignocaine metabolism (in line with the sex differences seen in metabolism). Extension of this work using delipidated and reconstituted microsomal proteins (Meftah & Skett, 1987a), showed that the sex difference in lignocaine N-deethylation could be explained by the presence of sex-specific lipids with female-derived lipids able to reduce the activity of male-derived enzymes to the level seen in the female. Such correlations between differences in drug metabolism and microsomal lipids have been postulated previously (Belina *et al.*, 1975; Turcan *et al.*, 1981).

The data in this study (Table 1) confirm the role of sex-dependent microsomal lipids in the control of lignocaine N-deethylation and also show that lipids derived from diabetic male rats can affect this enzyme. The lipids derived from the chronic (21 days) diabetic animals appeared to be more effective than that from the acute (3 day) diabetic animals. In this case, it is specifically the phospholipids which have been used, indicating that the sex-dependent effects of the microsomal lipids reside in this fraction. The diabetic state is known to decrease male-specific drug metabolism in the rat liver (Skett & Joels, 1985) and, indeed, has been shown to have a specific reductive effect on lignocaine N-deethylation in the male rat. The lower N-deethylase activity of the male-derived microsomal proteins in the presence of diabetic-derived lipids is, therefore, not unexpected and further enhances the idea of lipid control of sex-dependent drug metabolism. It is seen, therefore, that both female- and diabetes-derived microsomal phospholipids can decrease lignocaine N-deethylase activity.

In order to correlate these effects to phospholipid changes it was necessary to analyse the different phospholipid fractions. We analysed the phospholipids in terms of head group and fatty acid content. It was noted that female-derived phospholipids contained less total phospholipid (expressed g^{-1} liver tissue) than male-derived lipids, which was reflected in a lower content of PC and phosphatidylethanolamine (PE). The PC/PE ratio was markedly higher in females than in males. This confirms the findings of Belina *et al.* (1975). This was mimicked by the chronic diabetes-derived phospholipids except for the effect on the PC/PE ratio which was unaffected by the diabetic state. The phospholipids derived from the acute diabetic animals showed a pattern of lipids intermediate between the control male and control female. The actual amount of phospholipid per g liver is unimportant to this study as the same quantity of lipid was used in each reconstitution. What may be important, however, is the relative proportions of the different phospholipids. It has previously been noted that increasing the proportion of PE can enhance some drug metabolising enzyme activities (Ingelman-Sundberg & Glaumann, 1980) and, indeed, for the substrate used in this study, we have previously shown that N-deethylase activity of delipidated male-derived proteins is increased in the presence of PE (Meftah & Skett, 1987a). This is also seen for the activity of a purified, sex-specific cytochrome P-450 isozyme (cytochrome P-450 2c) indicating that we are examining a direct interaction between the lipid and the terminal oxidase enzyme (Meftah & Skett, 1987b). There is, however, no change in PC/PE ratio in the diabetes-derived phospholipids compared to control males, although this lipid mixture has a profound effect on N-deethylase activity (Table 1). It cannot simply be the PC/PE ratio that is causing the effects seen.

Analysis of the fatty acid content of the phospholipids (Table 3) showed that there were marked sex differences also in this parameter. There was a lowered linoleic (18:2) and arachidonic (20:4) acid and increased stearic (18:0) acid content in the female-derived phospholipids and, thus, the percentage of unsaturated fatty acids dropped from 48% to 37%. These differences are similar to those obtained by Feuer *et al.* (1980). Could any of these differences correlate to the effect on lignocaine N-deethylase activity? If we examine the fatty acid content of the

diabetes-derived phospholipids (which exert the same effect on N-deethylase activity), we see that the only effect of acute diabetes is a significant decrease in arachidonic acid ($P < 0.01$), whereas the chronic diabetic-derived lipids show a decrease in arachidonic and palmitoleic (16:1) acids and an increase in stearic, oleic (18:1) and linoleic (18:2) acids. In both cases the percentage unsaturated was intermediate between the control male and control female. These data are similar to those of Faas & Carter (1983) and Mercuri *et al.* (1967), except that the former group saw an increase in total phospholipid in diabetic animals. Faas & Carter (1980, 1983) suggested that the changes in fatty acid content were a consequence of the altered activity of the fatty acid desaturase enzymes. These differences between the female- and diabetic-derived phospholipids would seem to rule out linoleic acid as a candidate for the effector and, indeed, the overall percentage of unsaturated fatty acids in the phospholipids. We are, thus left with stearic and arachidonic acids as the possible factors affecting lignocaine N-deethylase activity. Attempts to correlate the activity of the lignocaine N-deethylase to all of the parameters so far measured showed that there was a weak correlation with PC and PE content (correlation coefficeint of 0.859 and 0.886 respectively), although this was negated by the use of the same amount of lipid from each sample in the reconstitution, and a strong correlation (correlation coefficient of 0.997) with the arachidonic acid content of the phospholipid. There were no other significant correlations seen. It seemed most likely, therefore, that the phospholipid content of arachidonic acid was the factor controlling N-deethylase activity.

In order to test this hypothesis further, delipidated male-derived proteins were reconstituted with synthetic PC species of known fatty acid content and the activity of the N-deethylase measured. Diarachidonyl-PC was by far the most efficient in reconstituting N-deethylase activity being 50% better than the PC containing saturated fatty acids (lauric, stearic and behenic (22:0) acids). Dioleoyl-PC (18:1) was significantly better than its saturated homologue (distearoyl-PC) ($P < 0.05$), whereas dicaproyl-PC (6:0) showed the lowest activity probably due to the formation of micelles by this PC rather than vesicles. (Dencher, 1986). This non-specific effect of dicaproyl-PC is further shown by the fact that it decreases 3-hydroxylase activity as well (an effect not noted for any of the other PC species). Thus, chain length above C-12 has little effect on N-deethylase activity but degree of unsaturation seems to be important and arachidonic acid, as noted above, seems to be the best side-chain in PC to reconstitute lignocaine N-deethylase activity. This would fit with the evidence of Marshall & McLean (1971) and Rowe & Wills (1976) that diets deficient in unsaturated fatty acids led to decreased drug metabolism and that unsaturated fatty acids can alter the spin-state of cytochrome P-450 and, thus, its efficiency as a terminal oxidase (Gibson & Tamburini, 1984).

All of the evidence, therefore, points heavily towards the phospholipid fraction of the endoplasmic reticulum membrane being important in the regulation of sex-dependent drug metabolism and that the arachidonic acid content of the phospholipid is, perhaps, the important factor in this control. This may relate to the control of sex dependent metabolism of other drugs in man but, due to the lesser importance of this phenomenon in this species, may be more related to a general phenomenon of regulation of drug metabolism by the microsomal membrane composition as well as the enzyme complement of the membrane.

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Pharmacological profile of leukotrienes E_4, N-acetyl E_4 and of four of their novel ω- and β-oxidative metabolites in airways of guinea-pig and man *in vitro*

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1 The biological effects of metabolites of leukotriene E_4 (LTE_4) i.e. N-acetyl LTE_4 (N-AcLTE_4), 20-COOH-LTE_4, 20-COOH-N-AcLTE_4, as well as 18-COOH-19,20-dinor-LTE_4 (dinor-LTE_4) and 16-COOH-17,18,19,20-tetranor-14,15-dihydro-LTE_4 (tetranor-LTE_4) were investigated on superfused strips of guinea-pig trachea (GPT) and lung parenchyma (GPP) *in vitro*.

2 The actions of LTE_4 were studied in isolated, superfused strips of human lung parenchyma (HP) and bronchus (HBr), in comparison with LTD_4 and histamine. Effects of N-AcLTE_4, the 20-carboxy metabolites, dinor-LTE_4 and tetranor-LTE_4 were also investigated in HBr.

3 N-AcLTE_4 (0.1–10 nmol) induced dose-related contractions of GPT and was approximately 100 times less active than LTD_4 (3–100 pmol).

4 In GPP, N-AcLTE_4 (0.01–3 nmol) was equiactive with LTE_4 (0.01–1 nmol) and approximately one order of magnitude less active than LTD_4 (1–300 pmol). Contractions caused by N-AcLTE_4 and LTE_4 were very similar and approximately twice as sustained as those due to LTD_4.

5 LTE_4 (0.1–30 nmol) contracted strips of HP and HBr and was about 2–3 orders of magnitude less active than LTD_4. As in GPP, the effect of LTE_4 was more protracted than that of LTD_4. Actions of N-AcLTE_4 were similar to those of LTE_4 in HBr.

6 20-carboxy-LTE_4, 20-carboxy-N-AcLTE_4, dinor-LTE_4 and tetranor-LTE_4, all at 0.3–30 nmol, were inactive in GPT, GPP and HBr.

7 Indomethacin (2.8 μM) potentiated the effect of N-AcLTE_4 in GPT, inhibited its contraction in GPP but did not affect that due to LTE_4 in either HP or HBr. FPL 55712 (1.9 μM) antagonised leukotriene-induced contractions in GPT, GPP and HBr.

8 In conclusion, the metabolism of LTD_4 to LTE_4 or N-AcLTE_4 may represent a detoxification but not an inactivation of cysteinyl-containing leukotrienes, since both metabolites still retain considerable biological activity in guinea-pig and human airways *in vitro*. However, further metabolism of LTE_4 and N-AcLTE_4 appears to result in inactivation of leukotrienes.

Introduction

Metabolism of cysteinyl-containing leukotrienes *in vivo* was thought to proceed predominantly via modification of the peptide moiety at C-6, with little or no change in the fatty acid part of the molecule. Leukotriene E_4 (LTE_4) was described as the main end-product of defined structure, generated in plasma, bile and urine of several species, including man, following exogenous administration of LTC_4 (Örning *et al.*, 1985; Denzlinger *et al.*, 1986; Ezra *et al.*, 1987). However, one exception is the rat, where LTE_4 can further undergo N-acetylation resulting in the synthesis of a novel leukotriene, N-acetyl LTE_4 (N-AcLTE_4), which is the major stable metabolite of LTC_4, characterized in bile as well as in subcellular fractions of liver and microsomal preparations of spleen, kidney, skin and lung (Hagmann *et al.*, 1986; Bernström & Hammarström, 1986). Metabolism of LTC_4 leading to formation of N-AcLTE_4 seems to occur via the mercapturic acid pathway, which is the established detoxification pathway of many xenobiotics before their excretion from the body. More recently, another route of degradation of LTE_4, via

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alteration of the fatty acid part of the molecule, was shown to occur *in vitro* and *in vivo*. Thus, novel oxidative metabolites, which are thought to arise following N-acetylation of LTE_4 via ω-oxidation and β-oxidation, were identified in rat bile and in preparations of liver microsomes and hepatocytes in this species (Keppler *et al.*, 1988; Stene & Murphy, 1989). It is of interest that such polar metabolites do not exclusively occur in the rat since ω-hydroxy-LTE_4 and ω-carboxy-LTE_4, for instance, were also detected in the bile of the monkey *Macaca fascicularis* (Keppler *et al.*, 1988). We have previously shown that LTC_4 and LTD_4 are very potent contractile agents of guinea-pig isolated airways and that their metabolite, LTE_4, although less potent than either parent molecule, retains considerable biological activity in this species. However, LTE_4 has a longer duration of action, causing contractions which are at least twice as protracted as those due to LTC_4, LTD_4 or, indeed, LTB_4 on superfused strips of guinea-pig lung parenchyma *in vitro* (Piper & Samhoun, 1983).

