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Muscarinic receptors mediating suppression of the M-current in guinea-pig olfactory cortex neurones may be of the M_2-subtype

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Guinea-pig olfactory cortical neurones *in vitro* were voltage clamped by means of a single intracellular microelectrode technique. Hyperpolarizing voltage commands from holding potentials between −40 to −50 mV produced slow inward current relaxations reflecting deactivation of the M-current (I_M). I_M was reversibly suppressed by 30 μM muscarine or carbachol; this suppression was insensitive to pirenzepine (up to 300 nM) but was inhibited by gallamine (10–20 μM) or 4-diphenylacetoxy-N-methylpiperidine (100, 500 nM), suggesting the involvement of the M_2-type muscarinic receptor.

Introduction There has been much interest recently in the possible subdivision of muscarinic receptors into M_1 and M_2 subtypes, based on their relative affinities for the competitive antagonist pirenzepine (Hammer *et al.*, 1980; Hammer & Giachetti, 1982). M_1-receptors exhibit a high binding affinity towards pirenzepine ($K_D \sim 10–20$ nM) and are found predominantly on neural tissue (e.g. autonomic ganglia), whereas the M_2-type show a low affinity towards this agent ($K_D \sim 200–800$ nM), and are considered to exist on both neural tissue and peripheral organs such as heart and ileal smooth muscle (see Hammer & Giachetti, 1984). A further subclassification of the peripheral M_2-receptor has also been suggested in view of the fact that certain skeletal neuromuscular blockers e.g. gallamine, show more selective anti-muscarinic effects on the heart (Nedoma *et al.*, 1985), whereas antagonists such as 4-diphenyl-acetoxy-N-methylpiperidine (4-DAMP) show a relatively higher affinity for the ileal muscarinic receptor (Barlow *et al.*, 1976). Recent intracellular studies on mammalian central neurones have suggested that both excitatory and inhibitory actions of cholinoceptor agonists can be mediated via M_2-muscarinic receptors (Egan & North, 1985; 1986; McCormick & Prince, 1986a). In the present study, we have attempted to characterize the muscarinic receptor subtype underlying the slow muscarinic depolarization of single olfactory cortical neurones *in vitro*. Under voltage clamp, a persistent, time-and-voltage-dependent potassium current, the M-current (I_M) exists at holding potentials more positive than −60 mV; this current can be specifically suppressed by muscarinic agonists (Constanti & Galvan, 1983b). We show here that the neuronal receptor mediating this suppression of I_M is relatively insensitive to pirenzepine, and therefore may be of the M_2- subtype.

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Methods Intracellular recordings were made from guinea-pig olfactory cortical neurones *in vitro* (23–25°C) using rostro-caudal slices of olfactory cortex (~500 μm thick; Constanti & Sim, 1984) cut with an Oxford vibratome. Slices were held between two nylon meshes (completely submerged) and superfused with oxygenated Krebs solution containing (mM): NaCl 118, KCl 3, $CaCl_2$ 1.5, $NaHCO_3$ 25, $NaH_2PO_4.2H_2O$ 1.2, $MgCl_2.6H_2O$ 1 and D-glucose 11; (bubbled with 95% O_2: 5% CO_2, pH 7.4). Microelectrodes were filled with 4M K acetate (50–80 MΩ) and coupled to an Axoclamp-2, single microelectrode current/voltage clamp preamplifier (2–3 kHz switching frequency, 30% duty cycle: see Constanti & Galvan, 1983a, for method of optimal adjustment). Sampled membrane currents (filtered at 300 Hz, low pass 48db per octave) and voltage were recorded on a Gould Brush 2400 pen recorder. All drugs (BDH Analar, or Sigma) were prepared in Krebs solution and applied via the superfusate (bath-exchange time ~30 s). 4-DAMP methylbromide was a gift from Dr R. B. Barlow (Bristol). The results described below are based on data pooled from five neurones (average impalement time ~3 h).

Results Figure 1 shows typical membrane currents recorded from a single olfactory cortex neurone, voltage clamped at a holding potential of −45 mV. On applying a 10 mV (500 ms) negative voltage jump from this potential, the membrane current showed an initial (instantaneous) step followed by a slow inward relaxation to a steady level. Stepping back to −45 mV

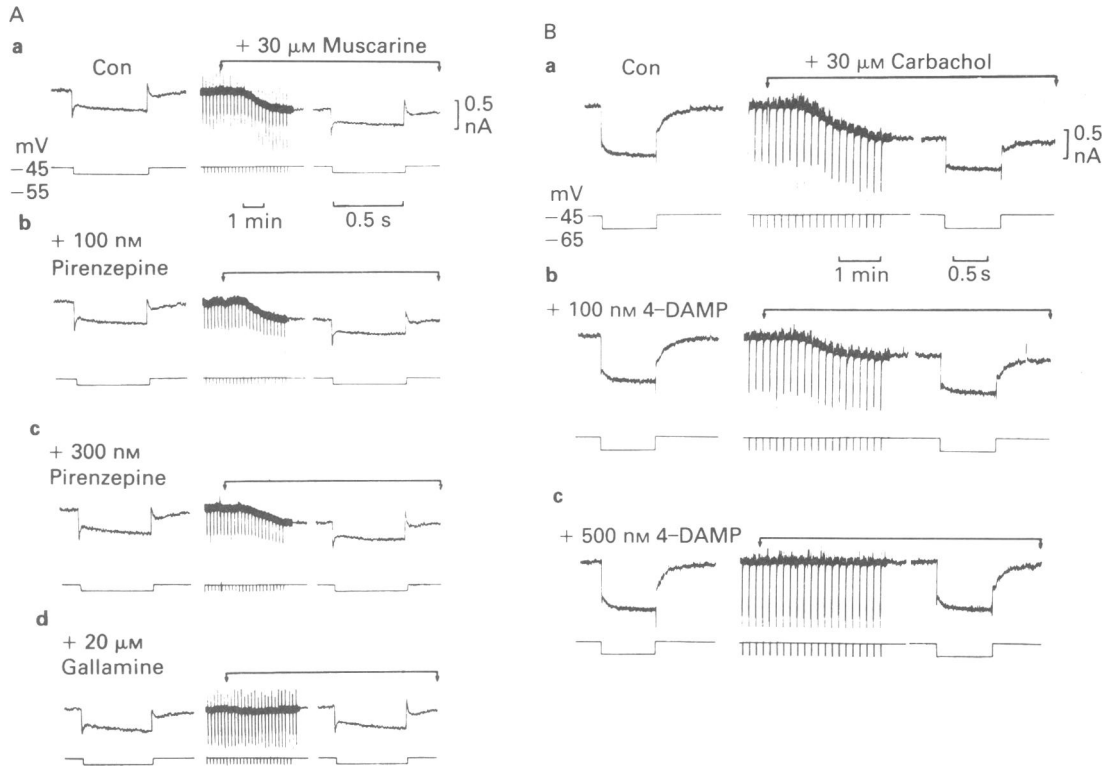

Figure 1 (A) Pirenzepine does not prevent the inhibition of the M-current (I_M) by muscarine. (a–d): Continuous chart record of clamp currents measured in a single olfactory cortex neurone under voltage clamp (holding potential = −45 mV). In each row, upper traces show membrane current; lower traces, voltage. (a): Inhibition of I_M by 30 μM muscarine recorded in control Krebs solution (Con). Record shows initially, the inward current relaxation in response to a −10 mV (500 ms) voltage jump (0.1 Hz). Chart speed was then slowed during drug application and speeded up after 3 min in muscarine. The effect of muscarine was still apparent in 100 nM (b) or 300 nM pirenzepine (c) but was abolished in the presence of 20 μM gallamine (d). Muscarine applications were followed by a 15 min washout period (not shown). Pirenzepine and gallamine were applied for 20 min and 10 min respectively, before testing muscarine. (B) 4–DAMP blocks the inhibition of I_M by carbachol (different neurone; V_{hold} = −45 mV). (a) Inhibition of I_M and inward shift in holding current induced by 30 μM carbachol in control solution (Con) (voltage jumps = −20 mV, 800 ms, 0.1 Hz). (b) Effect of carbachol was partially blocked after 10 min in 100 nM 4-diphenyl-acetoxy-N-methylpiperidine (4-DAMP), and abolished after 10 min in 500 nM DAMP (c). No recovery from antagonism was obtained after a 30 min wash. Each carbachol application was followed by a 25 min washout period (not shown).

evoked a smaller instantaneous step, followed by a decaying inward relaxation to the holding current level. These slow relaxations reflect the deactivation and reactivation respectively, of a 'background' outward K^+ current, I_M (Constanti & Galvan, 1983b).

Bath-application of (±)-muscarine (30 μM) (Figure 1Aa) induced a slow inward shift in holding current and a clear (reversible) reduction in the I_M relaxation amplitude revealed during the negative voltage command. Under current-clamp, this action would be manifest as a slow membrane depolarization and increase in cell input resistance. The muscarinic suppression of I_M was not blocked by low concentrations of pirenzepine (up to 300 nM), pre-applied for at least 20 min (Figure 1A b,c), whereas gallamine (20 μM), a 'cardioselective' M_2-receptor antagonist (Hammer & Giachetti, 1984; Nedoma *et al.*, 1985), produced a clear inhibition of the action of muscarine (Figure 1Ad). Higher concentrations of pirenzepine (500 nM–1 μM) did, however, exhibit an anti-muscarinic effect (not illustrated).

Further support for an M_2-mediated inhibition of I_M was obtained with 4-DAMP. Figure 1Ba shows (in a different cell) that the inward current and I_M

suppression induced by 30 μM carbachol could be partially reduced then completely antagonized in the presence of 100 and 500 nM 4-DAMP respectively. Pirenzepine, gallamine or 4-DAMP applied alone, had no notable effect on I_M or on the holding current level.

Discussion These preliminary results provide the first evidence obtained under voltage clamp, that the receptor mediating muscarinic inhibition of the M-current on a mammalian cortical neurone may be of the M_2-subtype. In view of the relatively slow recovery of I_M relaxations to their control amplitude after washout of muscarinic agonists, it was impractical to construct dose-response relationships for muscarinic suppression of I_M on the same cell. We could not therefore, at present, estimate K_D values for the muscarinic blocking action of the antagonists on single cells, nor can we speculate on their underlying modes of action.

Since Brown *et al.* (1980) showed that pirenzepine was a potent inhibitor of slow muscarinic (I_M-mediated?) depolarizations in rat ganglia, the notion has developed that the slow cholinergic depolarization of central pyramidal neurones mediated by I_M suppression may also involve an M_1-receptor (see McCormick & Prince, 1986b). Our results clearly indicate that unlike the ganglionic M_1-receptor, the cortical muscarinic receptor linked with I_M shows a low sensitivity to pirenzepine, but is sensitive to 'cardioselective' and 'ileum-selective' muscarinic M_2 antagonists, suggesting that the cortical 'M_2' receptor may be different from the cardiac and ileal receptor types. The availability of more specific M_2-antagonists in the future should help to establish any fundamental differences between central neuronal and peripheral M_2-receptors.

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Electrophysiological studies of the effects of the general anaesthetic etomidate on frog myelinated nerve fibre

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1 The effects of the general anaesthetic etomidate (0.1 to 1 mM) upon the node of Ranvier of frog isolated nerve fibres were investigated under current and voltage clamp conditions.

2 When added to the external solution, etomidate reversibly decreased the amplitude of the action potential. The action potential block, induced by the drug, was reversed by increasing the membrane potential.

3 Etomidate rapidly and reversibly blocked the Na current with an apparent dissociation constant of 0.6 mM. In the presence of the drug, the steady-state inactivation-voltage curve of the Na current was shifted towards negative voltages.

4 The block of Na current by etomidate was partially removed by repetitive depolarization preceded by a 50 ms period of hyperpolarization. In contrast, the block was enhanced when the repetitive depolarization was not preceded by hyperpolarization. This suggests that Na channels were preferentially blocked by the drug in the inactivated state.

5 The K current was reversibly blocked by etomidate with an apparent dissociation constant of 0.2 mM. In the presence of the drug, the K current showed an apparent fast inactivation suggesting that K channels were blocked in the open state.

6 We conclude that at higher concentrations than those attainable in the mammalian brain following single anaesthetic doses the general anaesthetic etomidate has a 'local anaesthetic-like' action on the peripheral nervous system.