We found it of interest to extend our previous studies of the bronchoconstrictor actions of leukotrienes in the guinea-pig and man. Hence, in this paper we describe the effect of N-AcLTE_4 on isolated preparations of guinea-pig trachea (GPT) and lung parenchyma (GPP) and of human bronchus (HBr). Initial results with LTE_4 on strips of human lung parenchyma (HP) and HBr suggested that this leukotriene possesses actions which are similar to those obtained in guinea-pig airways *in vitro*. These findings have been extended in this work. In addition, the availability of four novel oxidative leukotriene metabolites, products of ω- and β-oxidation of cysteinyl-containing leukotrienes, namely 20-COOH-LTE_4, 18-COOH-19,20-dinor-LTE_4 (dinor-LTE_4), 16-COOH-17,18,19,20-tetranor-14,15-dihydro-LTE_4 (tetranor-LTE_4) and 20-COOH-N-AcLTE_4, allowed us to perform further studies in preparations of GPT, GPP and HBr.

Part of the work describing the actions of N-AcLTE_4 in guinea-pig isolated airways was previously communicated to the British Pharmacological Society (Conroy *et al.*, 1987).

Methods

Guinea-pig tissues

Tracheal strips Male guinea-pigs (Dunkin Hartley, 500–550 g) were killed by a blow on the head, the trachea excised and trimmed of fat and connective tissue. Each GPT was then opened by a longitudinal cut on the ventral site and cut in a zigzag fashion, with alternate incisions made every 2–3 cartilage rings (Emmerson & Mackay, 1979).

Lung parenchymal strips These were prepared from large lobes as previously described (Piper & Samhoun, 1981).

Human tissues

Macroscopically normal tissue was obtained from patients undergoing lung resection for treatment of bronchial carcinoma or from donors at organ transplant operations. They were collected in Tyrode solution and used on arrival at the laboratory.

Lung parenchymal strips Segments of parenchyma were cut from the periphery of lobes and trimmed into strips measuring approximately 5 × 3 × 3 cm.

Bronchial strips Bronchi, of mainly second and third generation, were dissected, trimmed of connective tissue and then spirally cut into strips.

All tissues were suspended in a superfusion cascade system, in two banks of 2 to 3 tissues each, under a tension of 1 g, with the exception of some strips of human bronchi which required 2 g. Preparations were continuously superfused at 37°C at a rate of 5 ml min^{-1}, with oxygenated Tyrode solution composed of (mM): NaCl 138, KCl 2.7, $CaCl_2$-$6H_2O$ 1.8, $MgCl_2\,6H_2O$ 0.1, $NaH_2PO_4.2H_2O$ 0.4, $NaHCO_3$ 11.9 and D-glucose 5.6. The time required for agents to pass from the first to the third tissue was approximately 2 s.

With the exception of studies designed to compare effects of histamine and leukotrienes, tissues used in all other experiments were continuously superfused with a mixture of 'combined antagonists' of the following composition (μM): mepyramine 0.35, hyoscine 0.34, methysergide 0.55, phenoxybenzamine 0.49 and propranolol 8.8. Changes in tissue length were measured with Harvard smooth muscle transducers, with auxotonic levers (Paton, 1957) and the responses displayed on a Graphtec Linearcorder Mk VII. In some experiments, the comparative duration of action of N-AcLTE_4, LTD_4 and histamine on GPP were assessed by measuring areas under curves (in mm^2) with a Buxco data logger (model DL-12).

Agonists, i.e. leukotrienes and histamine, were administered as bolus injections (3–300 μl) into the superfusion fluid. Where applicable, FPL 55712 (LTD_4/LTE_4 antagonist) and/or indomethacin (cyclo-oxygenase inhibitor) were given as a continuous infusion over the tissues. Owing to the scarcity of human respiratory tissues, the effects of indomethacin and FPL 55712 could not be examined on actions of N-AcLTE_4. U-44069, ((15S)-hydroxy-9α, 11α-(epoxymethano)prosta-5Z,13E-dienoic acid), a

stable prostaglandin endoperoxide with thromboxane A_2-like actions, was used as a standard agonist on all preparations.

Materials

Histamine acid phosphate and hyoscine hydrobromide (BDH), mepyramine maleate (May & Baker), methysergide maleate (Sandoz), phenoxybenzamine hydrochloride and propranolol hydrochloride (ICI), indomethacin (MSD); the following were gifts: FPL 55712 (sodium 7-[3-(4-acetyl-3-hydroxy-2-propylphenoxy)-2-hydroxyproboxy]-4-oxo-8-propyl-4-H-1-benzopyran-2-carboxylate, Fisons), U-44069 (the Upjohn Company) and synthetic leukotrienes (Merck Frosst, Canada).

Statistical methods

Results are expressed as the mean ± s.e.mean from n experiments, where n represents either a different guine-pig trachea, lung or human lung sample.

Results

Action of N-AcLTE$_4$ on guinea-pig isolated trachea

N-AcLTE$_4$ (0.1–10 nmol) was approximately 2 orders of magnitude less active than LTD$_4$ (3–100 pmol) in causing dose-related contractions of GPT (Figure 1). Contractions induced by N-AcLTE$_4$ were of similar duration to those elicited by LTD$_4$ and longer-lasting than those due to histamine (1–30 nmol; $n = 3$). Tachyphylaxis was not observed.

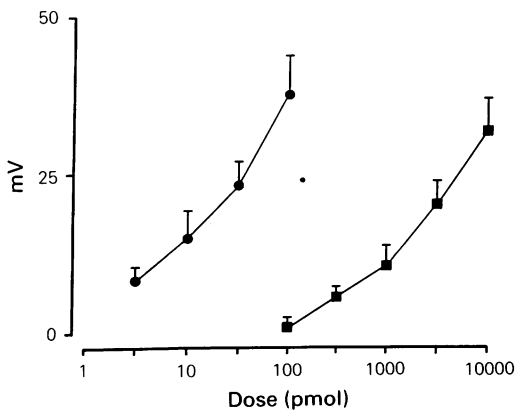

Figure 1 Actions of leukotriene D_4 (LTD$_4$) and N-AcLTE$_4$ on the same strips of guinea-pig trachea (GPT). N-AcLTE$_4$ (100–10,000 pmol, ■) was about 2 orders of magnitude less active than LTD$_4$ (3–100 pmol, ●) in inducing dose-related contractions of GPT. Bars represent s.e.mean from 5–9 experiments. Abscissa scale: doses of LTD$_4$ and N-AcLTE$_4$ (pmol).

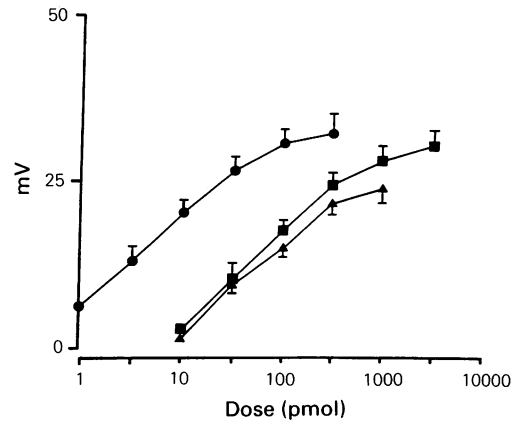

Figure 2 Comparative actions of N-acetyl leukotriene E_4 (N-AcLTE$_4$), LTE$_4$ and LTD$_4$ on strips of guinea-pig lung parenchyma (GPP). N-AcLTE$_4$ (10–3000 pmol, ■) was approximately equiactive with LTE$_4$ (10–1000 pmol, ▲) and one order of magnitude less active than LTD$_4$ (1–300 pmol, ●) in contracting GPP. Bars represent s.e.mean from 5–15 experiments. Abscissa scale: doses of leukotrienes (pmol).

In the presence of indomethacin (2.8 μM), responses caused by N-AcLTE$_4$ and LTD$_4$ were considerably enhanced. Contractions obtained following administration of 30 pmol LTD$_4$ and 1 nmol N-AcLTE$_4$ were increased 2.7 ± 0.2 fold and 3.2 ± 0.6 fold respectively.

Action of N-AcLTE$_4$ on strips of guinea-pig lung parenchyma

N-AcLTE$_4$ contracted strips of GPP in a dose-dependent manner when administered at doses ranging between 0.01 to 3 nmol. As shown in Figure 2, it was approximately equiactive with LTE$_4$ (0.01–1 nmol) and 10 times less active than LTD$_4$ (1–300 pmol). Further, contractions caused by both LTE$_4$ and N-AcLTE$_4$ were almost identical to each other and were more sustained than those produced by either LTD$_4$, histamine (3–30 nmol) or U-44069 (1–10 nmol). As previously demonstrated with LTD$_4$ and LTE$_4$, tachyphylaxis did not occur following repeated administration of N-AcLTE$_4$. Typical results obtained on GPP, with LTD$_4$, N-AcLTE$_4$ and histamine are shown in Figure 3.

Effect of indomethacin In the presence of indomethacin (2.8 μM), contractions induced by N-AcLTE$_4$ were greatly reduced, as in the case of LTD$_4$ and LTE$_4$ (Figure 4a). Contractions elicited by 100 and 300 pmol of the leukotrienes were inhibited by 77 ± 6 and 58 ± 7% and by 77 ± 4 and

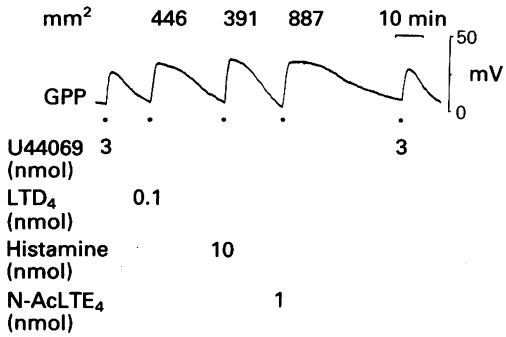

Figure 3 Duration of action of N-acetyl leukotriene E_4 (N-AcLTE$_4$), LTD_4 and histamine on a strip of guinea-pig lung parenchyma (GPP). N-AcLTE$_4$, 1 nmol, caused a contraction of a similar height to that induced by 0.1 nmol of LTD_4 and 10 nmol of histamine. Contraction elicited by N-AcLTE$_4$ (887 mm^2) was more protracted, lasting about twice as long as that due either to LTD_4 (446 mm^2) or histamine (391 mm^2).