Introduction

Etomidate (Figure 1) is a carboxylated imidazole which when intravenously administered has been used clinically to produce a rapid induction anaesthesia of short duration. Its use is associated with a rapid onset and recovery (Kay, 1976). No significant, or minimal, adverse effects of etomidate have been demonstrated on the cardiovascular system (Criado *et al.*, 1980; Vickers *et al.*, 1984) or respiratory system (Marquardt *et al.*, 1977). Research *in vitro* and microiontophoretic experiments *in vivo* indicated that etomidate has a γ-aminobutyric acid (GABA)-mimetic action (Evans & Hill, 1978). Recently interest has been directed to the suppressive effects on adrenal function first observed in the rat (Preziosi & Vacca, 1982) and found detrimental when etomidate was given as a prolonged continuous infusion (Ledingham *et al.*, 1983), as well as a prolactin-lowering ability of the drug (Preziosi *et al.*, 1983).

The aim of the present work was to study the effects of etomidate on the frog node of Ranvier.This preparation was used to elucidate the mode of action of the drug on excitable membranes, since membrane potential and ionic currents can be measured accurately under current and voltage clamp conditions.

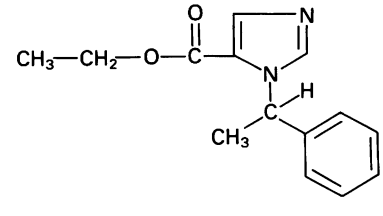

Figure 1 Chemical structure of etomidate.

[1] Correspondence.

Methods

Experiments were carried out on an isolated single myelinated nerve fibre from the frog *Rana esculenta*. The membrane potential and membrane currents were recorded under current and voltage clamp conditions using the method of Nonner (1969). The normal resting potential of fibres (− 70 mV) was defined as the potential at which fast Na inactivation occurred by 30%. Na current was monitored with a conventional two pulse protocol. For Na current recordings, a test pulse of various amplitudes was preceded by a 50 ms pulse to − 120 mV. For the steady-state inactivation-voltage ($h_\infty - V$) analysis of the peak Na current, a test pulse to 0 mV was preceded by a 50 ms pulse of various amplitudes. Membrane currents were calculated assuming an axoplasmic resistance of 10 MΩ. Linear leakage and capacity currents were analogically subtracted from the total current. The series resistance was not compensated for (See Chiu, 1977; Benoit *et al.*, 1985).

The Ringer solution (pH 7.4) contained (in mM): NaCl 111.5, KCl 2.5, $CaCl_2$ 1.8, $NaHCO_3$ 2.4. When recording action potentials or K current, the fibre ends were cut in a solution containing 120 mM KCl, which was used in the end pools throughout the experiments. When measuring Na current, K current was suppressed by replacing the end pool solution with 110 mM CsCl + 10 mM NaCl and adding 10 mM tetraethylammonium to the external solution. The temperature was 15–16°C.

In the present experiments D-etomidate, the active isomer of the etomidate molecule, was tested (Heykants *et al.*, 1975).

Results

Effects of etomidate on action potential

Figure 2 shows the effect of external application of 0.25 and 0.5 mM etomidate on the action potential. In the presence of the drug (0.25 mM), action potential duration increased and amplitude decreased (Figure 2b). The action potential was fully blocked after the addition of 0.5 mM etomidate to the external solution, whatever the amplitude of injected current (Figure 2e(i)). The action potential block, induced by 0.5 mM etomidate, was reversed by hyperpolarizing the membrane by about 20 mV (Figure 2e(ii)). These effects appeared 1 to 2 min after the application of etomidate and were reversed about 5 min after washing out the

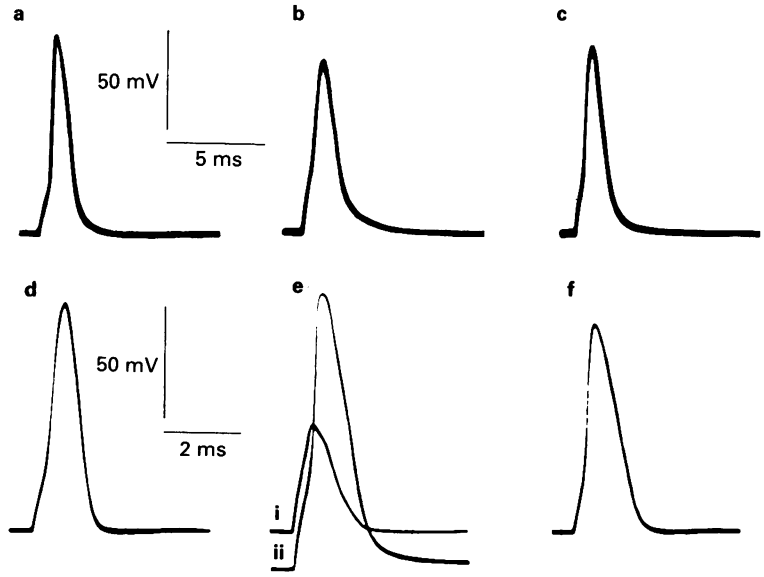

Figure 2 Effects of etomidate on action potential recorded from a single myelinated nerve fibre. (a and d) Control action potentials in Ringer solution. (b and e) Action potentials recorded 1 to 2 min after the addition of 0.25 mM (b) and 0.5 mM (e) etomidate to Ringer solution. (e,ii) The membrane was hyperpolarized by about 20 mV. (c and f) Action potentials recorded about 5 min after washout of the drug with Ringer solution. Fibres: 28–03–85 (a, b and c) and 3–07–85 (d, e and f).

drug (Figure 2c and f).

Effects of etomidate on Na current

The action of 0.5 mM etomidate on Na current is presented in Figure 3. About 1 to 2 min after the addition of etomidate to the external solution, Na current was decreased to about one third of its control value without significant changes either in its time course (Figure 3A) or in the shape of peak current-voltage relationships (Figure 3B). However, the maximum peak current was shifted by about 15 mV towards positive values (Figure 3B). This may be due to a series resistance artefact (Neumcke & Stämpfli, 1983). The effects of the drug on Na current were almost totally reversed about 3 to 5 min after washing out the drug with Ringer solution.

Figure 4 shows the effects of 0.5 mM etomidate on the steady-state inactivation-voltage ($h_\infty - V$) curve of the peak Na current. In the presence of etomidate (added to the external solution 1 min before), the curve was shifted along the voltage axis towards negative values without significant changes in its shape. The voltage corresponding to half maximum steady-state inactivation was about 15 mV more negative in the presence of 0.5 mM etomidate than under control conditions. In other experiments with 0.25 mM etomidate, the $h_\infty - V$ curve was shifted by about 5 mV towards negative values. The shift of the steady-state inactivation-voltage curve was reversed after about 5 min washing with etomidate-free solution (Figure 4).

From the preceding observations, it appears that the effects of etomidate resemble those of local anaesthetics (Hille, 1977). Most local anaesthetics exhibit a frequency-dependent block of Na current (Strichartz, 1973; Hille, 1977). In order to see whether the block of Na current induced by etomidate was also dependent on the frequency of stimulation, we performed the following experiment. In the presence of 0.5 mM etomidate, the fibre was stimulated at frequencies of 1 or 10 Hz, by 19 ms test pulses to either 0 or 30 mV, preceded or not by 50 ms hyperpolarization to −120 mV (Figure 5). In the absence of etomidate, the peak Na current was constant during a prolonged period of stimulation at 1 or 10 Hz. In the presence of etomidate, in order to allow a complete recovery (i.e., return to resting block), the membrane was held between each period of stimulation at a potential of −70 mV for 1 min. The peak Na current measured during each test depolarization, was normalized with respect to the corresponding value measured during the first test pulse of each period of stimulation. When

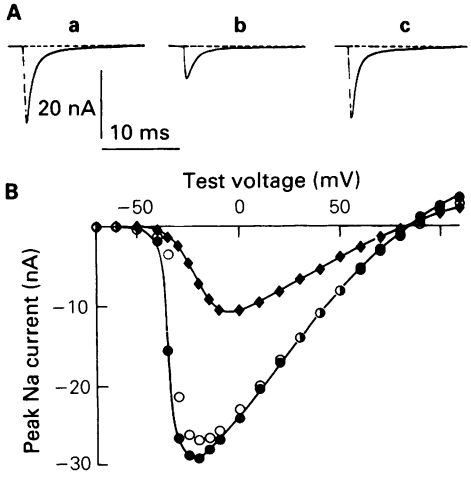

Figure 3 Effects of etomidate on Na current. (A) Na current recorded under control conditions (a), 1 min after the addition of 0.5 mM etomidate to the external solution (b) and 5 min after washout of the drug (c), during depolarization to 0 mV preceded by 50 ms hyperpolarization to −120 mV. (B) Current-voltage relationship of peak Na current, under control conditions (●), in the presence of 0.5 mM etomidate (◆) and after washout of the drug (○), during periods of depolarization of various amplitudes preceded by 50 ms hyperpolarization to −120 mV. Fibre: 13-03-85.

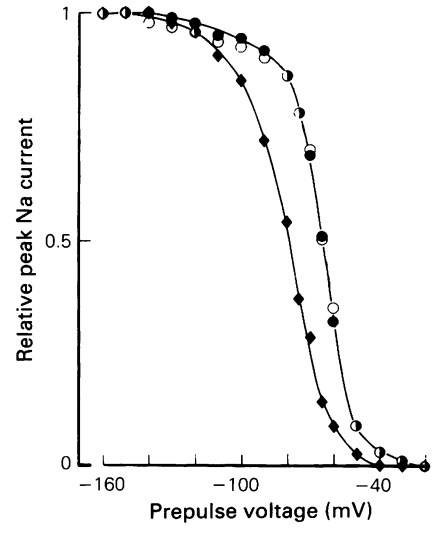

Figure 4 Effects of etomidate on the steady-state inactivation-voltage curve of the peak Na current. Na current was recorded under control conditions (●), in the presence of 0.5 mM etomidate (◆) and after washout of the drug (○), during depolarization to 0 mV preceded by 50 ms pulses of various amplitudes. Fibre: 13-03-85.

test pulses were preceded by a 50 ms period of hyperpolarization to −120 mV, the etomidate-induced block of Na current was partially removed by pulses of stimulation. Block removal was enhanced by an increased number of pulses and was larger when pulses were applied at 10 Hz than at 1 Hz (Figure 5a). Block removal was identical with test pulses to 0 or 30 mV (Figure 5b). In contrast, the Na current was increasingly blocked by etomidate as the pulse number was increased when test pulses were not preceded by hyperpolarization (Figure 5c).

Effects of etomidate on K current

External application of 0.5 mM etomidate reversibly blocked the K current in about 1 to 2 min (Figures 6 and 7). The K current was reduced by etomidate in a time-dependent manner showing an apparent fast incomplete inactivation (Figure 6b). This apparent inactivation could be described by an exponential function (●, in Figure 6b and d), whose extrapolation to time of depolarization gave a value almost equal to that of the steady-state K current recorded in the absence of the drug (□, in Figure 6a and d). The time-dependent block of the K current by etomidate was consistently observed in three different fibres.

In the presence of the drug, the steady-state K current was reduced to about 25% of its control value and the current-voltage curve appears to change from a sigmoid to a linear relationship (Figure 7; see Discussion). The effects of etomidate on the K current were dependent neither on the frequency of stimulation nor on the membrane potential. They were fully reversed about 5 min after washing out the drug with Ringer solution (Figures 6c and 7).

Dose-response curves for the effects of etomidate on membrane currents

Dose-response curves for the steady-state inhibition of K and Na currents by etomidate (0.1 to 1 mM) are presented in Figure 8a. The steady-state K and peak Na currents were normalized with respect to their corresponding values measured before the application of the drug. Assuming that the inhibition of Na and K currents by the drug is based on a one-to-one reaction between ionic channels and etomidate molecules, the apparent dissociation constants are 0.2 and 0.6 mM for K and Na currents, respectively. However, the theoretical curve deviates considerably from the

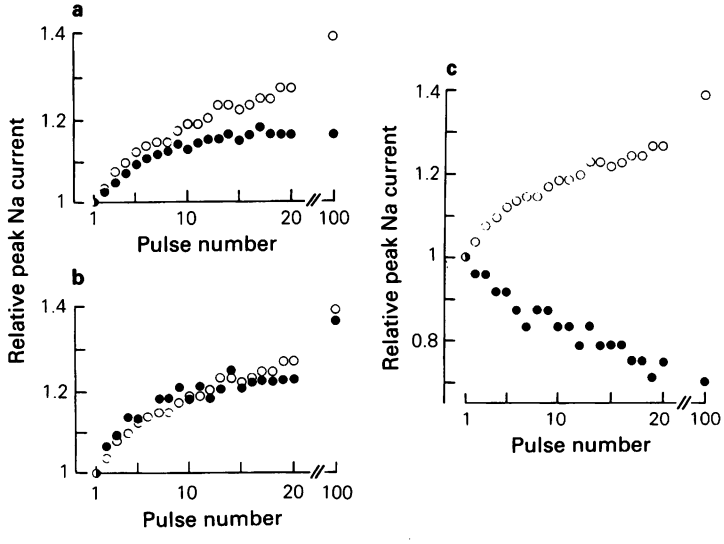

Figure 5 Effects of stimulation frequency and membrane potential on the peak Na current block induced by etomidate. The Na current was recorded in the presence of 0.5 mM etomidate. Between each period of stimulation, the membrane was held at a potential of −70 mV for 1 min. The peak Na current was measured during each 19 ms period of test depolarization and was normalized relative to the corresponding value measured during the first test pulse of each period of stimulation. (a) The fibre was stimulated at a frequency of either 1 Hz (●) or 10 Hz (○). The Na current was recorded during depolarization to 0 mV preceded by 50 ms hyperpolarization to −120 mV. (b and c) The fibre was stimulated at a frequency of 10 Hz. (b) The Na current was recorded during depolarization to either 0 mV (○) or 30 mV (●) preceded by 50 ms hyperpolarization to −120 mV. (c) The Na current was recorded during depolarization to 0 mV preceded (○) or not (●) by 50 ms hyperpolarization to −120 mV. Fibre: 12-03-85.

points for Na current. In order to establish whether a one-to-one interaction between ionic channels and etomidate molecules had occurred, we did plots in double logarithmic co-ordinates (Hill plots) of the points of the experiment of Figure 8a and for seven other experiments in which the drug concentration was changed from 0.1 to 1 mM (Figure 8b). Hill coefficients (n_H) of 1.42 and 1.19 and apparent dissociation constants of 0.48 and 0.19 mM were calculated for peak Na and steady-state K currents, respectively. It should be noted, especially for Na currents that n_H values are likely to be more accurate (but see Discussion).

Discussion

The results show that the general anaesthetic etomidate (0.1 to 1 mM) blocks the action potential and ionic currents at the frog node of Ranvier.

Etomidate has two major effects on Na permeability. It decreases the maximum Na conductance and shifts the steady-state Na current inactivation curve towards negative voltages. Moreover, the inhibition of the Na current is frequency-dependent. The block is decreased by an increased number and frequency of depolarizing pulses when depolarization is preceded by hyperpolarization which removes the resting Na inactivation. In contrast, when depolarization is not preceded by hyperpolarization, i.e., when the Na permeability is partially inactivated before pulses, the inhibition is enhanced by an increased number of depolarizing pulses.

In some respects, the effects of etomidate on the Na current resemble those of local anaesthetics (Strichartz, 1973; Hille, 1977). However, the effects of most

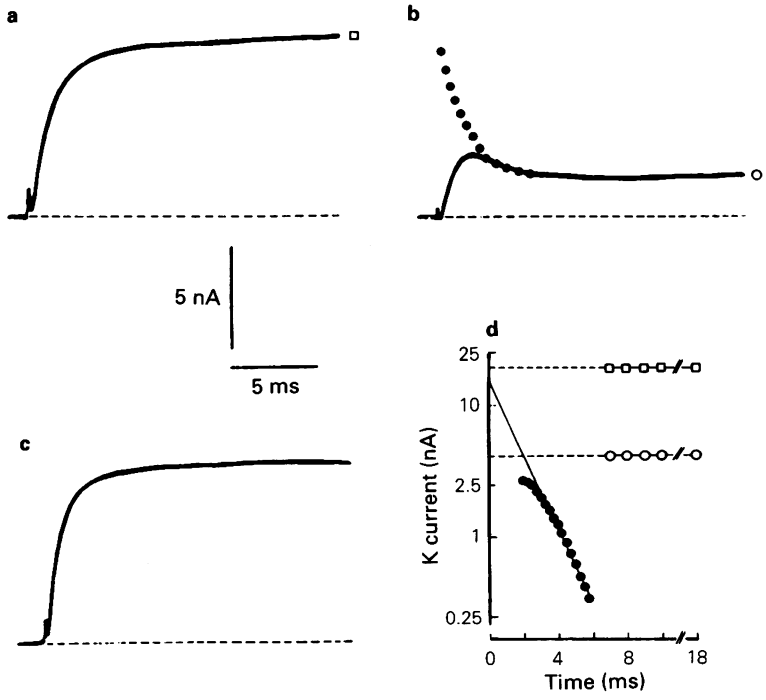

Figure 6 Effects of etomidate on K current. (a, b and c) K current recorded in Ringer solution (a), in the presence of 0.5 mM etomidate (b) and after washout of the drug (c), during an 18 ms period of depolarization to + 50 mV preceded by 50 ms hyperpolarization to − 120 mV. In (b), (●) represent the exponential extrapolation, to time of depolarization, of the apparent incomplete inactivation, induced by etomidate, whose semilog representation is shown in (d). In (d), (●) represent the K current, in the presence of 0.5 mM etomidate, during its inactivation phase minus the steady-state K current (○) in (b) and (d)). In (a) and (d), (□) represent the steady-state K current under control conditions. Extrapolation to time of depolarization of the etomidate-induced inactivation (straight line through (●)) gave 13.59 nA and was determined by a linear regression analysis using the log of the measured values ($r = 0.99$). The steady-state K current, under control conditions and in the presence of etomidate, was 19.08 nA and 4.14 nA, respectively. Fibre: 28–03–85.

local anaesthetics are enhanced by repetitive depolarization and these drugs are assumed to block open channels preferentially (Strichartz, 1973). In contrast, the effects of repetitive depolarization on the inhibition of Na current induced by etomidate can be explained if one assumes that the drug preferentially binds to inactivated channels, either during periods of test depolarization or between periods of test depolarization when the resting Na inactivation is not removed. When depolarizing pulses are preceded by hyperpolarization, etomidate would bind to channels during depolarization and dissociate from channels during hyperpolarization. According to this assumption, the inhibition would be removed by repetitive pulses if the dissociation during hyperpolarization is greater than binding during depolarization. In the absence of a period of prehyperpolarization repetitive depolarization would induce cumulative binding and inhibition.

The removal of the effects of etomidate on the Na current by hyperpolarization also explains the restoration of the action potential by increasing the resting membrane potential, in the presence of 0.5 mM etomidate (see Figure 2e).

From this point of view, the mode of action of etomidate would be similar to that of unprotonated local anaesthetics and of the antiarrhythmic agent ajmaline (Khodorov *et al.*, 1976; Khodorov & Zaborovskaya, 1983).

Etomidate appears to be more efficient in blocking potassium than sodium currents (See Figure 8). This is in contrast with local anaesthetics which preferentially block Na currents (Århem & Frankenhaeuser, 1974).

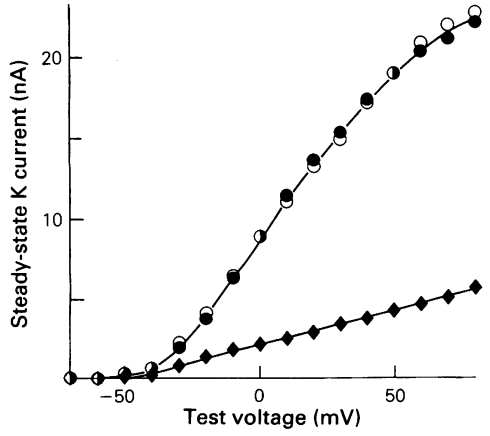

Figure 7 Current-voltage relationships of the steady-state K current. The K current was measured at the end of an 18 ms period of depolarization of various amplitudes preceded by 50 ms hyperpolarization to −120 mV under control conditions (●), in the presence of 0.5 mM etomidate (◆) and after washout of the drug (○). Fibre: 28-03-85.

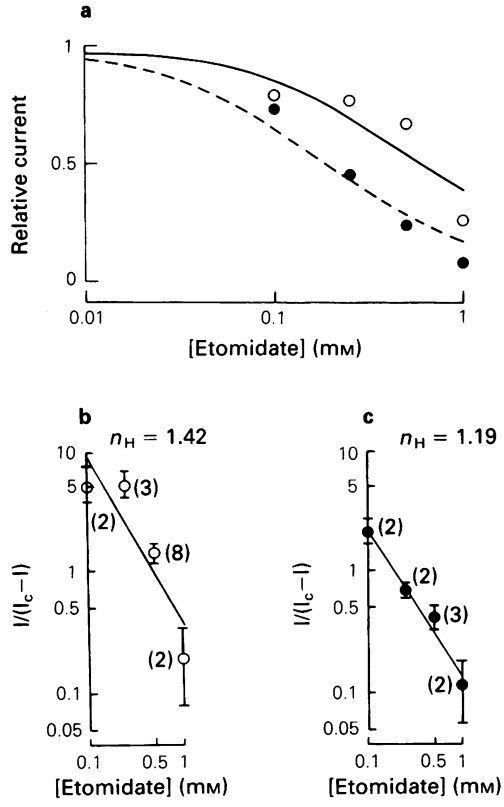

Figure 8 Etomidate dose-response relationship for membrane currents. Peak Na current (○) and steady-state K current (●) were recorded during or at the end of an 18 ms period of depolarization to 0 mV preceded by 50 ms hyperpolarization to −120 mV. (a) Etomidate log dose-response curves for peak Na (continuous line) and steady-state K (broken line) currents. The currents were normalized relative to their respective values in the absence of drug. The curves represent the block of currents assuming a one-to-one reaction between channels and etomidate molecules. Fibre: 28-03-85. (b and c) Hill plots of the effects of etomidate on peak Na and steady-state K currents, according to the equation: $\log (I/(I_c - I)) = \log (K^{-n}{}_H - n_H \log [\text{etomidate}])$, where K is the apparent dissociation constant and n_H the Hill coefficient. The currents were measured in the presence (I) and in the absence (I_c) of various concentrations of etomidate. Mean values and standard errors of the mean obtained in 2-8 experiments (numbers in parentheses). The straight lines were obtained from linear regression through the points. The correlation coefficients (r) were 0.81 (Na current) and 0.97 (K current).

Similar to derivatives of tetraethylammonium (Armstrong & Hille, 1972), quaternary derivatives of local anaesthetics (Strichartz, 1973), strychnine (Shapiro, 1977), quinidine (Revenko *et al.*, 1982), capsaicin (Dubois, 1982) and the antiarrhythmic agent ajmaline (Khodorov & Zaborovskaya, 1983), etomidate blocks the K current in a time-dependent manner. Taking into account that the etomidate molecule is neutral at physiological pH ($pK_a = 4.10$) and the effects of the drug are rapidly reversed upon washing, the receptor site for etomidate associated with K channels should be located at the external face of the membrane and its affinity (or accessibility) should be larger when K channels are in the open rather than in the resting state.

In the presence of etomidate, the K current-voltage curve appears to change from a sigmoid to a linear relationship (Figure 7) and the etomidate dose-response curves, specially for Na current, are poorly described by a one-to-one interaction between ionic channels and drug molecules (Figure 8), but are better fitted if one assumes that more than one molecule of drug is necessary to block one channel. There are various interpretations of these effects. The reduction of the K current by etomidate should lead to a decrease in K accumulation in the nodal gap and consequently indirectly modify the current (Dubois, 1981a; 1983). Moreover, in the frog node of Ranvier, K and Na currents are made up of several components having different voltage sensitivities and pharmacological properties (Dubois, 1981b; 1983; Benoit *et al.*, 1985; Benoit & Dubois, 1986). Etomidate might be more efficient in blocking one of these components. Furthermore, as shown for tetrodotoxin and niflumic acid (Benoit *et al.*, 1985; Benoit & Dubois, 1986), more than one molecule of drug may be necessary to block one channel. Further experiments would be needed to clarify these points.

In conclusion, the effects of the general anaesthetic etomidate on the myelinated nerve fibres resemble those of local anaesthetics. However, while most local anaesthetics are thought to interact preferentially with the open Na channels, etomidate may bind preferentially to open K channels and to inactivated Na channels.