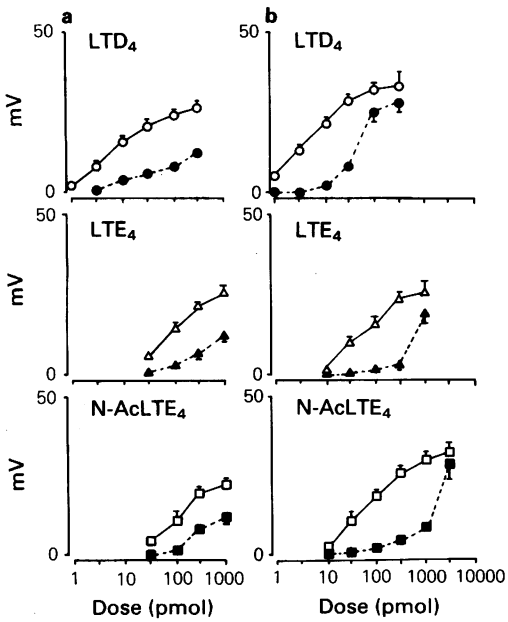

Figure 4 Effects of (a) indomethacin and (b) FPL 55712 on strips of guinea-pig lung parenchyma (n = 5–15 in all cases). (a) Indomethacin (2.8 μM) inhibited, contractions induced by N-acetyl leukotriene E_4 (N-AcLTE$_4$, 30–1000 pmol, ■) (lower panel), as well as those due to LTD_4 (3–300 pmol, ●) and LTE_4 (30–1000 pmol, ▲) (upper and middle panels, respectively). (b) Dose-related contractions produced by LTD_4 (1–300 pmol, ●), LTE_4 (10–1000 pmol, ▲) and N-AcLTE$_4$ (10–3000 pmol, ■) (upper, middle and lower panels, respectively) were greatly reduced in the presence of FPL 55712 (1.9 μM). Abscissae: doses of leukotrienes (pmol).

68 ± 7% in the case of N-AcLTE$_4$ and LTE_4 respectively. Contractions induced by U-44069 were not reduced by indomethacin.

Effect of FPL 55712 When tested in a series of experiments to compare the actions of N-AcLTE$_4$ with those of LTD_4 and LTE_4, FPL 55712 (1.9 μM) antagonised responses induced by all the leukotrienes but not those elicited by U-44069. This effect of FPL 55712 was overcome after administration of larger doses of all the leukotrienes used (Figure 4b).

The putative metabolites, 20-COOH-LTE_4, 20-COOH- N-AcLTE$_4$, dinor-LTE_4 and tetranor-LTE_4, were inactive on strips of guinea-pig trachea and lung parenchyma on the GPP and GPT at doses ranging between 0.3 and 30 nmol, in contrast to LTE_4 and N-AcLTE$_4$ which produced their characteristic contractions on the tracheal and lung strips when given at low doses (10–100 pmol) in the same series of experiments (n = 3–5).

In preparations of GPT or GPP, the leukotriene-induced responses of second and third tissues in series in the cascade were unchanged, regardless of whether agonists were administered above the first tissue (and, subsequently, to the tissues below) or directly to lower tissues thus by-passing the upper preparation(s).

Comparative actions of LTD_4, LTE_4, N-AcLTE$_4$, 20-COOH-LTE_4, 20-COOH-N-AcLTE$_4$, dinor-LTE_4 and tetranor-LTE_4 on human respiratory smooth muscle in vitro

Strips of human lung parenchyma LTE_4 (0.1–30 nmol) was tested in comparison with LTD_4 (10 pmol–30 nmol) on strips of HP in six experiments and was 2–3 orders of magnitude less active than LTD_4. LTE_4 contracted the tissues only at high doses, in excess of 1 nmol, in contrast to LTD_4 which was active at 30 pmol. However, contractions induced by LTE_4 were longer-lasting than those due to LTD_4. As in the case of LTD_4, responses caused by LTE_4 were stable in the presence of indomethacin (2.8 μM). U-44069 induced dose-related contractions at doses ranging between 0.03–3 nmol.

Strips of human bronchus LTD_4 and LTE_4 were considerably more active on HBr than on HP. Further, LTD_4 was about 2 orders of magnitude more active in contracting HBr than LTE_4. Relative potencies of LTD_4 (3–100 pmol) and LTE_4 (0.3–30 nmol) are shown in Figure 5. Responses induced by LTE_4 differed from those due to LTD_4 by being notably more protracted and lasting at least twice as long (Figure 6). In another series of experiments, N-AcLTE$_4$ (0.1–3 nmol, n = 4), tested in comparison

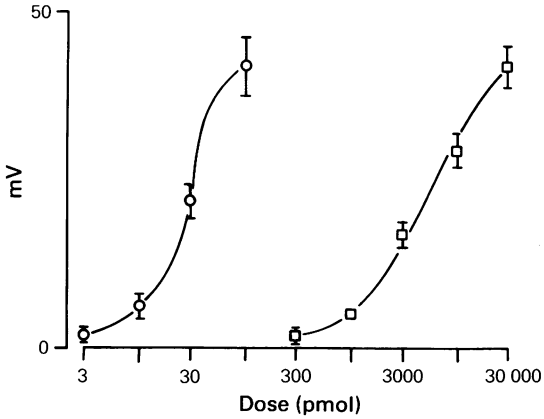

Figure 5 Dose-response curves elicited by leukotriene D_4 (LTD_4, 3–100 pmol, ○) and LTE_4 (300–30,000 pmol, □) on the same preparations of human bronchus. Bars represent s.e.mean from 5–11 experiments. Abscissae: doses of leukotrienes (pmol).

with LTD_4 and LTE_4, displayed a profile of responses which was almost identical with that of LTE_4 in terms of potency and duration of action. Histamine (0.3–20 nmol, $n = 3$) was approximately one order of magnitude less active than either LTE_4 or N-AcLTE_4 and elicited contractions which were of a shorter duration than those due to any leukotriene (data not shown). Tachyphylaxis was not observed following administration of leukotrienes to either HP or HBr.

In five experiments, 20-COOH-LTE_4, 20-COOH-N-AcLTE_4 as well as dinor- and tetranor-LTE_4, all administered at 0.3–30 nmol, were inactive on HBr. These bronchial strips, however, contracted following administration of either LTD_4 (30 and 100 pmol), LTE_4 (0.5–1 nmol) or U-44069 (0.03–3 nmol). In all experiments, contractions produced on lower preparations of HP or HBr were unaltered, whether leukotrienes were administered to the top tissue (and subsequently superfused the tissues below) or directly to the lower tissue(s) in the cascade.

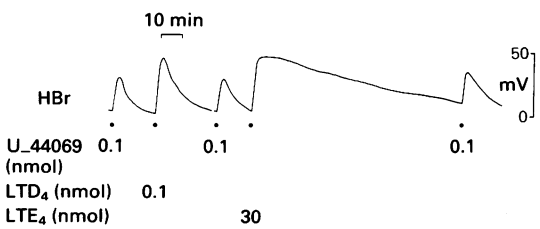

Figure 6 Comparative effects of leukotriene E_4 (LTE_4) and LTD_4 on a strip of human bronchus. LTE_4 (30 nmol) caused a response of a similar height to that due to 0.1 nmol LTD_4. The LTE_4-induced contraction was notably more protracted, lasting about four times as long as that due either to LTD_4 or to U-44069 (0.1 nmol).

Effect of indomethacin and FPL 55712 LTE_4-induced contractions remained unchanged and reproducible during treatment with indomethacin (2.8 μM), even when this was maintained for up to 4 h ($n = 4$). When tested in the presence of FPL 55712 (1.9 μM), responses due to LTE_4 were greatly reduced ($n = 3$) (data not shown).

Discussion

This paper describes the comparative biological activities of various leukotriene metabolites, arising from the enzymatic degradation of LTC_4, in guinea-pig and human airway smooth muscle, *in vitro*. It is now established that LTC_4 rapidly undergoes cleavage of γ-glutamic acid at C-6, by the action of the ubiquitous γ-glutamyltranspeptidase, with subsequent formation of LTD_4. However, this does not result in loss of biological activity in airways since LTC_4 and LTD_4 are equipotent in a variety of species, including man (Piper & Samhoun, 1987). In contrast, reduced biological activity occurs following further cleavage of the peptide moiety and generation of LTE_4, the cysteinyl leukotriene, which, as shown above, was considerably less active than LTD_4 in causing dose-related contractions of both guinea-pig and human respiratory tissues. N-AcLTE_4 displayed a pharmacological profile which was identical to that of LTE_4 in GPP, which represents a model of small airways, but also contains blood vessels, *in vitro*. Thus, both leukotrienes were about equiactive, one order of magnitude less active than LTD_4 and elicited long-lasting contractions which were reduced in the presence of both FPL 55712 and indomethacin. Inhibition by indomethacin indicates that N-AcLTE_4 partly acts by causing the release of cyclo-oxygenase products with bronchoconstrictor properties, such as thromboxane A_2, which will in turn augment the leukotriene-induced contractile activity, as previously demonstrated for LTB_4, LTC_4, LTD_4 and LTE_4 (Piper & Samhoun, 1982; 1983). Antagonism by FPL 55712 suggests that N-AcLTE_4, like LTE_4, may be activating a similar receptor population to that occupied by LTD_4. However, this hypothesis requires further investigations which do not fall within the scope of this paper. Also recent studies in guinea-pig lung membranes indicate that LTE_4 may selectively bind to only a subset of LTD_4 receptors, those in the high-affinity state (Aharony *et al.*, 1988).

In GPT, a model of large airways *in vitro*, N-AcLTE_4 was also considerably less active than LTD_4 but its action was enhanced by indomethacin, which is in agreement with previous results with LTE_4 (Samhoun & Piper, 1986). Indomethacin has been shown to potentiate the actions of LTC_4 as well as those of other contractile agents in the guinea-pig isolated trachea, which could be attributed to inhibition of endogenous relaxing or inhibitory cyclo-oxygenase products (Hay *et al.*, 1987; Folkerts *et al.*, 1989).

It is of interest that cyclo-oxygenase products formed in upper preparations of GPP and GPT in the cascade do not appear to influence myotropic activity of other tissues superfused in series, since responses of lower preparations did not vary, regardless of whether the leukotrienes were administered directly to these lower tissues or, in the usual manner, to the upper tissues. In the case of LTD_4, stability of contractions produced on lower preparations of GPT and GPP, as well as HP and HBr, further indicates lack of metabolism of this leukotriene over the short period of time (2 s) which is necessary for agonists to pass from the first to the last tissue. Indeed, it is unlikely that LTD_4 is metabolised under these experimental conditions, since LTD_4 predominantly was shown to accumulate in the first 5 min following incubation of guinea-pig and human airway preparations with either LTC_4 or LTD_4 (Aharony *et al.*, 1985).