Etomidate penetrates into the brain very rapidly, since the peak level occurred less than 1 min after injection: $1.50 \pm 0.35\,\mu g$ of drug per g of brain (0.004 mM) was sufficient to produce general anaesthesia in mammals. With a dose ($9.1\,mg\,kg^{-1}$), nearly 30 times that employed in humans ($0.3\,mg\,kg^{-1}$), concentrations of about $15\,\mu g$ per g of brain (0.04 mM) were found (Heykants *et al.*, 1975). In the present research, drug concentrations (0.1–1 mM) 25–250 times higher than are needed in the mammalian brain to obtain anaesthesia with etomidate were employed. On the other hand, on the node of Ranvier several anaesthetic molecules are active at the same dose range (Hille, 1977; Carratù *et al.*, 1983). However, we must point out that extrapolation of our results to mammals may not be justified in view of the importance of species differences.

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Dopamine receptor-mediated spinal antinociception in the normal and haloperidol pretreated rat: effects of sulpiride and SCH 23390

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1 Nociceptive tail flick latencies (TFL) were recorded in response to noxious thermal stimuli applied to lightly anaesthetized rats. The effects of intrathecally administered dopamine receptor agonists alone and combined with dopamine receptor antagonists were examined upon the TFL. Experiments were repeated on animals made supersensitive to dopamine following withdrawal from 28 day administration of haloperidol.

2 In untreated animals the D_2-receptor agonist LY 171555 and apomorphine produced an increase in TFL. In contrast, the D_1-receptor agonist SKF 38393 had no significant effect on TFL.

3 Following haloperidol-induced dopamine-supersensitivity, SKF 38393 produced an increase in TFL. In contrast, LY 171555 and apomorphine had minimal effects on TFL in this preparation.

4 In animals not treated with haloperidol, the dopamine receptor antagonists SCH 23390 and (±)-sulpiride both blocked the increase in TFL produced by the D_2-agonists.

5 SCH 23390 and (±)-sulpiride also blocked the increase in TFL produced by SKF 38393 in haloperidol-supersensitized animals.

6 The antinociceptive action of intrathecally administered dopamine agonists appears to be mediated via D_2-receptors. Whether the antinociception produced by SKF 38393 is exclusively contingent upon the activation of D_1-receptors in the dopamine-supersensitive animal is as yet unresolved.

Introduction

It is generally accepted that several neurotransmitter systems are involved in the modulation of nociceptive information at the level of the spinal cord (Yaksh *et al.*, 1981). There is accumulating evidence that enhancement of dopamine function produces antinociception (Saarnivaara, 1969; Paalzow & Paalzow, 1975; 1983; Barasi & Duggal, 1985; Jensen & Smith, 1982; 1983a,b; Jensen & Yaksh, 1984; Fleetwood-Walker *et al.*, 1984), whilst there are also reports of hyperalgesia following L-DOPA, apomorphine (Tulunay *et al.*, 1975) and nomifensine (Gonzalez *et al.*, 1980) treatments. However, these divergent reports appear to be dependent on the different species, routes of drug administration and noxious stimuli employed. It is of particular interest that Paalzow & Paalzow (1983) have demonstrated different effects of apomorphine on nociceptive vocalization threshold induced by electrical stimulation in rats, where low doses produced hyperalgesia and high doses antinociception, indicating the possibility of two dopamine receptor sub-types mediating these responses. In other studies electrical stimulation of the rat substantia nigra (SN) was reported to produce antinociception (Sandberg & Segal, 1978). However, Duggal & Barasi (1985) were unable to replicate this observation and concluded that the previously reported antinociceptive effects were not due to discrete activation of the SN alone but resulted from current spread to neighbouring structures. More recently electrical stimulation in the region of the A11 dopamine cell group has been shown to suppress selectively nociceptive responses recorded from dorsal horn neurones (Fleetwood-Walker & Hope, 1985); this correlates well with anatomical evidence for the origin of a descending dopaminergic pathway (Bjorklund & Skagerberg, 1979; Skagerberg & Lindvall, 1983).

In previous studies we have shown that apomorphine elevates the thermal tail flick latency (TFL) in

barbiturate-anaesthetized rats (Barasi & Duggal, 1985). Moreover it was shown that in mice the reputed D_2-agonist LY 141865 (Stoof & Kebabian, 1981) produced antinociceptive activity after intracerebroventricular injection whilst the D_1-agonist SKF 38393 (Setler *et al.*, 1978) was inactive (Ben-Sreti *et al.*, 1983a). The lack of effect of SKF 38393 on the nociceptive threshold might be explained by the idea that this compound may stimulate only supersensitive dopamine receptors (Setler *et al.*, 1978). Furthermore in chronically morpine-treated rats undergoing withdrawal, which is a model of dopaminergic supersensitivity (Lal, 1975; Sicuteri *et al.*, 1980), SKF 38393 produced an overall intensification of the withdrawal syndrome as shown by the increased incidence of dopamine mediated withdrawal signs (Ben-Sreti *et al.*, 1983b).

The present study examines the effects of the dopamine agonists apomorphine, SKF 38393 and LY 171555 (the laevorotatory isomer of LY 141865) on nociceptive sensitivity in naïve rats and in rats with haloperidol-induced dopamine supersensitivity. In addition, the reputedly specific dopamine antagonists SCH 23390 (Hyttel, 1983) and sulpiride (Elliott *et al.*, 1977; Woodruff *et al.*, 1980) were used to characterize the dopamine receptor type mediating changes in nociception produced by the dopamine agonists. Preliminary results have been presented to the British Pharmacological Society (Barasi *et al.*, 1985a,b).

Methods

Animals and environment

Male Wistar rats (UWIST breeding stock) weighing between 200–300 g were used in all experiments. They were allowed free access to standard rat and mouse breeding diet (Grain Harvesters, Wigham, Kent) and tap water, both being withdrawn 2 h before experimentation. Animal house and laboratory conditions were maintained on a 12 h light-dark cycle at a constant temperature of 21°C.

Drugs and injections

All drug solutions were made up immediately before injection. The following drugs were dissolved in apyrogenic 0.9% (w/v) sodium chloride: LY 171555 (trans-(−)-4aR-4,4a,5,6,7,8,8a, 9-octahydro-5-propyl-2H-pyrazolo [3,4-g] quinoline monohydrochloride, Eli Lilly); apomorphine hydrochloride (Sigma). SKF 38393 (2,3,4,5-tetrahydro-7,8-dihydroxy-1-phenyl-1H-3-benzazepine) was dissolved in sterile water for injection. Sulpiride (Ravizza), haloperidol (Searle) and SCH 23390 ((R)-(+)-8-chloro-2,3,4,5-tetrahydro-3-methyl-5-phenyl-1H-3-benzazepin-7-ol hemimaleate) (Schering) were dissolved in 1% tartaric acid and the pH was adjusted to approximate neutrality. A commerical preparation of pentobarbitone (Saggital, May and Baker) was used as anaesthetic. Anaesthesia was maintained by injecting small (approx. 0.02ml) volumes of dilute barbiturate ($12\,mg\,ml^{-1}$) at appropriate intervals throughout the experiment.

Two routes were used for drug administration. Peripheral injections via the femoral vein in a volume of 0.2 ml were administered over a period of 2 min. Similarly intrathecal injections were made into the lumbar subarachnoid space at 15 µl dose volume followed by 10 µl of 0.9% saline to flush the cannula. Animals were subjected to a post-mortem examination to determine the location of the intrathecal cannula.

Earlier studies from this and other laboratories (Jensen & Smith, 1982; Jensen & Yaksh, 1984; Barasi & Duggal, 1985) have demonstrated the dose-related nature of the effect of the currently used dopamine agonists on tail flick latencies following spinal administration. On the basis of these previous studies, appropriate doses of agonist were selected.

Nociceptive testing

Nociceptive threshold in rats lightly anaesthetized with pentobarbitone were determined as tail flick latencies (TFL) in seconds elicited in response to noxious radiant heat (D'Amour & Smith, 1941; Barasi & Duggal, 1985). Briefly, radiant heat was applied by means of a 75W projector bulb to the underside of the tail which had previously been blackened with Indian ink. After establishing a steady nociceptive baseline latency (ranging from 1.5 to 2.5 s) TFLs were determined at 4 min intervals following drug administration. A cut-off latency of 6 s was imposed after which the stimulus was terminated, and that particular site of stimulus was excluded from further study. The noxious stimulus was applied to successive sites along the middle 5 cm of the tail in order to reduce any changes in the sensitivity of cutaneous nociceptors following repeated exposure to the radiant heat. In previous studies simultaneous recordings of femoral arterial blood pressure and tail-skin temperatures showed that with this technique the increases in nociceptive sensitivity detected were not related to changes in tail blood flow (Barasi & Duggal, 1983).

Intravenous and intrathecal cannulations

The femoral vein was cannulated to enable dilute anaesthetic and drugs to be injected. To reduce dead space a three-way tap was not used. An intrathecal cannula was introduced as described previously (Barasi & Duggal, 1985) and the spinal location checked by post mortem examination. Briefly, a

7.5 cm length of Portex PP10 tube was inserted through a small slit in the atlanto-occipital membrane. Drugs were introduced by a manual injection system.

Induction of dopamine supersensitivity

Dopamine supersensitivity was induced by the method of Muller & Seeman (1978) whereby haloperidol was administered in tap water (50 mg litre^{-1}) as the sole source of fluid intake for 28 days. Experiments on nociceptive sensitivity were performed 4 days after withdrawal from haloperidol. Based on the average fluid consumption of each animal, the daily intake of haloperidol per rat ranged between 3 mg kg^{-1} at the start of the schedule to approximately 12 mg kg^{-1} at the end of the schedule. To check the degree of dopamine supersensitivity which had been achieved, a group of rats was challenged with apomorphine 0.25 mg kg^{-1} i.p. four days after haloperidol withdrawal. The incidence of stereotype behaviour was assessed by use of the rating scale devised by Creese & Iversen (1973).

Assessment of data

Comparison of the nociceptive sensitivity of different treatment groups was achieved by converting tail flicks latencies to an Index of Analgesia (IA) as follows;

$$IA = \frac{TFL - \text{mean control TFL}}{\text{max TFL} - \text{mean control TFL}}$$

Calculations of statistical significance were performed using the Mann-Whitney U-test (Siegel, 1956).

Results

Effect of dopamine agonists on tail flick latency in untreated rats

Apomorphine (75 μg kg^{-1}) and LY171555 (75 μg kg^{-1}) injected intrathecally (i.t.) were found to significantly increase the TFL for periods between 20 to 30 min. Initially the responses exceeded the 6 s cut-off, gradually returning to the vehicle control baseline latencies (Figure 1). In contrast to these responses, administration of SKF38393 (150 μg kg^{-1}, i.t.) produced no significant change from the control TFL. There was a statistically insignificant reduction in the TFL following injection of the D_1-agonist.

Effect of dopamine agonists on TFL in dopamine-supersensitive rats

Administration of SKF38393 (75 μg kg^{-1}, i.t.) to rats withdrawn from chronic haloperidol treatment

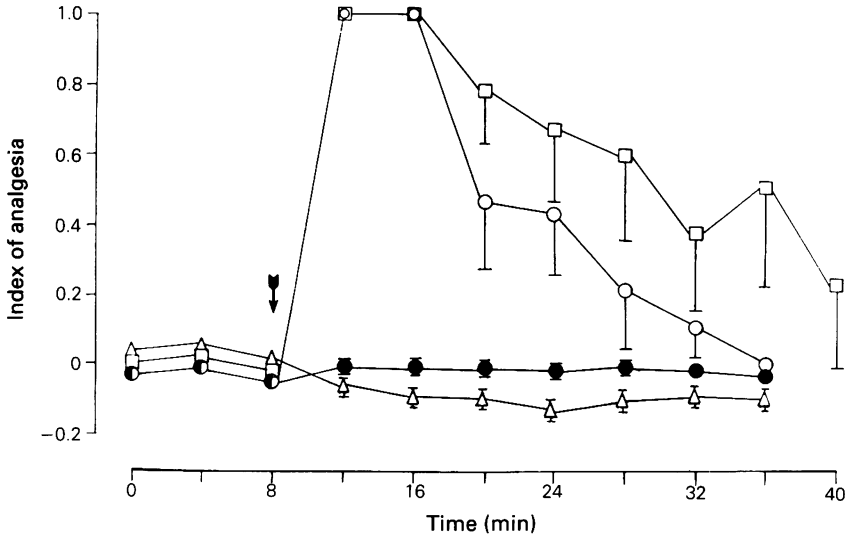

Figure 1 The effects of intrathecal (i.t.) injections of dopamine agonists on tail-flick latencies expressed as Index of Analgesia in vehicle pretreated (i.e. dopamine normosensitive) rats. After establishment of a steady baseline the following agents were injected at time = 8 min; apomorphine (75 μg kg^{-1} i.t.) (□), LY171555 (75 μg kg^{-1}, i.t.) (○), SKF38393 (150 μg kg^{-1}, i.t.) (△) and 0.9% w/v saline (15 μl per rat, i.t.) (●).