In contrast to its effects in the guinea-pig, indomethacin did not alter contractions caused by either LTD_4 or LTE_4 in HP and HBr indicating that, in human airways, leukotrienes exert direct actions which are independent of the cyclo-oxygenase pathway of arachidonic acid metabolism. It is of interest that LTE_4 and N-AcLTE_4 were less active than LTD_4 in the human preparations used, indicating the importance of the cysteinyl-glycinyl residue present in the LTD_4 molecule for potent bronchoconstrictor activity. Nevertheless, as in GPP, both LTE_4 and N-AcLTE_4 still retained considerable biological activity and were at least one order of magnitude more active than histamine. Further, they elicited contractions which were of a longer duration than those due to any of the other agents used, including LTD_4. This protracted action could either indicate that LTE_4 and N-AcLTE_4 bind more firmly to receptors, e.g. a high-affinity receptor population, or that they are less readily metabolised than LTD_4 in pulmonary tissue. Recently, we have demonstrated that LTE_4 is formed following incubation of HP and HBr with either LTC_4 or LTD_4. However, further metabolism beyond LTE_4 could not be detected, suggesting that it represents the stable end product of leukotriene metabolism in human isolated airways (Conroy *et al.*, 1989). N-AcLTE_4 has not yet been detected in healthy man or primates but was characterized as a urinary product in patients with the hepatorenal syndrome (Keppler *et al.*, 1988). In contrast to LTE_4 and N-AcLTE_4, their ω-oxidative products, 20-carboxy-LTE_4 and 20-carboxy-N-AcLTE_4, as well as dinor- and tetranor-LTE_4 which are formed following sequential β-oxidation, were inactive in doses exceeding by far the active doses of their parent metabolites in guinea-pig and human airways. These oxidative metabolites seem unlikely, therefore, to play an important role in airway smooth muscle and have been suggested as suitable markers, in urine or bile of various species, for formation of leukotrienes *in vivo* (Keppler *et al.*, 1988; Delorme *et al.*, 1988).

In the event of the generation of cysteinyl-containing leukotrienes in airways following inflammatory or allergic stimuli, LTE_4 is likely to be present in tissues and fluids as their stable metabolite. Thus, in addition to its detection in various body fluids in normal man, LTE_4 has recently been characterized in urine of patients during acute asthma and allergic rhinitis (Taylor *et al.*, 1989). It is of interest that inhaled LTE_4 was also shown to cause long-lasting bronchoconstriction in normal humans (Barnes *et al.*, 1984). Owing to its stability and sustained action, LTE_4 may, therefore, persist for a longer time than either LTC_4 or LTD_4 in respiratory tissues.

Our findings indicate that, while enzymatic cleavage of the leukotriene peptide side chain to a single amino acid or modification of the latter, as in the case of LTE_4 and N-AcLTE_4, may represent a detoxification step, it still confers on these leukotrienes appreciable contractile activity on airway smooth muscle. However, further metabolic steps, involving ω- and β-oxidation, with subsequent shortening of the fatty acid chain of the molecule, result in inactivation of leukotrienes in both guinea-pig and human respiratory tissues *in vitro*.

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Selective antagonism to succinylcholine-induced depolarization by α-bungarotoxin with respect to the mode of action of depolarizing agents

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1 The interactions of α-bungarotoxin or tubocurarine with the neuromuscular block and endplate depolarization induced by succinylcholine (SCh) in the phrenic nerve-diaphragm preparation of mice were studied in order to elucidate the role of depolarization by SCh in the neuromuscular blockade.

2 The SCh concentrations required to depress the indirect twitch response by 20% and the evoked endplate potential in cut muscle preparations by 80% were 10 μM and 6 μM, respectively, while only 2 μM SCh was needed to induce maximal endplate depolarization from −80 mV to about −60 mV.

3 SCh blocked the neuromuscular transmission synergistically with either α-bungarotoxin or tubocurarine. There was an initial partial reversal of the neuromuscular inhibition caused by tubocurarine, but not that by α-bungarotoxin.

4 α-Bungarotoxin (0.025 μM) antagonized SCh (10 μM)-induced depolarization more effectively than it depressed miniature endplate potentials and the antagonism was insurmountable by increasing SCh concentration. By contrast, tubocurarine preferentially depressed miniature endplate potentials and antagonized SCh-depolarization competitively.

5 The above difference was attributed to the irreversible nature of α-bungarotoxin binding to acetylcholine receptors, to the slow diffusion of the toxin molecule into the synaptic cleft and thus to the more rapid binding with perijunctional receptors compared with junctional ones.

6 It is concluded that the sustained depolarization of the endplate by SCh results largely from an action on the perijunctional receptor in mice and, unlike cats, the neuromuscular block by SCh is not due to the depolarization *per se* but rather to a direct attenuation of endplate potential.

Introduction

The mechanism of neuromuscular blockade by agonist-type depolarizing agents has been disputed for a long time (for comprehensive literatures, see Zaimis, 1976; Zaimis & Head, 1976; Colquhoun, 1986). It is generally attributed, at least for the initial phase of action, to the inactivation of voltage-dependent sodium channels at the endplate zone resulting from a sustained depolarization (Burns & Paton, 1951; Zaimis, 1976; Zaimis & Head 1976; Colquhoun, 1986). On the other hand, Thesleff (1955a,b) suggested that the neuromuscular block by succinylcholine (SCh) and decamethonium was largely a result of desensitization of the acetylcholine (ACh) receptor, but not of depolarization *per se*, in frog, guinea-pig, rabbit, rat and cat muscles. Agonist-type muscle relaxants are also believed to block neuromuscular transmission by a dual mechanism, i.e., initially by depolarization and then, after a prolonged action, changing to a non-depolarizing block, presumably by desensitization of the receptor or by competitive inhibition (Zaimis, 1976). Since desensitization is an inactive closed state of the ACh-receptor channel occurring in the presence of an agonist, the outcome of desensitization is difficult to

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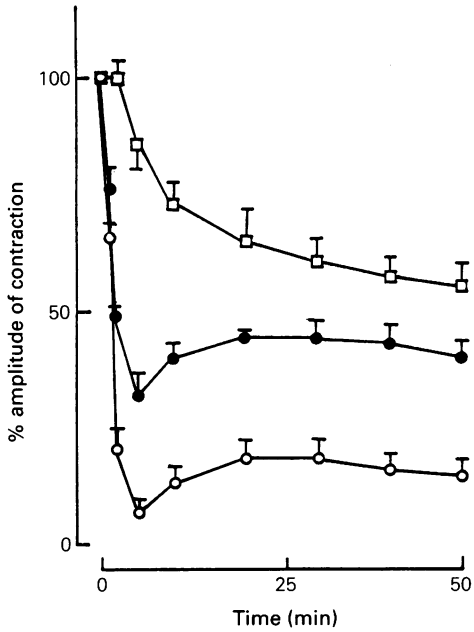

Figure 1 Time-course of succinylcholine-induced block of indirect twitch responses of the mouse phrenic nerve diaphragm preparation: (□) 20 μM; (●) 30 μM; (○) 50 μM. Points are mean values from 4 or more preparations; vertical bars show s.e.

discriminate from competitive blockade (Colquhoun, 1986). In addition to the postsynaptic effect, a presynaptic action has been reported for SCh (Edwards & Ikeda, 1962; Hubbard *et al.*, 1965; Standaert & Adams, 1965) but this mechanism is unlikely to be essential for the block of neuromuscular transmission (Colquhoun, 1986).

Acetylcholine receptors on the motor endplate, in addition to those subsynaptic junctional ones, have been shown to extend to the perijunctional area by as far as 25–50 μm with decreasing densities (Salpeter *et al.*, 1988). The perijunctional receptor will be more easily accessible than the junctional one to exogenously applied agonists whereas the reverse is true for the neurally-released ACh. It has been shown that exogenous ACh preferentially affects extrajunctional receptors in chick biventer cervicis muscle with intact acetylcholinesterase and the response can be preferentially blocked by α-bungarotoxin (Chang & Tang, 1974; Chang & Su, 1975; Chang *et al.*, 1985). In this paper we show that α-bungarotoxin, but not tubocurarine, antagonized the SCh-induced depolarization more markedly than the depression of miniature endplate potential (m.e.p.p.) amplitude while the neuromuscular block by SCh was enhanced. These results shed more light on the role of endplate depolarization and mode of action of SCh in neuromuscular blockade.

Methods

Twitch contractions

Hemidiaphragms of both right and left sides with the phrenic nerve attached were isolated from 20–25 g mice (ICR) of either sex after a blow on the head and bleeding. The organ bath contained 20 ml Tyrode solution (composition in mM: NaCl 137, KCl 2.8, $CaCl_2$ 1.8, $MgCl_2$ 1.1, NaH_2PO_4 0.33, $NaHCO_3$ 11.9 and glucose 11.2) maintained at 37 ± 0.5°C and oxygenated with 95% O_2 + 5% CO_2. The resting tension was 0.5 g. The nerve was stimulated with supramaximal pulses of 0.05 ms at 0.2 Hz in order to elicit indirect single twitch contractions. The diaphragm muscle was stimulated with a pair of long silver electrodes sandwiching the diaphragm at the margin of the rib end with submaximal pulses of 0.5 ms at 0.2 Hz to elicit direct twitch contractions. Contractions were recorded isometrically through a transducer (BG 25, Gould) coupled to a rectilinear physiorecorder (Gould 2200S).

Electrophysiological recordings

Experiments were carried out at 33 ± 0.5°C on the mouse phrenic nerve diaphragm preparation bathed in 12 ml Tyrode solution. Intracellular transmembrane potentials were recorded with glass capillary microelectrodes filled with 3 M KCl (5–10 MΩ) coupled to a high impedance d.c. amplifier (Grass P6). Microelectrodes were inserted into the endplate region of the diaphragm to record the resting transmembrane potential, spontaneous miniature endplate potentials (m.e.p.ps) and evoked endplate potentials (e.p.ps). M.e.p.ps or e.p.ps with rise times slower than 0.8 ms were rejected. The cut muscle preparation (Barstad & Lilleheil, 1968) of mouse diaphragm was equilibrated in the Tyrode solution for 2 h until the resting membrane potential declined to about −40 mV when the muscle fibre became incapable of generating action potentials, and evoked e.p.ps could be easily recorded. The amplitudes of m.e.p.ps and e.p.ps in the presence of SCh were corrected for the depolarization as previously described (Chang *et al.*, 1986).

Drugs

Succinylcholine chloride and tubocurarine chloride were obtained from Sigma Chemicals and α-

bungarotoxin was isolated from the crude venom of *Bungarus multicinctus* as previously described (Lee *et al.*, 1972).

Results

Succinylcholine-induced neuromuscular block and depolarization

In the isolated mouse phrenic nerve-diaphragm preparation, SCh did not depress the indirect twitch responses evoked by stimulation of the nerve at 0.2 Hz at concentrations lower than 10 μM. At concentrations of 10–20 μM, neuromuscular transmission was partially blocked progressively in 60 min. At concentrations of 30 μM or higher, SCh caused a marked and rapid block within 5 min of application followed by a slight spontaneous recovery in the next 20–40 min before the second phase block took place (Figure 1). Figure 2 illustrates the relation between SCh concentrations and percentage neuromuscular blockade. The concentrations for 20% and 50% inhibition (IC_{20}, IC_{50}) at 40 min after SCh administration were 12 and 25 μM, respectively. The mouse muscle is thus more than ten times as resistant to SCh as the cat muscle (Zaimis & Head, 1976).

The endplate area was rapidly and maximally depolarized from −80 mV to about −55 mV within 2–3 min after addition of SCh to the organ bath at concentrations 2 μM and higher. The membrane potential was slightly restored to −60 mV in about 5 min and stayed at this level for more than 40 min (Figure 3). The median effective concentration (EC_{50}) of SCh for depolarization was about 0.6 μM (Figure 2), being 20 times lower than the IC_{20} for the neuromuscular block. Whether SCh-induced depolarization results in a substantial inactivation of the sodium channel and thus leads to a neuromuscular block was studied by testing the effect of SCh on the twitch response to direct stimulation at the rib end of the diaphragm muscle. SCh showed no inhibitory effect on the direct twitch response at all (data not shown) even at concentrations up to 50 μM which markedly disturbed the indirect twitch response.

Effects of succinylcholine on m.e.p.ps and e.p.ps

M.e.p.ps recorded from the uncut muscle preparations were reduced in amplitude dose-dependently by SCh with IC_{50} being about 0.76 μM (Figure 2). This value is quite close to the SCh concentration which caused 50% maximal depolar-

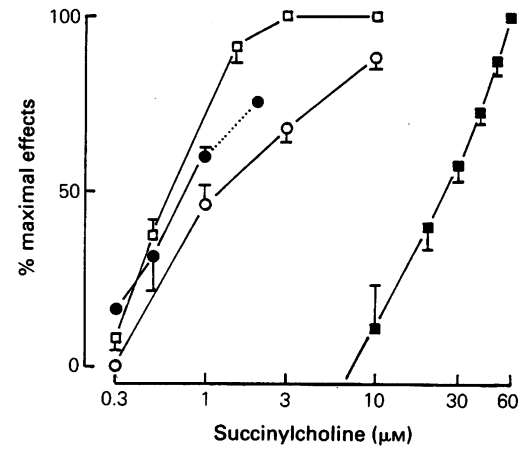

Figure 2 Concentration-response curves of succinylcholine (SCh) for inducing neuromuscular blockade and endplate depolarization and for inhibiting m.e.p.ps and e.p.ps. M.e.p.ps (●), endplate depolarization (□) and neuromuscular block of indirect twitch responses (■) were measured in intact muscle preparations with resting membrane potential of about −80 mV. E.p.ps (○) were measured in cut muscles with resting membrane potential of about −40 mV. The effects of SCh were expressed as percentage taking complete inhibition as 100% except for depolarization where maximal depolarization by SCh (from −80 mV to −60 mV) was taken as 100%. Mean from 4 preparations or from >15 endplates; s.e. shown by vertical bars.

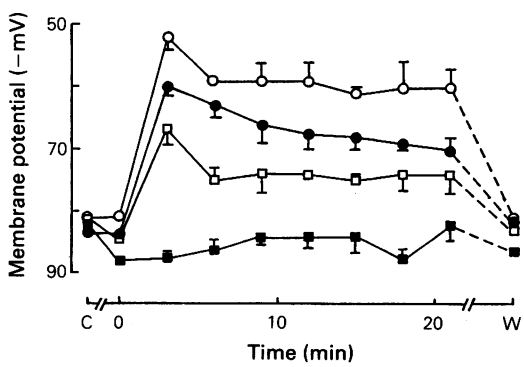

Figure 3 Time-course of endplate depolarization by succinylcholine (SCh) and effects of α-bungarotoxin and tubocurarine. SCh (10 μM) was added at 0 min; (○) SCh only; (●) in the presence of tubocurarine (0.2 μM); (□, ■) pretreated with 0.025 μM α-bungarotoxin for 30 and 70 min, respectively. C denotes control. W denotes washout of SCh. Mean values from >20 endplates in 4 preparations; vertical bars show s.e.

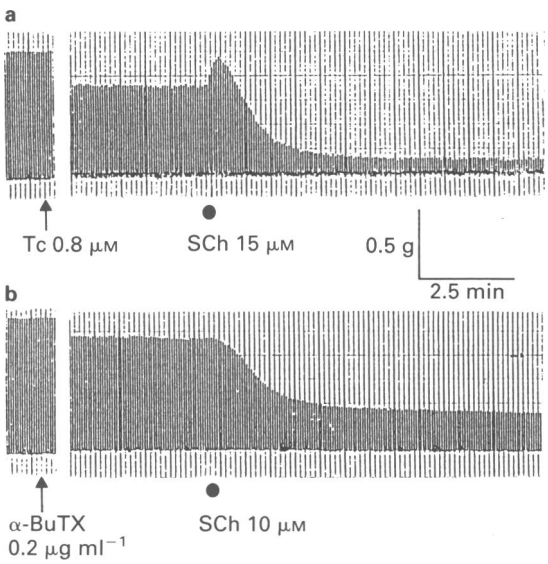

Figure 4 Effects of succinylcholine (SCh) on the neuromuscular transmission partially blocked by tubocurarine (Tc) or α-bungarotoxin (α-BuTx). Left panels show the control twitch response. In (a) tubocurarine was added at 0.8 μM throughout, while in (b) the preparation was pretreated with α-bungarotoxin (0.025 μM) for 40 min and washed out. At (●) SCh (15 μM in a, 10 μM in b) was added.

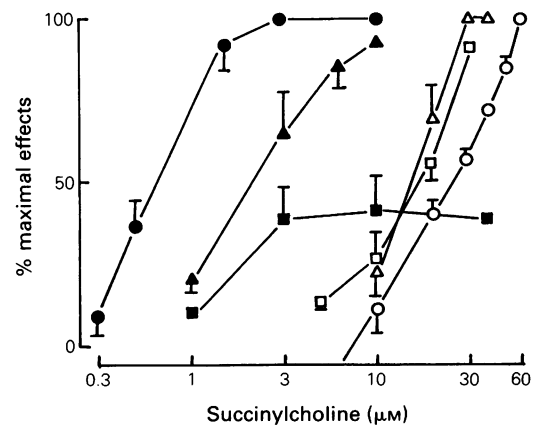

Figure 5 Effects of α-bungarotoxin and tubocurarine on the concentration-effect curves of succinylcholine (SCh) for neuromuscular block and endplate depolarization. Neuromuscular block by SCh in control (○), in the presence of 0.2 μM tubocurarine (△) and in preparations pretreated with 0.025 μM α-bungarotoxin (□) for 45 min and washout. Depolarization of endplate by SCh, as percentage of maximal depolarization (from −80 mV to −60 mV), in control (●), tubocurarine (▲) and α-bungarotoxin (■)-pretreated preparations. Mean from 4 preparations or >15 endplates; vertical bars show s.e.

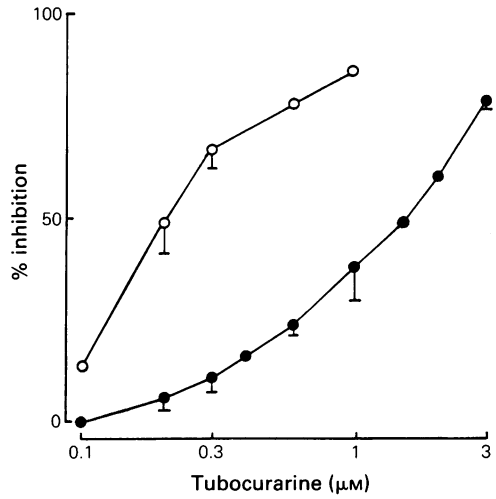

Figure 6 Inhibition of m.e.p.ps and succinylcholine (SCh)-induced depolarization by various concentrations of tubocurarine. The mouse diaphragm preparations were pretreated with tubocurarine for 30 min. The m.e.p.p. amplitudes (○) and SCh (10 μM)-induced depolarizations (●) in the presence of tubocurarine were expressed as percentage of control. Mean from >20 endplates in 3–4 preparations; vertical bars show s.e.

ization. On the other hand, e.p.ps recorded from the cut muscle preparations were depressed by SCh at somewhat higher concentrations (Figure 2). To inhibit the e.p.p. amplitude by 50 and 80%, 1.2 and 6 μM SCh were needed, respectively. The latter concentration is close to the minimal effective concentration (IC_{20}) required to impair neuromuscular transmission.

Interaction with tubocurarine

Attempts to see whether tubocurarine (at various concentrations) could antagonize the neuromuscular blocking action of SCh in mice, as it does in cats (Zaimis & Head, 1976), were unsuccessful. It was found that SCh only temporarily antagonized the partial block caused by tubocurarine (0.8 μM) (Figure 4a). The neuromuscular block, however, was enhanced in the presence of tubocurarine. The IC_{50} was reduced from 25 μM to 18 μM in the presence of 0.2 μM tubocurarine (Figure 5). The spontaneous recovery following the initial block (cf. Figure 1) was no longer observed. On the other hand, the depolarization of the endplate caused by SCh was reduced dose-dependently by tubocurarine at 0.2–3 μM (Figure 6) and the concentration-depolarization

curve for SCh was shifted to the right in parallel by tubocurarine (Figure 5), suggesting the competitive nature of the antagonism between tubocurarine and SCh in depolarizing the endplate. The effect of tubocurarine on m.e.p.ps was compared with that on endplate depolarization caused by SCh at 10 μM, the minimal concentration to impair neuromuscular transmission (Figure 6). The amplitude of m.e.p.p. was depressed by 50% by tubocurarine at 0.2 μM, a concentration very close to its dissociation constant (Colquhoun, 1986), whereas 1.5 μM of tubocurarine was needed to attenuate by 50% the depolarization induced by SCh at 10 μM.

Interaction with α-bungarotoxin

In preparations pretreated with α-bungarotoxin (0.2 μg ml^{-1} = 0.025 μM) for 45 min when the indirect twitch response was slightly inhibited, SCh (10 μM) caused synergistic augmentation of neuromuscular block without any initial transient reversal (Figure 4b) as seen in the tubocurarine-treated preparation. The median concentration of SCh to cause neuromuscular block was decreased from 25 μM in control preparations to 18 μM in the α-bungarotoxin-pretreated preparation (Figure 5). Figure 7 compares the effects of α-bungarotoxin after 15 to 60 min incubation on the amplitude of m.e.p.ps and on the SCh (10 μM)-induced depolarization. Unlike tubocurarine, the SCh-induced depolarization was almost completely antagonized after 60 min incubation with α-bungarotoxin when the m.e.p.p. amplitude was depressed by only 50–60%. This result indicates that the SCh-depolarization was more vulnerable to the block by α-bungarotoxin than the response to neurally-released ACh. This situation is in contrast to that observed with tubocurarine. Also unlike tubocurarine, the depression of SCh-depolarization by α-bungarotoxin was not surmountable by increasing SCh concentrations (Figure 5).

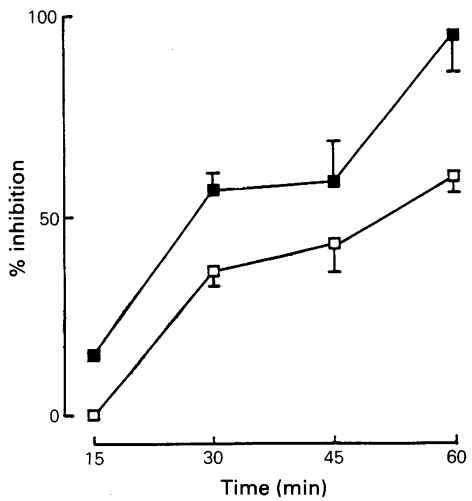

Figure 7 Inhibition by α-bungarotoxin of m.e.p.ps and succinylcholine (SCh)-induced depolarization. The mouse diaphragm preparations were incubated with 0.025 μM α-bungarotoxin for various times and washed out. The m.e.p.p. amplitude (□) and SCh (10 μM)-induced depolarization (■) after toxin treatment were expressed as percentage of control. Mean from >20 endplates in 3–4 preparations; vertical bars show s.e.