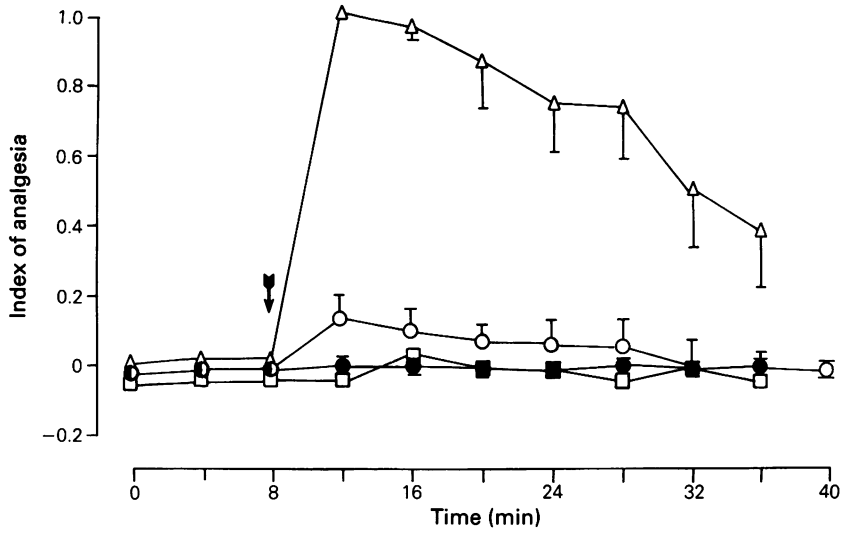

Figure 2 The effects of intrathecal (i.t.) injections of dopamine agonists on tail-flick latencies (expressed as Index of Analgesia) in rats with haloperidol-induced dopamine supersensitivity. After establishment of a steady baseline the following agents were injected at time = 8 min; SKF38393 (75 $\mu g\,kg^{-1}$, i.t.) (△), LY171555 (75 $\mu g\,kg^{-1}$, i.t.) (○), apomorphine (75 $\mu g\,kg^{-1}$, i.t.) (□) and 0.9% w/v saline (15 μl per rat) (●).

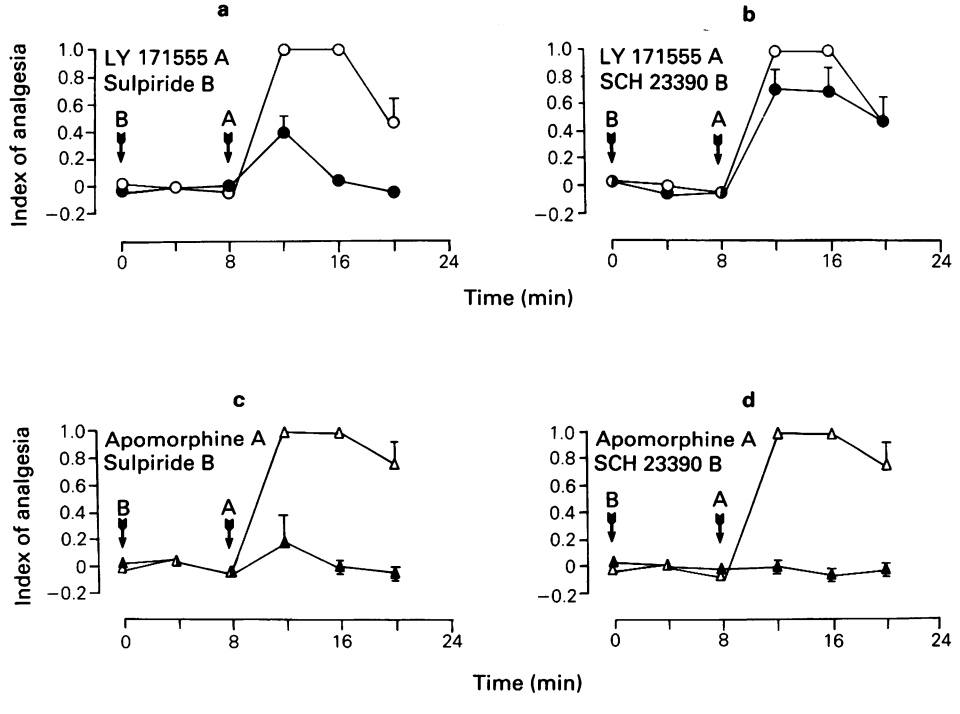

Figure 3 The effects of intrathecal (i.t.) injections of LY 171555 (75 $\mu g\,kg^{-1}$) or apomorphine (75 $\mu g\,kg^{-1}$) alone (open symbols) or in combination with intravenous (i.v.) injections of sulpiride (10 $mg\,kg^{-1}$) or SCH23390 (250 $\mu g\,kg^{-1}$) (closed symbols) on tail-flick latencies in vehicle pretreated (dopamine normosensitive) rats.

produced a significant elevation of TFL compared to the control baseline (Figure 2). The first TFL reading after injection of SKF 38393 attained the cut-off value and subsequently diminished over the duration of the experiment. This contrasts with the lack of effect when examined in untreated rats. In addition, the effect of LY 171555 (75 $\mu g\,kg^{-1}$, i.t.) on TFL was substantially reduced in the dopamine supersensitized animals compared with the cut-off response in dopamine naïve rats. Similarly, administration of apomorphine (75 $\mu g\,kg^{-1}$, i.t.) produced no change in TFL and response latencies were of the same order of magnitude as those of vehicle controls (Figure 2).

Effects of sulpiride and SCH 23390 on the apomorphine and LY 171555 elevated TFL responses in dopamine-naïve rats

The effects of intravenous administration of the dopamine antagonists sulpiride and SCH 23390 at doses corresponding to those used by ourselves and other workers (Gonzalez *et al.*, 1985; Pugh *et al.*, 1985) were examined on the protracted increase in TFL produced by apomorphine and LY 171555 in dopamine-naïve rats (Figure 3). Both sulpiride (10 $mg\,kg^{-1}$, i.v.) and SCH 23390 (250 $\mu g\,kg^{-1}$, i.v.) blocked the increased TFL produced following the i.t. administration of LY 171555 (75 $\mu g\,kg^{-1}$). The blockade produced by sulpiride was almost complete whilst that associated with SCH 23390 was considerably less extensive although it achieved statistical significance (Figure 3a,b). Furthermore, in subsequent experiments, treatment with either sulpiride or SCH 23390 (doses as above) totally abolished the increase in TFL produced by i.t. administration of apomorphine (Figure 3c,d). In the presence of either of these blockers the response to apomorphine was comparable to that produced by vehicle treatment.

Effects of sulpiride and SCH 23390 on the increase in TFL produced by SKF 38393 in dopamine-supersensitive rats

The effects of intravenous administration of sulpiride and SCH 23390 were examined on the increase in TFL produced by SKF 38393 in rats with haloperidol-withdrawal-induced dopamine supersensitivity (Figure 4). Administration of both sulpiride (10 $mg\,kg^{-1}$, i.v.) and SCH 23390 (250 $\mu g\,kg^{-1}$, i.v.) completely blocked the TFL responses produced by SKF 38393 (75 $\mu g\,kg^{-1}$, i.t.). In the presence of the antagonists the SKF 38393 response had returned to the baseline control level (Figure 4).

Assessment of maximum dopamine supersensitivity

Following withdrawal from haloperidol, rats became progressively more sensitive over a period of several days in terms of certain behavioural effects. By challenging the animals with apomorphine (0.25 $mg\,kg^{-1}$, i.p.) following four haloperidol-free days, it was noted that maximal stereotyped gnawing, licking and yawning was observed between 40 and 80 min after the agonist was injected. Thus in experiments with sensitized animals, drug studies were performed on the fourth day after haloperidol treatment had been discontinued.

Discussion

Chronic administration of haloperidol and other neuroleptics is an established method for inducing supersensitivity in dopaminergic systems, though supersensitivity in other aminergic systems has also been shown (Muller & Seeman, 1978). The schedule employed in the present study involving 28 day

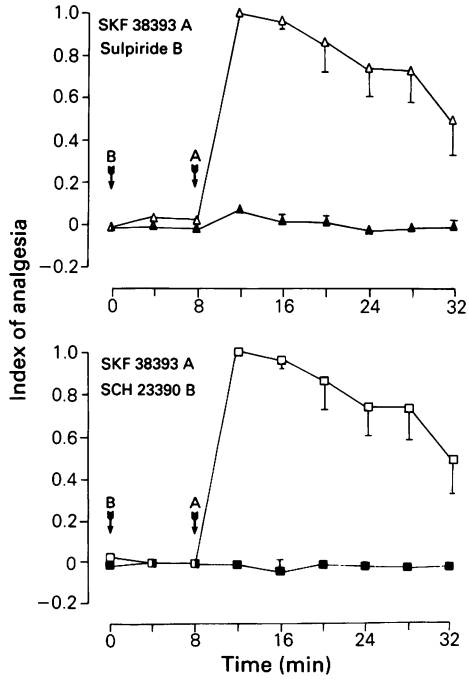

Figure 4 The effects of intrathecal (i.t.) injections of SKF 38393 (75 $\mu g\,kg^{-1}$, i.t.) alone (open symbols) or in combination with intravenous injections (i.v.) of either sulpiride (10 $mg\,kg^{-1}$) or SCH 23390 (250 $\mu g\,kg^{-1}$) (closed symbols) on tail-flick latencies in rats with haloperidol-induced dopamine-supersensitivity.

administration of haloperidol in the drinking water and the subsequent 4 day drug-free period before testing was shown to induce a characteristic pattern of stereotypic behaviour after apomorphine challenge. This demonstrates that the haloperidol dosing produced a measurable and marked degree of functional dopamine supersensitivity which accords with a 40% increase in D_2-binding sites (Mackenzie & Zigmond, 1985).

The present results clearly highlight the ability of different groups of dopamine agonists to influence spinal nociceptive reflexes. These antinociceptive profiles are correlated with the presence or absence of functional dopamine receptor supersensitivity. Thus it has been demonstrated that the putative D_2-agonists apomorphine and LY 171555 (Kebabian & Calne, 1979; Stoof & Kebabian, 1981) produced a significant elevation of the TFL in untreated naïve rats. In addition, under identical conditions the agonist SKF 38393, which is reputedly specific for D_1-receptors (Stoof & Kebabian, 1981), produced a marginal but insignificant hyperalgesia manifested by a reduction in TFL. In contrast to these results, following withdrawal from chronic exposure to haloperidol, SKF 38393 produced a pronounced elevation of the TFL whereas in untreated rats no such increase in the nociceptive response latency was recorded. Likewise in these supersensitive rats the antinociceptive response produced by LY 171555 was substantially reduced whilst that of apomorphine was abolished completely. The data obtained with untreated rats are in agreement with other reports concerning the effects of the application of dopamine agonist drugs to the spinal cord and their modification of nociceptive responses. For example, in studies by Jensen & Smith (1982, 1983a,b) using spinalized rats or following pretreatment with 5-hydroxytryptamine receptor or noradrenoceptor blocking drugs, apomorphine was found to produce an antinociceptive effect. These authors noted that apomorphine had no effect on TFL in untreated rats; the effects of the anaesthetic used in the present study may be analogous to the spinalised preparation used by Jensen & Smith. The ability of SKF 38393 to produce an antinociceptive response in the dopamine-supersensitive but not the untreated rats appears to reflect the specificity of SKF 38393 for supersensitive dopamine receptor sites as previously suggested by Setler and co-workers (1978). These authors reported SKF 38393 to produce contralateral rotation in rats with nigrostriatal lesions but not in the intact animal. In other studies it was shown that SKF 38393, which was inactive behaviourally, exacerbated withdrawl signs in chronically morphine-treated rats (Ben-Sreti *et al.*, 1983b). This state of opioid withdrawal is believed to be associated with dopamine-supersensitivity (Lal, 1975).