Discussion

A comparison of the effective concentrations of SCh in causing endplate depolarization (EC_{50} = 0.6 μM; EC_{80} = 1.2 μM), in inhibiting m.e.p.ps (IC_{50} = 0.76 μM) and e.p.ps (IC_{50} = 1.2 μM; IC_{80} = 6 μM) and in causing a block of neuromuscular transmission (threshold concentration: IC_{20} = 12 μM) indicates that the cause for neuromuscular block induced by SCh in mice cannot be depolarization. This inference is further supported by the findings that the maximal depolarization reached only −60 mV and that the muscle action potential could still propagate through the endplate area during maximal depolarization indicating that the muscle sodium channel was not sufficiently inactivated to cause a neuromuscular blockade. These effects of SCh in mice are quite different from those in cats where the endplate can be depolarized to lower than −50 mV, which results in complete inactivation of the sodium channel and a depression of muscle contraction to direct stimulation (Zaimis & Head, 1976). Evidently, the difference in the magnitude of SCh-depolarization may result in different modes of neuromuscular blocking action by SCh and different potency in different species. Indeed, the mouse ACh receptors appear to have equal affinity for SCh as the cat receptors although they are much more resistant to its neuromuscular block. The cause of neuromuscular block by SCh in mice seems to be more probably the inhibition of e.p.ps as judged from the effective concentrations, considering a safety factor of 3 to 5 for murine diaphragm neuromuscular transmission (Chang *et al.*, 1975). The present data also show that m.e.p.ps were depressed slightly more than e.p.ps at low concentrations of SCh, implying that the quantal content of e.p.ps may be slightly increased. This is in line with the view that neurally-released ACh has a positive feedback on the release of neurotransmitter (Bowman *et al.*, 1986; Chang *et al.*, 1988).

Both tubocurarine and α-bungarotoxin are well known antagonists of the nicotinic ACh receptor of the motor endplate. They display qualitatively the same mode of neuromuscular blocking action except that tubocurarine, but not α-bungarotoxin, may additionally affect the open ACh channel (Katz & Miledi, 1978) and that the action of α-bungarotoxin is practically irreversible (Chang, 1979). The present study revealed that there are substantial differences in the interactions of α-bungarotoxin and tubocurarine with SCh. α-Bungarotoxin at 0.025 μM and tubocurarine at 0.2 μM were equipotent in inhibiting the amplitude of m.e.p.ps, which represents the response of junctional ACh receptor to neurally-released quanta of ACh, and in enhancing the neuromuscular block by SCh. On the other hand, the depolarizing response to SCh at the minimal concentration (10 μM) to impair neuromuscular transmission was more sensitive than m.e.p.ps to α-bungarotoxin and less sensitive to tubocurarine. The relationships between the inhibition of SCh-depolarization and the inhibition of m.e.p.p. by tubocurarine and by α-bungarotoxin, as summarized in Figure 8, are markedly different. These results suggest that SCh induces depolarization by acting on receptors different from those for m.e.p.ps. The difference in the actions of tubocurarine on the SCh-depolarization and m.e.p.ps may be explained on the basis of pharmacokinetics. Despite the fact that the binding of tubocurarine with the nicotinic receptor is reversible, practically no competition or displacement of tubocurarine from the nicotinic receptor can take place by the neurally-released ACh (cf. Colquhoun, 1986) because of the limited space in the synaptic cleft and the time available. Unlike neurally-released ACh, exogenous SCh has plenty of space and time available in competing with tubocurarine for inducing depolarization. The preferential block of SCh-depolarization by α-bungarotoxin may be due to a slow binding with the junctional receptor, since, at the toxin concentration used (0.025 μM), the free toxin molecule existing in the space of the synaptic cleft of one endplate (not more than 1 μm^3) is less than 15 at equilibrium in the face of 10^7 receptors for binding. It is evident that the binding of α-bungarotoxin with junctional receptors would be much slowed compared to the binding with exposed perijunctional receptors, as previously shown in the chick muscle (Chang & Tang, 1974; Chang & Su, 1975).

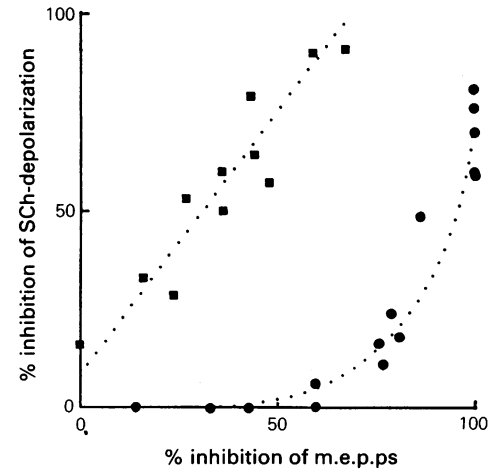

Figure 8 The relationships between the suppression of m.e.p.ps and succinylcholine (SCh)-induced depolarization by tubocurarine or by α-bungarotoxin. The preparations were either preincubated with α-bungarotoxin (0.025 μM) for various times and washed out (■) or treated with various concentrations of tubocurarine (●). Data from Figures 6 and 7.

Finally it may be inferred that SCh causes depolarization of the endplate by acting on perijunctional ACh receptors whereas the block of neuromuscular transmission in the mouse muscle by SCh is due to its effect on the junctional receptor. The latter effect is apparently not accompanied by sustained depolarization. The present results incidentally raise the possibility that the junctional ACh receptor of the endplate reacts with agonists differently from perijunctional ones in that the depolarization seems not to be maintained and is more prone to desensitization.

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Direct evidence for the atypical nature of functional β-adrenoceptors in rat adipocytes

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1 The nature of the rat epididymal adipocyte β-adrenoceptor was investigated by studying the effects of β_1- and β_2-selective antagonists on lipolysis induced by (−)-isoprenaline and the lipolytically selective agonist BRL 37344.

2 From 10 nM to 10 μM, the potent and highly selective β_1-adrenoceptor antagonist CGP 20712A did not influence the concentration-response curve (CRC) of BRL 37344 whereas small but consistent shifts to the right of the (−)-isoprenaline-induced CRC were observed. Clear rightward shifts of the CRCs induced by both (−)-isoprenaline and BRL 37344 were produced only at 100 μM CGP 20712A with the corresponding pA_2 values being 4.80 and 4.61, respectively.

3 When the β_2-selective antagonist ICI 118,551 was used at 10 μM and higher, clear and concentration-dependent shifts to the right of the CRCs of both agonists were observed. The slopes of the Schild plots did not deviate significantly from unity, the pA_2 values being 5.49 and 5.33 against (−)-isoprenaline and BRL 37344, respectively.

4 The results demonstrate that (−)-isoprenaline-induced lipolysis in rat white adipocytes is mediated predominantly by atypical β-adrenoceptors, whereas the typical β_1-adrenoceptors play a small, subordinate role. The lipolytically selective agonist BRL 37344 acts solely through atypical β-adrenoceptors.

Introduction

The classification of the β-adrenoceptor mediating lipolysis in rat adipocytes has been the subject of much debate. Originally, Lands *et al.* (1967), using a series of catecholamine agonists, classified it as a β_1-adrenoceptor. However, Harms *et al.* (1974), and De Vente *et al.* (1980) suggested a hybrid or atypical type of β-adrenoceptor mediating lipolysis with low affinity for both β_1- and β_2-selective and non-selective antagonists. Moreover, fat cell β-adrenoceptors showed lower stereoselectivity ratios for antagonist enantiomers than typical β_1-(cardiac) and β_2-(skeletal muscle) adrenoceptors (Harms *et al.*, 1977).

Bojanic & Nahorski (1983) and Bojanic *et al.* (1985) more recently provided evidence for the co-existence of at least two subtypes of β-adrenoceptors on adipocyte membranes. In radioligand binding studies in which subnanomolar concentrations of [^{125}I]-cyanopindolol were used, they found predominantly typical β_1-adrenoceptors, while in functional experiments they found the β-adrenoceptor mediating adenylyl cyclase activation to be clearly atypical, resembling that of intact cells mediating lipolysis. This conclusion was strengthened by the observation that the irreversible photo-affinity β-antagonist *p*-aminobenzylcarazolol, in concentrations that almost completely inhibited adenylyl cyclase activation of rat reticulocyte membranes, was virtually ineffective in adipocyte membranes (Bojanic & Nahorski, 1984). Furthermore, the introduction of a novel series of β-adrenoceptor agonists that selectively stimulate lipolysis, provided important support for the concept of an atypical β-adrenoceptor mediating lipolysis (Arch *et al.*, 1984).

However, more recently Bahouth & Malbon (1988) disputed these claims. They studied the β_2-selective antagonist ICI 118,551 and the new 10,000-fold β_1-selective antagonist CGP 20712A (Dooley *et al.*, 1986) on lipolysis, adenosine 3:5-cyclic monophosphate (cyclic AMP) accumulation

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and radioligand binding displacement in rat adipocytes and membranes. The binding experiments confirmed that the adrenoceptor sites occupied by low concentrations of the radioligand are predominantly of the β_1-type. Since their functional studies also showed that CGP 20712A was a more effective antagonist than ICI 118,551 it was concluded that the character of the rat fat cell β-adrenoceptor is solely β_1. However, close inspection of the functional data revealed that the potency difference between the two antagonists did not agree at all with the binding affinities for a typical β_1-adrenoceptor. Therefore we decided to investigate in more detail CGP 20712A and ICI 118,551 for antagonism of lipolysis induced by isoprenaline and by the lipolytically selective β-adrenoceptor agonist BRL 37344. The results show unequivocally that a small part of the lipolysis mediated by isoprenaline is through its action at typical β_1-adrenoceptors, but the major part is through atypical β-adrenoceptors, while the lipolytically selective compound BRL 37344 acts solely through the atypical β-adrenoceptor.

Methods

Epididymal adipocytes from male Wistar rats (210–230 g) were isolated essentially according to Rodbell (1964). The rats were killed by a blow on the head. Fat pads were removed and placed in Krebs-Henseleit buffer composed of (mM): NaCl 117.5, KCl 5.6, $MgSO_4$ 1.18, $CaCl_2$ 2.52, $NaHPO_4$ 1.28, $NaHCO_3$ 25.0 and glucose 5.5, at room temperature, pregassed with 5% CO_2 in O_2, pH 7.4. After removal of the major blood vessels the fat pads were chopped in 600 μm slices with a McIlwain tissue chopper. Cells were then isolated by incubation in a shaking waterbath, in a Teflon vessel containing 3 ml Krebs-Henseleit solution per fat pad, 0.33 mg ml^{-1} collagenase and 1% bovine serum albumin, at 37°C under an atmosphere of 5% CO_2 in O_2, pH 7.4. As proposed by Honnor *et al.* (1985), digestion was performed in the presence of 200 nM adenosine to reduce day-to-day variability and to diminish cell lysis (see Hollenga *et al.*, 1989). After 1 h, cells were filtered through nylon cloth (100 μm), and washed 2–3 times with Krebs-Henseleit buffer. Adipocytes were incubated in small Teflon vessels in Krebs-Henseleit buffer at pH 7.4, containing 2% bovine serum albumin, at 37°C in a shaking waterbath gassed with 5% CO_2 in O_2. In each vessel about 50,000 cells ml^{-1} were used, as determined with a Bürker-Türk counting chamber. Before addition of the agonist, cells were preincubated for 15 min with the antagonist under investigation or with vehicle. In the case of (−)-isoprenaline, $Na_2S_2O_5$ 50 μg ml^{-1} was added to the incubation vessel as anti-oxidant. In each experiment 1, 3 and 10 μM (−)-isoprenaline was used to determine maximum lipolysis. After 90 min, the incubation was terminated by extraction with 1-propanol:*n*-heptane:1N H_2SO_4 (40:20:1). After centrifugation for 5 min at 2000 r.p.m. in a Hettich Rotixa/KS centrifuge, the upper layer was used for free fatty acid (FFA)-determination. FFA-production was measured with an auto-analyzer essentially according to Antonis (1965), as described by Hollenga *et al.* (1989). All experiments were performed in duplicate.