From the results of dopamine receptor-binding studies the benzazepine derivative SCH 23390 has been characterized as a selective antagonist at D_1-receptors (Hyttel, 1983; Christensen *et al.*, 1984). The results of the present studies with SCH 23390 and sulpiride may suggest a possible lack of dopamine receptor selectivity in the mechanisms of the antinociceptive responses produced by the dopamine agonists used. Both dopamine antagonists blocked the increase in TFL evoked by LY 171555 and apomorphine in untreated rats and also that of SKF 38393 in the supersensitive model. However, in recent publications it has been shown that SCH 23390 antagonizes amphetamine-induced locomotor activity and selected apomorphine stereotypes, behaviours associated with D_2-receptor stimulation (Christensen *et al.*, 1984; Molloy & Waddington, 1984; Arnt, 1985a). It is not surprising therefore that SCH 23390 effectively blocks the antinociceptive effect produced by apomorphine in this study (Figure 3). In pharmacokinetic studies (Schulz *et al.*, 1985), it has been shown that whilst SCH 23390 was cleared rapidly from the periphery, high levels were maintained in the CNS for comparatively long periods of time, in contrast to other neuroleptics. However, it has been observed that ligands which interact specifically with D_1-receptors may influence the expression of dopamine-mediated behaviours with a possible functional interaction between D_1- and D_2-receptors (Arnt, 1985a,b). In the light of the present results the possibility exists that SCH 23390 may influence the expression of D_2-mediated suppression of nociception. The predominantly D_2-dopamine receptor antagonist sulpiride, which was found to block the increased TFL produced by SKF 38393 in chronic haloperidol-withdrawn rats, may also block the activation of supersensitive dopamine receptor sites by D_1-agonists. In a recent report, sulpiride has been resolved into stereoisomers with the (−)-isomer showing a marked selectivity for D_2-receptors (Leff *et al.*, 1984). It may be possible using the (−)-isomer to determine whether blockade of D_1-sites by sulpiride contributes towards the overall antagonism of the SKF 38393 response produced in the supersensitive model.

The results of this study provide further evidence that the antinociceptive action of intrathecally administered dopamine agonists in lightly anaesthetized rats is mediated via D_2-receptors. D_1-agonists appear to have no antinociceptive activity in normal rats. In complete contrast, D_2-agonists have minimal antinociceptive activity in rats with supersensitive dopamine receptors. It must be added that in addition to the well characterized dopamine supersensitivity following chronic neuroleptic treatment, the antidopamine effect of brief exposure to haloperidol may persist for several weeks (Campbell *et al.*, 1985), and this may account for the apparent lack of effect of D_2-agonists in the supersensitive animal. However, in

these animals the D_1-agonist SKF 38393 increases TFL, but whether this antinociception is exclusively contingent upon the activation of D_1-receptors in the neuroleptic pretreated rats is as yet unresolved.

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Antagonism between (−)-N^6-phenylisopropyl-adenosine and the calcium channel facilitator Bay K 8644, on guinea-pig isolated atria

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1 Antagonism between (−)-N^6-phenylisopropyladenosine (PIA) and the dihydropyridine calcium channel facilitator Bay K 8644 was investigated in guinea-pig spontaneously beating or electrically driven isolated atria, taken from normal and from reserpine-treated animals.

2 PIA (3–100 nM) produced a dose-dependent decrease in contractile tension and frequency in spontaneously beating atria being more effective in reserpinized preparations.

3 Bay K 8644 (5–200 nM) produced an increase in contractile tension in both normal and reserpinized atria. In electrically driven left atria the positive inotropic effect of Bay K 8644 was similar to that in spontaneously beating preparations. The positive chronotropic effect of Bay K 8644 was slight and variable.

4 PIA produced a rightward parallel shift of the concentration-response curves for the positive inotropic effects of Bay K 8644 in all experimental conditions. In spontaneously beating atria from normal guinea-pigs, the Schild regression plot was linear and its slope near to unity; pA_2 of PIA 8.63 ± 0.05 (IC_{50} 2.35 ± 0.25 nM). In electrically driven atria the antagonism by PIA of the effects of Bay K 8644 was apparently competitive, and the IC_{50} of PIA was 18.6 ± 0.4 nM. PIA antagonized the positive chronotropic effect of Bay K 8644 in spontaneously beating preparations, both from normal and from reserpine-treated animals.

5 Carbachol did not modify the positive inotropic effects of Bay K 8644.

6 These data indicate that PIA may interact with Bay K 8644 at the level of the slow calcium channels, and may decrease the transmembrane calcium flux into the cell.

Introduction

Adenosine is continuously produced by the working heart (Rubio *et al.*, 1979). This nucleoside directly depresses the rate of firing of the sino-atrial pacemaker and the force of atrial contraction thus yielding negative chronotropic and inotropic effects in various animal species (for reviews see: Baer & Drummond, 1979; Burnstock, 1980; Daly *et al.*, 1983; Berne *et al.*, 1983; Schütz & Freissmuth, 1985; Stone, 1985). There is much controversy on the mechanism responsible for the cardiac effects of adenosine (for reviews see: Endoh *et al.*, 1983; Schütz & Freissmuth, 1985). A first hypothesis suggests that adenosine reduces the catecholamine-induced increase in calcium inward current mediated by adenosine 3′:5′-cyclic monophosphate (cyclic AMP) accumulation (Baumann *et al.*, 1981; Dobson, 1983; Dobson & Schrader, 1984; Isenberg & Belardinelli, 1984; Böhm *et al.*, 1985). Alternatively, adenosine reduces the synthesis of cyclic AMP through the activation of inhibitory adenosine receptors (R_i/A_1) coupled to adenylate cyclase (Evans *et al.*, 1982; Collis, 1983; Leung *et al.*, 1983). Data indicating that adenosine may act by changing K^+ and/or Ca^{2+} conductance directly, by a mechanism independent of cyclic AMP have also been presented (De Gubareff & Sleator, 1965; Schrader *et al.*, 1975; Hartzell, 1979; Tuganowski *et al.*, 1980; Belardinelli & Isenberg, 1983; Endoh *et al.*, 1983; Hughes & Stone, 1983). However, the effector of this mechanism is still unknown. Preliminary results suggested a role of adenosine in modulating slow Ca^{2+} channels (Caparrotta *et al.*, 1985). We investigated this hypothesis by studying the interaction of a stable analogue of adenosine, (−)-N^6-phenylisopropyladenosine, with a calcium channel facilitator, Bay K 8644, on guinea-pig isolated atria.

Bay K 8644 is a 1,4-dihydropyridine derivative

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which enhances myocardial contractility and constricts blood vessels (Schramm *et al.*, 1983a,b; Wada *et al.*, 1985) by increasing the transmembrane calcium current through the slow channels. Bay K 8644 binds to the same dihydropyridine binding site as does nifedipine, in or near voltage-operated slow calcium channels (Schramm *et al.*, 1983a,b; Thomas *et al.*, 1984; Ishii *et al.*, 1985). However, in contrast to nifedipine, Bay K 8644 promotes the influx of calcium ions, thus increasing heart contractility.

(−)-N^6-phenylisopropyladenosine (PIA, Londos *et al.*, 1980) was used as a stable analogue of adenosine, acting on the cell surface R adenosine receptors functionally linked to adenylate cyclase.

The effect of PIA on the positive inotropic and chronotropic effects of Bay K 8644 was studied on guinea-pig isolated atria, spontaneously beating or electrically driven, taken from normal and from reserpine-treated animals in order to exclude any interference by endogenous catecholamines.

Methods

Hearts were removed from guinea-pigs of either sex (400–600 g) and placed in physiological solution (29°C) of the following composition (mM): NaCl 120 KCl 2.7, $CaCl_2$ 1.36, $MgCl_2$ 0.9, $NaHCO_3$ 11.9, NaH_2PO_4 0.4, glucose 5.5, bubbled with 95% O_2 and 5% CO_2. The K^+ concentration was increased without isotonic compensation to 22 mM to depolarize electrically driven left atria, in order to inactivate the fast sodium channels and to generate responses supported by slow inward Ca^{2+} fluxes.

The atria were dissected, suspended in a 30 ml organ bath and connected to a high sensitive transducer (Basile, type DYO). An initial tension of 1 g was applied to the tissues and changes in isometric tension were recorded by a writing oscillograph (Basile, Unirecord System, mod 7050). The control developed tension ranged from 0.8 to 1.3 mN. Left atria were mounted on punctate electrodes with a load of 0.5 g and stimulated by square wave electrical pulses of 3 ms duration and a voltage 10% to 20% greater than threshold by a Grass stimulator (Mod. 24KR). The control developed tension ranged from 0.09 to 0.20 mN.

An equilibration period of 60 min was allowed before experiments were started. Concentration-response curves were constructed by cumulative addition of Bay K 8644, 5 nM to 200 nM. Responses were allowed to stabilize before the concentration was increased. The effect of Bay K 8644 was slow in onset (20–30 min) and required a prolonged period of washing for reversal (at least 2 h).

PIA 3 to 50 nM was added to the bath and allowed to equilibrate, unless otherwise stated, for 6–8 min before a cumulative Bay K 8644 concentration-response curve was performed.

Cumulative concentration-response curves for the positive inotropic effects of Bay K 8644 were expressed as percentage of the maximum increase over control tension induced by Bay K 8644 at the plateau. The responses of Bay K 8644 in the presence of PIA were related to the maximum effect of Bay K 8644 (100%) in the absence of the inhibitor. Only one concentration of PIA was tested in a single preparation, due to the prolonged period of washing required after exposure to Bay K 8644.

The concentration-response curves for the positive chronotropic effects of Bay K 8644 were expressed as percentage increase from the basal level and not as percentage of the maximum effect, as the effect of Bay K 8644 on the atrial rate was low (+ 30%) and never reached a plateau at the concentrations used.

Reserpinized atria were obtained by treating guinea-pigs with reserpine 2.5 mg kg^{-1} i.p. twice, 48 and 12 h before the experiment.

Drugs and compounds used

Methyl-1, 4-dihydro-2, 6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate, Bay K 8644, was generously supplied by Dr G. Franckowiak from Bayer AG (Wuppertal, FRG). Bay K 8644, 1 mM, was freshly dissolved in absolute ethanol. This stock solution was diluted in appropriate amounts in pre-warmed and pre-aerated bathing solution to achieve the desired final concentration. As the drug was sensitive to light, all experiments were carried out in a dark room using red light.

(−)-N^6-phenylisopropyladenosine (PIA; Boehringer, Mannheim) was dissolved in ethanol to produce a stock solution of 10 mM and subsequently diluted with 50% ethanol-50% bathing solution to achieve the desired concentration. The total volume of ethanol added never exceeded 30 µl in a 30 ml organ bath.

Other drugs used: reserpine (Ciba-Geigy); isoprenaline chloride (Boehringer Ingelheim); noradrenaline

Table 1 Effects of (−)-N^6-phenylisopropyladenosine (PIA) on contractile tension and frequency in spontaneously beating atria from normal and reserpine-treated guinea-pigs

	PIA IC_{50} (nM)	
Group	*Contractile tension*	*Frequency*
Normal	15 ± 2.7	21 ± 3.0
Reserpine-treated	9 ± 0.8	13 ± 1.2
	($P < 0.01$)	($P < 0.01$)

Means ± s.e. mean of 10 preparations are shown.

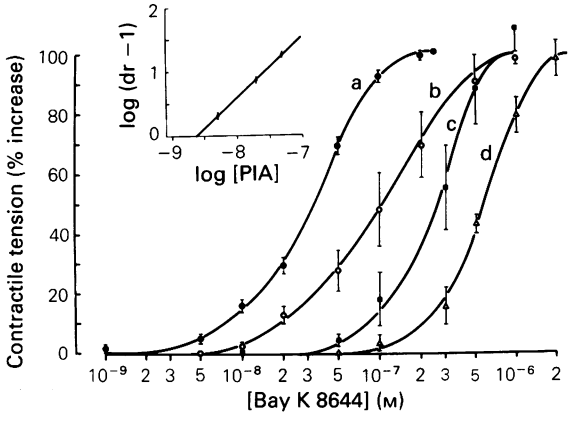

Figure 1 Inhibitory effect of (−)-N^6-phenylisopropyladenosine (PIA) on the positive inotropic effect of Bay K 8644 in normal atria. Cumulative concentration-response curves for Bay K 8644 in the absence (a, ●) (n = 14–21) and in presence of PIA 5×10^{-9}M (b, ○) (n = 7–9), 2×10^{-8}M (c, ■) (n = 4-6), 5×10^{-8}M (d, △) (n = 4–6). The Bay K 8644-induced contraction in the presence of PIA is related to the maximum effect of Bay K 8644 (100%) in the absence of the inhibitor. Each point is the mean and vertical lines indicate s.e. mean. Inset: Schild regression for PIA with Bay K 8644 as the agonist (dr = dose-ratio). The slope of the regression line was 0.92 ± 0.02 (r = 0.99), and the apparent pA_2 value was 8.63 ± 0.05.

bitartrate (Sigma); carbamylcholine chloride (carbachol, Sigma).