Data analysis

All dose-response curves were expressed relative to (−)-isoprenaline maximum.

Schild plots were constructed according to Arunlakshana & Schild (1959). When the slopes did not significantly differ from unity (two-tailed Student's *t* test, $\alpha = 0.05$), pA_2 values were calculated for each concentration of antagonist according to: $pA_2 = -\log \{[\text{antagonist}]/(DR-1)\}$ (Mackay, 1978).

ICI 118,551 concentrations were corrected for protein binding as described by Bojanic *et al.* (1985).

All data are given as mean ± s.e.mean.

Materials

Collagenase (type II, 390 u mg^{-1}) and (−)-isoprenaline hydrochloride were purchased from Sigma (St. Louis, USA). Demineralized bovine serum albumin was from Organon Technika (Oss, The Netherlands). Adenosine research grade was from Serva (Heidelberg, FRG). BRL 37344 (4-[2-[(2-hydroxy-2-(3-chlorophenyl)ethyl)amino]propyl]-phenoxyacetic acid), ICI 118,551 (erythro-1-(7-methylindan-4-yloxy)-3-(isopropylamine)-butan-2-ol), and CGP 20712A (1-[2-((3-carbamoyl-4-hydroxy)phenoxy)ethylamino]-3-[4-(1-methyl-4-trifluoromethyl-2-imidazolyl)phenoxy]-propan-2-ol) were kind gifts from Beecham (Epsom, U.K.), ICI (Macclesfield, U.K.) and Ciba-Geigy (Basel, Switzerland). All buffer salts and analytical reagents were from Merck (Amsterdam, The Netherlands).

Results

Antagonism of (−)-isoprenaline-induced lipolysis by CGP 20712A is shown in Figure 1a. With low concentrations of the antagonist, ranging from 10 nM to 10 μM, small rightward shifts of the lipolysis curves were consistently found. Only at 100 μM CGP 20712A a clear rightward shift was obtained. The Schild plot (inset) shows, as expected, a clear biphasic character. The 10 μM and 100 μM data points corresponded with a pA_2 value for CGP 20712A of 4.80 ± 0.09 ($n = 6$).

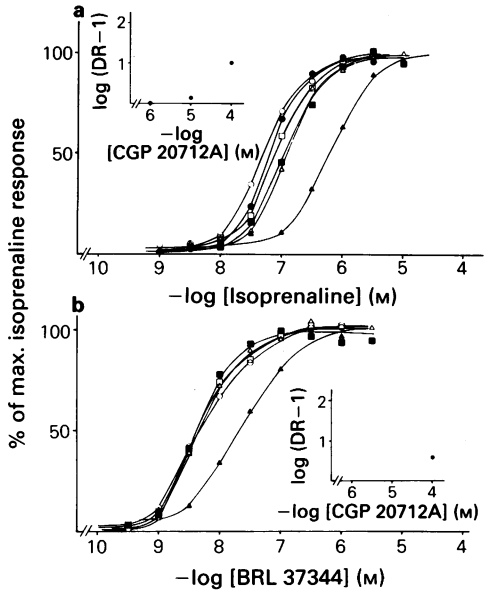

Figure 1 Antagonism of (−)-isoprenaline- (a) and BRL 37344- (b) induced lipolysis by CGP 20712A. Control (○); CGP 20712A 10 nM (●), 100 nM (□), 1 μM (■), 10 μM (△) and 100 μM (▲). Lipolysis is expressed as % relative to (−)-isoprenaline maximum. Shown are the mean of three to four experiments each performed in duplicate. For clarity standard errors are not shown, but they are less than 10%. The insets show corresponding Schild plots derived from the mean curves.

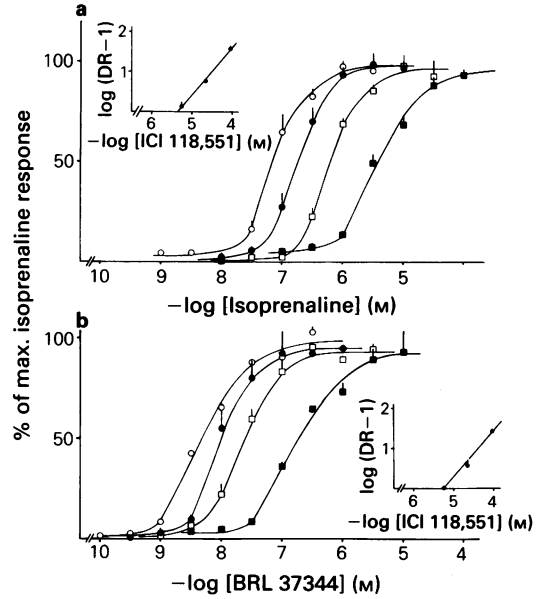

Figure 2 Antagonism of (−)-isoprenaline- (a) and BRL 37344- (b) induced lipolysis by ICI 118,551. Control (○); ICI 118,551 10 μM (●), 30 μM (□), and 100 μM (■). Lipolysis is expressed as % relative to isoprenaline maximum. Shown are the mean curves of three to four experiments each performed in duplicate. The insets show Schild plots calculated from the individual experiments. The slopes are 1.16 ± 0.07 ((−)-isoprenaline) and 1.18 ± 0.04 (BRL 37344), not significantly different from unity.

CGP 20712A did not shift BRL 37344-induced lipolysis curves, until the antagonist concentration reached 100 μM (Figure 1b). From the shift to the right induced by this concentration, a pA_2 value for CGP 20712A of 4.61 ± 0.10 ($n = 3$) was calculated.

Figure 2a shows (−)-isoprenaline-induced lipolysis antagonized by ICI 118,551. The Schild plot had a slope not significantly different from unity. The pA_2 value of ICI 118,551 was 5.49 ± 0.06 ($n = 10$).

Antagonism of BRL 37344-induced lipolysis by ICI 118,551 is shown in Figure 2b. Again, the slope of the Schild plot was not significantly different from unity, and the pA_2 value for ICI 118,551 was found to be 5.33 ± 0.06 ($n = 9$).

Discussion

Several studies with antagonists as well as with non-catecholamine agonists have cast considerable doubts on the lipolytic β-adrenoceptor being of the β_1-type, as classified by Lands *et al.* (1967) using catecholamine agonists (for a review see Zaagsma *et al.*, 1985). Thus, atypically low potencies and stereoselectivities have been found with β-adrenoceptor antagonists, irrespective of their β_1- or β_2- or non-selective properties. This was found not only in lipolysis experiments, but also with cyclic AMP-accumulation in intact cells and adenylyl cyclase in adipocyte membranes (Bojanic *et al.*, 1985). The early suggestion of a hybrid nature of the adipocyte β-adrenoceptor (Harms *et al.*, 1974) was studied in more detail by De Vente *et al.* (1980) in an elaborate study using a series of tolamolol-type antagonists. The results agreed with the hypothesis that the receptor area interacting with the aromatic moiety of β-adrenoceptor ligands is β_2 in nature while the site interacting with the alkanolamine side chain has β_1-characteristics. However, a similar approach using a series of non-catecholamine β-agonists did not confirm unequivocally the mixed β_1/β_2-character. A typical β_1-nature was not found either, even when the correlation analysis was performed with partial agonists only (i.e. the absence of receptor reserve), in agreement with previous non-catecholamine agonist studies (Zaagsma *et al.*, 1985

and references cited therein). In addition, it was established that the atypical behaviour of antagonists was not due to a heterogeneous population of both β_1- and β_2-adrenoceptors (De Vente *et al.*, 1980; Bojanic *et al.*, 1985).

In sharp contrast, radioligand displacement studies on adipocyte membranes have revealed only typical β-adrenoceptor binding sites, predominantly β_1, with high and low affinity for β_1- and β_2-selective antagonists respectively and with normal (high) stereoselectivities of enantiomer pairs (Bojanic & Nahorski, 1983). These results were recently confirmed by Bahouth & Malbon (1988) in a study in which up to four different nonselective radioligands were used. However, in all displacement experiments low radioligand concentrations, slightly above the K_d values for typical β_1- or β_2-receptors, but clearly below the atypical concentrations required to inhibit lipolysis, were used. As discussed previously (Zaagsma *et al.*, 1985), such experimental conditions will only detect the presence of typical β_1 and β_2 binding sites. In an elegant study by Bojanic & Nahorski (1984), it was indeed demonstrated that irreversible blockade of the typical β_1-receptor population was virtually without consequence on the isoprenaline-induced adenylyl cyclase activation in adipocyte membranes, implying again that the functional adipocyte response is mediated by atypical β-adrenoceptors. In addition to radioligand binding, Bahouth & Malbon (1988) also studied the inhibition of lipolysis and cyclic AMP accumulation, induced by 2 μM adrenaline, by the same two β_1- and β_2-selective antagonists, CGP 20712A and ICI 118,551 respectively, as were used in the radioligand displacement studies. The dose-inhibition curves for glycerol release appeared parallel to each other, CGP 20712A being about twice as potent as ICI 118,551. With cyclic AMP accumulation the inhibition curves were not parallel, but converged at 30 μM where both antagonists produced the same amount (50%) of inhibition. Because the onset of inhibition by CGP 20712A was at lower concentrations than with ICI 118,551 and because of the potency difference seen with glycerol release, Bahouth & Malbon (1988) concluded, on the basis of their binding and functional experiments, that the character of the rat adipocyte β-adrenoceptor is solely β_1. It was not realized, however, that atypically high (micromolar) concentrations of both antagonists were required for significant inhibition of the functional responses and also that the strong potency differences observed in the binding experiments with CGP 20712A having considerably lower K_d values for β_1-sites than ICI 118,551 were not observed at all in functional experiments.

In the present study the same antagonists were investigated for inhibition of rat adipocyte lipolysis by performing complete concentration-response curves with the agonists isoprenaline and BRL 37344. The latter compound, introduced by Arch and coworkers (1984), was reported to be 400 and 20 fold selective for rat brown adipocyte lipolysis as compared to atrial β_1- and tracheal β_2-adrenoceptor responses, respectively, the lipolytic potency being 5–6 times higher than isoprenaline. The present results show that the potency difference on rat white (epididymal) adipocytes is even higher: the control curves in Figures 1 and 2 reveal BRL 37344 to be over 10 times more potent than (−)-isoprenaline.

In these experiments CGP 20712A did not antagonize BRL 37344-induced lipolysis at concentrations up to 10 μM although at 100 μM a clear rightward shift was observed. With (−)-isoprenaline as the agonist, all CGP 20712A concentrations below 100 μM induced in a concentration-dependent manner a small but consistent shift to the right which increased dramatically at 100 μM and the resulting Schild plot was clearly biphasic. Because CGP 20712A is a very potent and highly β_1-selective antagonist, these results demonstrate (1) that BRL 37344-induced lipolysis is not mediated by typical β_1-adrenoceptors but solely by atypical β-adrenoceptors, (2) that (−)-isoprenaline-induced lipolysis is predominantly mediated by atypical receptors at all concentrations but to a small extent also by β_1-adrenoceptors and (3) that CGP 20712A up to 10 μM has no affinity for atypical adipocyte β-adrenoceptors. A comparison of the pA_2 value of CGP 20712A for rat sinoatrial β_1-adrenoceptors (9.44) as reported by Kaumann (1986), with the value for atypical rat adipocyte β-adrenoceptors found at 100 μM CGP 20172A with BRL 37344 as the agonist (4.61), reveals a 67,000 fold higher affinity of CGP 20712A for typical β_1-adrenoceptors over the atypical adipocyte β-adrenoceptors.

The experiments with ICI 118,551 (Figure 2) show that this β_2-selective antagonist also acts on adipocytes almost exclusively via atypical receptors; parallel, concentration-dependent shifts were observed, against both agonists, the slopes of the Schild plots being not significantly different from unity in both cases. The pA_2 values found (5.49 and 5.33) are well below the pA_2 values found on β_2-adrenoceptor-mediated tracheal relaxation (8.72) and on β_1-adrenoceptor-mediated atrial rate (7.19) (Arch *et al.*, 1984). Furthermore, the slightly higher (DR − 1) values observed with (−)-isoprenaline compared with BRL 37344 at all concentrations of ICI 118,551 (Figure 2, insets) are in agreement with a minor participation of β_1-adrenoceptors in the (−)-isoprenaline-induced lipolysis.

In conclusion, the remarkable difference between the affinities of CGP 20712A for typical β_1-adrenoceptors and fat cell β-adrenoceptors, found

in the present study, provides strong support for the view that rat adipocyte lipolysis is mediated predominantly by an atypical β-adrenoceptor population whereas the β_1-receptors at most play a subordinate functional role.

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Sensory Nerves and Neuropeptides in Gastroenterology, Florence, Italy. (Fondazione Internazionale Menarini, Piazza del Carmino, 4-20121 Milan, Italy).

6–8 December 1989

Presynaptic Receptors — an Examination of Different Views, New York City, USA. (Conference Department, New York Academy of Sciences, 2 East 63rd Street, New York, NY 10021, U.S.A.)

10–14 December 1989

Sino-Brazilian Symposium on Chemistry and Pharmacology of Natural Products, Rio de Janeiro, Brazil. (Secretariat, Fundacao Oswaldo Cruz, Departmento de Farmacodinamica, Aremida, Brasil, 6365, Rio de Janeiro, Brazil).

3–5 January 1990

British Pharmacological Society Winter Meeting. London, U.K. (Closed meeting for members and guests)

14–17 January 1990

Fourth International Meeting on Biological Reactive Intermediates, Tucson, USA. (Dr I.G. Sipes, Dept. of Pharmacology, College of Pharmacy, University of Arizona, Tucson, USA.)

7 February 1990

Royal Society of Chemistry, Free Radicals in Biotechnology and Medicine, London, U.K. (Miss P.E. Hutchinson, The Analytical Division, Royal Society of Chemistry, Burlington House, London W1V 0BN).

26–28 March 1990

Brain Research Association National Meeting, Bristol, U.K. (Dr. I.C. Kilpatrick, Dept. of Pharmacology, The Medical School, University Walk, Bristol BS8 1TD).

1–6 April 1990

6th World Congress on Pain, Adelaide, Australia. (Int. Association for the Study of Pain, 909 NE 43rd Street, Suite 306, Seattle, WA 98105, U.S.A.)

2–4 April 1990

Central and Peripheral Significance of Neuropeptide Y and its Related Peptides, Baltimore, U.S.A. (Conference Department, New York Academy of Sciences, 2 East 63rd Street, New York, NY 10021, U.S.A.).

4–6 April 1990

New Concepts in Anxiety, Castres, France. (Martin Dehaye, Centre de Recherche Pierre Fabre, 17 avenue Jean Moulin, 81100 Castres, France).

18–20 April 1990

British Pharmacological Society Spring Meeting. Sheffield, U.K. (Closed meeting for members and guests)

6–10 May 1990

4th Swiss Workshop of Methodology in Receptor Research, Lucerne, Switzerland. (Receptor Workshop, c/o Bureco AG, Kaiserstrasse 5a, CH-4310 Rheinfelden, Switzerland).

8–10 May 1990

First ICR International Clinical Symposium, Edinburgh, Scotland. (CEP Consultants Ltd., 26–28 Albany Street, Edinburgh EH1 3QH.)

27–30 May 1990

Heterocycles in Bio-organic Chemistry, Solbacka, Sweden (Secretariat, PO Box 1136, S-11181 Stockholm, Sweden).

29 May–1 June 1990

Fondazione Giovanni Lorenzini Conference on Prostaglandins and Related Compounds, Florence, Italy (Fondazione Giovanni Lorenzini, Via Monte Napoleone, 23, 20121 Milan, Italy).

24–30 June 1990

2nd International Congress of Neuroendocrinology, Bordeaux, France. (2nd Int. Congress of Neuroendocrinology, Bordeaux Congress, 33300 Bordeaux-Lac, France).

26–29 June 1990

Presynaptic Receptors and Neuronal Transporters, Rouen, France. (Dr S.Z. Langer, Synthelabo Recherche (LERS), 58 rue de la Gaciere, 75013 Paris, France).

27–29 June 1990

3rd Manchester IUPHAR Satellite Symposium — The Pharmacology of Adrenoceptors, Manchester, UK. (Dr E. Szabadi, Department of Psychiatry, University of Manchester, Stopford Building, Oxford Road, Manchester M13 9PT.)

28–30 June 1990

IUPHAR Satellite — Effects of Nicotine on Biological Systems, Amsterdam, Netherlands. (Professor D.F. Adlkofer, Research Council on Smoking and Health, Harvestehuder Weg 88, D-2000 Hamburg 13, FRG.)

28–30 June 1990

IUPHAR Satellite — Mediators in Airway Hyperreactivity, The Hague, The Netherlands. (F.P. Nimkamp, Department of Pharmacology, PO Box 80176, 3508 TD Utrecht, Netherlands).

30 June–4 July 1990

6th Southeast Asian/W. Pacific Meeting of Pharmacologists, Hong Kong. (Secretary, Department of Pharmacology, Faculty of Medicine, University of Hong Kong, 5 Sassoon Road, Hong Kong).

27–30 June 1990

International Society for Heart Research XI European Section Meeting, Glasgow, UK. (Professor J.R. Parratt, Dept. of Physiology & Pharmacology, Royal College, University of Strathclyde, Glasgow G1 1XW).

1–6 July 1990

IUPHAR 11th International Congress of Pharmacology, Amsterdam, Netherlands (Contact to be advised).

12–13 July 1990

Synaptic Transmission — First Kelvin Conference, Glasgow, Scotland. (T.W. Stone, Department of Pharmacology, University of Glasgow, Glasgow G12 8QQ.)

2–7 September 1990

34th Harden Conference — Free Radicals: Cell Growth, Disease and Repair Mechanisms, Ashford, UK. (R. Dale, The Biochemical Society, 7 Warwick Court, London WC1R 5DB).

9–14 September 1990

35th Harden Conference — Cell–Cell Interations in the Nervous System, Ashford, U.K. (R. Dale, The Biochemical Society, 7 Warwick Court, London WC1R 5DB.

12–14 September 1990

British Pharmacological Society Autumn Meeting, Belfast, UK. (Closed meeting for members and guests).

19–21 December 1990

British Pharmacological Society Winter Meeting, London, UK. (Closed meeting for members and guests).

The Indices to Volumes 96, 97 and 98 together with the Cumulative Contents will be published with the January 1990 issue

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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. *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 re-submission 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.

Special Reports should normally occupy no more than two printed pages of the Journal; two illustrations (Figures or Tables, with legends) are permitted. As a guideline, with type face of 12 pitch and double-line spacing, a page of A4 paper could contain about 400 words. The absolute maximum length of the *Special Report* is 1700 words. For each Figure or Table, please deduct 200 words. The manuscript should comprise a Title page with key words (maximum of 10), a Summary consisting of a single short paragraph, followed by Introduction, Methods, Results, Discussion and References (maximum of 10). In all other respects, the requirements are the same as for Full Papers (see current 'Instructions to Authors').

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Erratum

Br. J. Pharmacol. (1989), **97**, 1067–1074.

The cardiovascular actions of clonidine and neuropeptide-Y in the ventrolateral medulla of the rat by M.A. McAuley, I.M. Macrae & J.L. Reid.

Figure 6 on page 1071 of this paper was misprinted. The correct version is given below.

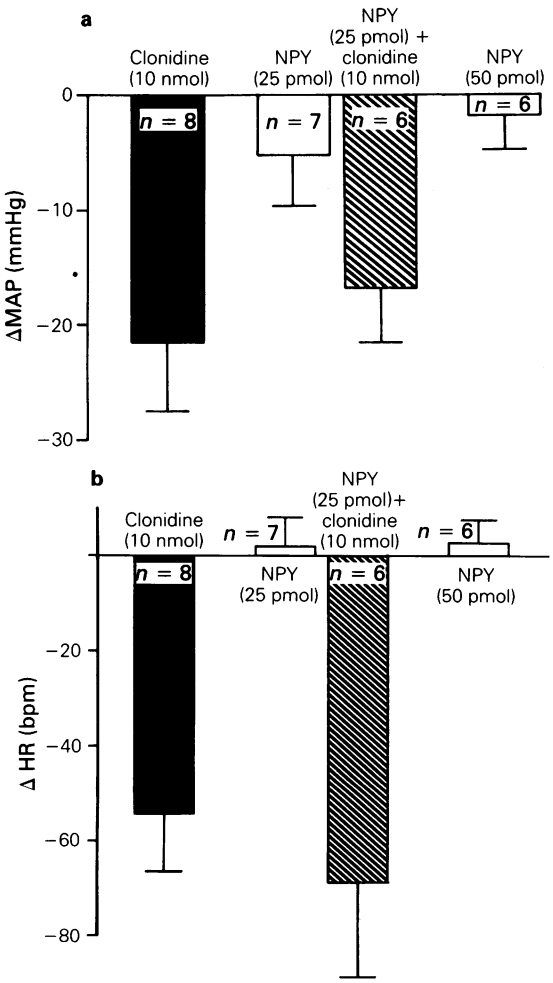

Figure 6 Co-administration of 25 pmol neuropeptide-Y (NPY) and 10 nmol clonidine into the rostral ventrolateral medulla. Columns show change in (a) mean arterial pressure (MAP) and (b) heart rate (HR) (with s.e.mean shown by vertical bars) 30 min post injection.

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