Analysis of results

Values presented are means ± s.e. mean. Statistical differences between mean values were analysed by use of Student's t test.

The Schild plot (Arunlakshana & Schild, 1959) was constructed by the calculated regression of log (dose-ratio − 1) on −log (concentration of PIA). Dose-ratios were calculated by using the points of the concentration-response curve from 20% to 80% level. The pA_2 extrapolated from the Schild plot gave the 'empirical' K_i of the antagonist PIA. The K_i thus obtained was compared with the IC_{50} calculated by the equation $K_i = IC_{50}/(1 + [C]/K_a)$.

Results

Effects of PIA on the basal contractile tension and frequency

PIA, the R-site adenosine receptor agonist produced a concentration-dependent decrease in contractile tension and frequency in spontaneously beating atria at concentrations 3–100 nM. The IC_{50} values for PIA, shown in Table 1, indicate that PIA was slightly but significantly more effective in atria from reserpine-treated animals. The maximal inhibition of both atrial parameters was 95 ± 3% at 100 nM PIA. This is in accordance with data of Evans *et al.*, (1982).

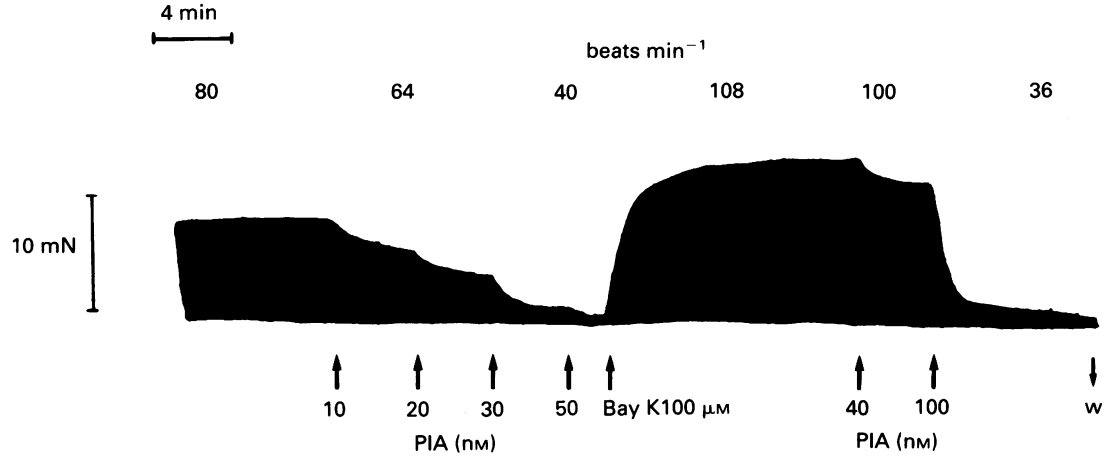

Figure 2 Depressant effects of (−)-N^6-phenylisopropyladenosine (PIA) on the contractile tension and frequency in spontaneously-beating atria from the guinea-pig. The effects of PIA were reversed by Bay K 8644. PIA was much less effective in the presence of Bay K 8644. Increasing concentrations of PIA were added cumulatively.

Effects of PIA on the positive inotropic actions of Bay K 8644 in different atrial preparations

In spontaneously beating atria, taken from normal guinea-pigs, Bay K 8644 produced a concentration-dependent increase in contractile tension (Figure 1). The positive inotropic effect was evident at 5 nM and reached a plateau at 100 nM, in accordance with data of Schramm *et al.*, (1983a) in guinea-pig isolated perfused hearts. The EC_{50} was 33 ± 3 nM. The maximum increase was 159 ± 14% over control level. The effect of Bay K 8644 was slow in onset and reached equilibrium within 10–30 min.

The effect of various concentrations of PIA on the dose-response curves for the positive inotropic effect of Bay K 8644 are shown in Figure 1. The three concentrations of PIA used, 5 nM, 20 nM and 50 nM, reduced the control tension by 15 ± 2% (n = 10), 55 ± 6% (n = 10) and 85 ± 7% (n = 5), respectively. PIA inhibited the positive inotropic effect of Bay K 8644 in a concentration-dependent manner. At all concentrations of PIA, the antagonism was fully surmountable by increasing the Bay K 8644 concentration to 1000 nM or more. Hence, PIA produced parallel rightward shifts in the Bay K 8644 concentration-response curves, indicative of a possible competitive antagonism. The IC_{50} of PIA was 2 ± 0.4 nM.

The Schild regression plot (Figure 1) was linear with a slope of 0.92 ± 0.02 (r = 0.99); 99% confidence limits = 0.88–0.96. The pA_2 value of the antagonism of Bay K 8644 by PIA was determined to be 8.63 ± 0.05 and the K_i 2.35 ± 0.25 nM, corresponding to the IC_{50} calculated according to the equation $K_i = IC_{50}/(1 + [C]/K_a)$. The negative effects of PIA on guinea-pig isolated atria were also antagonized by Bay K 8644 (Figure 2).

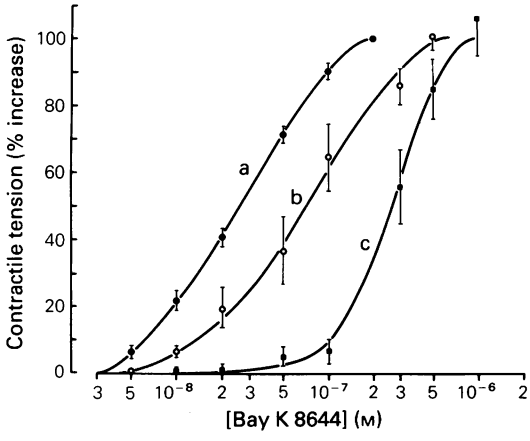

Figure 3 Inhibition by (−)-N⁶-phenylisopropyladenosine (PIA) of the positive inotropic effect of Bay K 8644 on spontaneously beating atria from reserpine-treated guinea-pigs. Cumulative concentration-response curves for Bay K 8644 in the absence (a, ●) (n = 14) and presence of PIA 4 × 10^{-9}M (b, ○) (n = 5–7) and 10^{-8}M (c, ■) (n = 5–6). Responses are expressed as in Figure 1. Each point is the mean and vertical lines show s.e. mean.

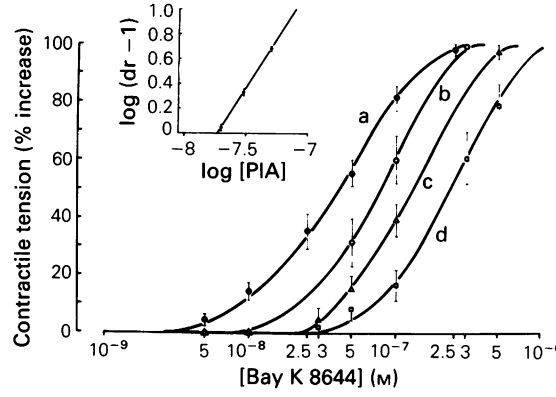

Figure 4 Inhibition by (−)-N⁶-phenylisopropyladenosine (PIA) of the positive inotropic effect of Bay K 8644 on electrically driven left atria. Cumulative concentration-response curves for Bay K 8644 in the absence (a, ●) (n = 8–11) and in the presence of PIA 2 × 10^{-8}M (b, ○) (n = 4–5); 3 × 10^{-8}M (c, △) (n = 4) and 5 × 10^{-8}M (d, □) (n = 8–9). Responses expressed as in Figure 1. Each point is the mean and vertical lines show s.e. mean. Inset: Schild regression for PIA with Bay K 8644 as the agonist (dr = dose-ratio). The slope of the regression line was 1.58 ± 0.02 (r = 0.99) and apparent pA_2 value 7.73 ± 0.02.

In spontaneously beating atria from reserpine-treated guinea-pigs, the positive inotropic effect of Bay K 8644 was very similar to that in atria from untreated animals (Figure 3). Here too, increasing concentrations of PIA produced a parallel shift to the right of the Bay K 8644 concentration-response curve. The IC_{50} of PIA was 1.5 ± 0.4 nM, not significantly different ($P > 0.20$) from that in untreated atria.

In electrically driven left atria, we eliminated the frequency parameter, that was strongly affected by PIA (Table 1). The positive inotropic effect of Bay K 8644 proportionally increased with the rate of stimulation (0.5 to 3 Hz). Thus we used 1.5 Hz as a fixed rate of stimulation.

The positive inotropic effect of Bay K 8644 was similar to that previously shown (Figure 4). PIA inhibited the positive inotropic effect of Bay K 8644 in a concentration-dependent manner and shifted the concentration-response curve of Bay K 8644 to the right. But, in these experimental conditions, the IC_{50} of PIA was 8.5 ± 0.2 nM, about 4 times higher than in spontaneously beating atria.

Table 2 pA_2 for (−)-N^6-phenylisopropyladenosine (PIA) as an antagonist of the positive inotropic effect of Bay K 8644 in guinea-pig atria

		Developed tension	
	Slope[1]	*pA_2*	*IC_{50}* (nM)
Spontaneously beating	0.92 ± 0.02	8.63 ± 0.05	2.35 ± 0.25
Electrically driven	1.58 ± 0.02*	7.73 ± 0.02*	18.60 ± 0.40*

*$P < 0.01$. [1]Slope of log (dose-ratio − 1) against log molar concentration of the antagonist, PIA.

The Schild regression plot (Figure 4) was linear with a slope of 1.58 ± 0.02 ($r = 0.99$); 99% confidence limits 1.54–1.64. The apparent pA_2 value, extrapolated from the Schild regression plot, was 7.73 ± 0.02 and the K_i 18.6 ± 0.4 nM (Table 2), higher than the IC_{50} calculated according to the equation $K_i = IC_{50}/(1 + [C]/K_a)$. This behaviour of the Schild regression may indicate a temporal or a thermodynamic disequilibrium. The diffusion, or drug-receptor interaction, may be rate-limiting (Kenakin, 1985). However, the slope was not modified by changing the incubation time (5 to 20 min) of PIA with the left atria (not shown).

Effect of PIA on the positive chronotropic effect of Bay K 8644.

The interaction between PIA and Bay K 8644 on the atrial frequency was difficult to investigate as the positive chronotropic effect of Bay K 8644 was very low and variable. The frequency increased over the control level of 14 ± 2% in normal and of 31 ± 4% in reserpinized atria but never reached a maximum at the concentrations used. Consequently, concentration-response curves for the chronotropic effect of Bay K 8644 in the absence and presence of PIA were incomplete and difficult to analyse. The positive chronotropic effect of Bay K 8644 was apparently antagonized by PIA, but the type of antagonism was difficult to define.

Lack of interaction between carbachol and Bay K 8644

The specificity of the antagonism of Bay K 8644 by PIA was tested by using carbachol which, like PIA, has negative inotropic and chronotropic effects on atrial preparations.

Carbachol was tested at concentrations of 7 nM, 10 nM and 20 nM, which reduced the atrial rate and contractility by 15%, 30% and 60% respectively. Carbachol did not modify the positive inotropic action of Bay K 8644 (Table 3), nor the chronotropic effect (not reported).

Effect of PIA in depolarized atria

We studied the effects of PIA on Ca^{2+}-dependent contraction in potassium-depolarized electrically stimulated left atria (Figure 5). PIA was able to counteract the contractile responses generated in these experimental conditions by the inward slow calcium current.

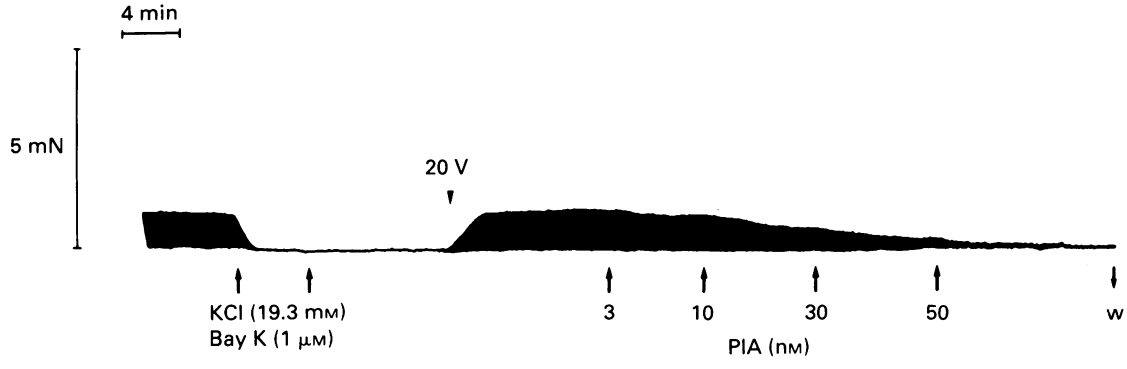

Figure 5 The effects of (−)-N^6-phenylisopropyladenosine (PIA) on the contractions induced in the potassium-depolarized, electrically-driven (1.5 Hz, 3 ms, 0.6 V) left atria of the guinea-pig. The medium contained a final K^+ concentration of 22 mM in order to inactivate the fast sodium channels (KCl 19.3 mM was added to obtain a final concentration of 22 mM). PIA was added cumulatively and the final concentrations in the medium are indicated.

Table 3 Lack of an interaction between carbachol and Bay K 8644

Carbachol	n	*Bay K 8644* 5 nM	10 nM	50 nM	100 nM	500 nM
—	12	8.3 ± 2.6	23.1 ± 4.1	74.9 ± 5.8	93.7 ± 3.5	97.9 ± 2.1
7 nM (IC_{15})	2	14	31	75	100	—
10 nM (IC_{30})	5	14.2 ± 5.7	29.4 ± 9.0	72.7 ± 6.5	93.2 ± 4.9	99.4 ± 0.6
20 nM (IC_{60})	5	18.6 ± 5.4	26.7 ± 5.2	76.8 ± 13.4	88.8 ± 8.0	100 ± 0

Carbachol was introduced in the medium 10 min before Bay K 8644. Data are expressed as percentage of the maximum developed tension to Bay K 8644. Mean ± s.e. mean of the number of preparations indicated (*n*) are shown.

Discussion

PIA, a stable analogue of adenosine, inhibits at nanomolar concentrations the positive inotropic effect of Bay K 8644, a calcium channel facilitator, in guinea-pig isolated atria. The antagonism is evident both in spontaneously beating preparations, taken from normal and from reserpine-treated animals, as well as in electrically driven left atria.

The antagonism by PIA of the effects of Bay K 8644 is specific as carbachol had no effect on the response to Bay K 8644. Cholinoceptor agonists are known (Belardinelli & Isenberg, 1983) to increase conductance of the membrane to K^+ in atrial myocytes, with a consequent shortening of the action potential and inhibition of the slow calcium current. The lack of interaction between carbachol and Bay K 8644 indicates that PIA acts by a mechanism different from the cholinoceptor agonists. It is also known (Schramm *et al.*, 1983a; Ishii *et al.*, 1985) that the positive inotropic effect of Bay K 8644 is neither the result of α- or ß-adrenoceptor stimulation nor of an interaction with dopamine, 5-hydroxytryptamine, histamine or opiate receptors.

In spontaneously beating atria, the antagonism of Bay K 8644 by PIA was apparently competitive. The IC_{50} of PIA was 2.3 ± 0.2 nM. The Schild plot for PIA against Bay K 8644 was linear with a slope near to unity, which suggests simple competition between an agonist and an antagonist with a homogeneous receptor population (Kenakin, 1985). These results indicate that PIA and Bay K 8644 may compete for a common receptor site in or near the slow calcium channel. Alternatively, the observed antagonism may be the result of an allosteric competition between PIA and Bay K 8644, i.e, the two drugs may interact with topologically distinct sites, but the binding of one drug induces a conformational change that prevents the binding of the other drug.

In electrically driven atria, PIA also produced parallel rightward shifts in the Bay K 8644 concentration-response curves; but the IC_{50} of PIA was 10 times higher than in spontaneously beating atria (18.6 ± 0.4 nM) and the Schild regression had a slope higher than unity (1.58 ± 0.02). This behaviour may indicate a temporal or a thermodynamic disequilibrium (Kenakin, 1985). The drug-receptor interaction may be rate-limiting, as the slope was not modified by varying the time (5 to 20 min) of equilibration of PIA with left atria.

Bay K 8644 apparently binds to the same binding site as nifedipine (Schramm *et al.*, 1983a, b; Thomas *et al.*, 1984; Ishii *et al.*, 1985) but these receptor sites can exist in low- and high-affinity states (Glossmann *et al.*, 1984). Further, the channel can exist in different conformations through which it can cycle in the intact cell (open, closed, inactivated) (Mestre *et al.*, 1985). The channel blocking drugs often do not interact with closed, or resting, channels. The functional kinetics of channel blocking drugs are thus difficult to define (Spedding, 1985). Recent findings (Thomas *et al.*, 1986) with Bay K 8644 in voltage-clamp experiments showed that Bay K 8644 binds only to the open state of the calcium channel preventing its closing as long as the compound is bound.

Adenosine (Belardinelli & Isenberg, 1983) and PIA (Böhm *et al.*, 1985) shorten the normal action potential in atrial preparations and hence reduce the entry of Ca^{2+} into the cell. Adenosine was also found to produce a dose-dependent slowing of the rate of sinus node cells, mainly by decreasing the rate of diastolic depolarization (West & Berlardinelli, 1985). This may decrease the probability for Bay K 8644 finding calcium channels in the open state, which is the only state responsive to Bay K 8644. In electrically driven atria, where the rate of depolarization is constant, and not decreased by the presence of PIA, the probability that Bay K 8644 binds to the open channel and develops its facilitator effect is increased. In these experimental conditions, the only action component of PIA is the one shortening action potential. This allows a simple explanation for the lower IC_{50} of PIA in electrically driven atria compared with the IC_{50} in spontaneously beating atria, and could also provide an explanation for the disequilibrium in drug-receptor interaction suggested by a slope higher than unity of

the Schild regression in electrically driven atria.

Although the present data do not allow us to choose between the various molecular mechanisms involved in the PIA-Bay K 8644 interaction, they indicate that this interaction takes place at the level of slow calcium channels.

Further evidence is obtained from the inhibitory effect of PIA on the contractile response generated by electrical stimulation in K^+-depolarized atria. This conclusion supports the hypothesis that adenosine acts in atria by a mechanism independent of cyclic AMP and indicates that the effector of this mechanism is the slow calcium channel. It is also substantiated by results showing a direct action of 2-chloroadenosine on the voltage-activated calcium currents in cultured rat dorsal root ganglia neurones (Dolphin & Scott, 1986).

A general consideration suggested by our data is whether an analogous situation may also occur *in vivo*. If so, adenosine might be considered as a physiological endogenous factor modulating slow calcium channel function.

In summary, the results presented here suggest that in guinea-pig atria PIA is able to interact at a site in or near the slow calcium channels, and that occupation of these sites by PIA may decrease the transmembrane calcium flux into the cell. This supports the hypothesis of an involvement of slow Ca^{2+} channels in the 'direct' action of adenosine, independent of cyclic AMP. If so, adenosine has to be considered as a putative endogenous factor modulating Ca^{2+} channel function *in vivo*.

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A facilitatory effect of bicuculline on the enteric neurones in the guinea-pig isolated colon

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1 Changes in the efficiency of the peristaltic reflex, acetylcholine (ACh) output and motor responses to transmural and periarterial nerve stimulation produced by bicuculline and γ-aminobutyric acid (GABA) receptor desensitization were investigated in the guinea-pig isolated colon.

2 Bicuculline, at concentrations unable to affect spontaneous colonic motility and lacking anticholinesterase activity, produced a dose-dependent increase of both the efficiency of the peristaltic reflex and the stimulated ACh output. Such effects could not be observed in GABA-desensitized preparations.

3 A frequency-dependent potentiation of the cholinergic excitatory and non-adrenergic non-cholinergic (NANC) inhibitory responses to transmural stimulation was also observed in the presence of bicuculline. Conversely bicuculline exhibited an inhibitory effect on the relaxation induced by periarterial nerve stimulation.

4 Acute GABA-desensitization was unable to affect the contractile responses to transmural stimulation, the ACh output and the efficiency of the peristaltic reflex. On the contrary, desensitization was able to mimic the effects of bicuculline on the inhibitory responses to both transmural and periarterial nerve stimulation.

5 Our results are consistent with a significant role played by an intrinsic GABAergic pathway in the modulation of both cholinergic excitatory and NANC inhibitory neurones. The hypothesis is advanced that a feed-back modulation carried out through bicuculline-sensitive GABAergic synapses could operate during the propagation of peristaltic motor activity.

Introduction

Since high affinity uptake of γ-aminobutyric acid (GABA) by enteric neurones has been demonstrated by Jessen *et al.* (1979), evidence has accumulated that GABA may serve as a transmitter in the enteric nervous system. Besides being widely distributed along the intestine together with its synthesizing and metabolic enzymes (Miki *et al.*, 1983; Krantis & Harding, 1986) and strongly taken up by intrinsic neurones (Jessen *et al.*, 1983), GABA has been shown to be released during depolarization of enteric neurones (Taniyama *et al.*, 1983a). Moreover enteric neurones have been shown to be sensitive to GABA receptor stimulation (Krantis *et al.*, 1980; Giotti *et al.*, 1983; Cherubini & North, 1984). However, the involvement of the postulated GABAergic pathway in the neurogenic control of intestinal motor function is still a matter of speculation. In particular little is known about the participation of the putative GABAergic neurones in the multineuronal pathways which subserve the intestinal intrinsic reflexes. All the hypotheses on the possible functional role of endogenous GABA are still mainly based on the analogy to the pharmacological effects of exogenous GABA (Giotti *et al.*, 1985). Little attention has been devoted to the study of the functional effects of GABA receptor blockade which has so far yielded conflicting results (Krantis *et al.*, 1980; Krantis & Kerr, 1981a; Ong & Kerr, 1983).

In the present paper the effects of the GABA receptor antagonist, bicuculline, and of the desensitization to exogenous GABA on the efficiency of the peristaltic reflex, acetylcholine output and nerve mediated responses of the longitudinal muscle have been investigated in the guinea-pig distal colon. In

addition, the ability of the motor enhancing concentrations of bicuculline to exhibit anticholinesterase activity was also tested.

Methods

Experiments were carried out in the guinea-pig isolated distal colon. The models used to measure the nerve-mediated motor activities were the peristaltic reflex and the responses of the longitudinal muscle to nerve stimulation. Moreover, the activity of cholinergic neurones was investigated by measuring the stimulated acetylcholine (ACh) output. Isolated specimens of terminal colon 5–7 cm long were taken from male guinea-pigs weighing 300–400 g, mounted in organ baths perfused with Tyrode solution and loaded with 1–1.5 g through an isotonic force transducer.

The peristaltic reflex was elicited by a radial distension of the lumen applied at the proximal end of the specimens by means of an intraluminal balloon. Longitudinal muscle movements and displacement of the balloon towards the distal end of the colon together with the surface electrical activity were recorded. Since the velocity of propulsion is dependent on the degree of distension (Frigo & Lecchini, 1970), supramaximal stimulation was employed and the maximal velocity of propulsion taken as a measure of the efficiency of the peristaltic reflex. The improvement or the inhibition of peristalsis was indicated as the percentage variation of the propulsion velocity with respect to the control value and was estimated as described by Frigo *et al.* (1984). Electrical activity was recorded from the serosal surface by means of two extracellular glass electrodes (Frigo *et al.*, 1972; Tonini *et al.*, 1974).

ACh release was measured by incubating the specimens in a 3 ml organ bath with physostigmine sulphate (1.5×10^{-5} M) during 20 min collection periods, and by applying rectangular pulses of supramaximal strength, 1 ms duration and 1 Hz frequency delivered by coaxial silver wire electrodes for the first 10 min in a way similar to that described by Frigo *et al.* (1984). The 1 Hz frequency of stimulation was chosen because of its relative selectivity in stimulating the enteric cholinergic neurones (Cowie *et al.*, 1978). In order to measure ACh release from GABA-desensitized preparations, a preincubation period of 20 min with GABA (1×10^{-4} M) was allowed before starting the collection of the ACh and GABA (1×10^{-4} M) was then present throughout the experiment. ACh was assayed on the guinea-pig isolated ileum incubated with morphine sulphate (6.6×10^{-6} M) and physostigmine sulphate (7.7×10^{-9} M) in a way similar to that described by Paton & Vizi (1969). To allow for any effects that modifying drugs might have on the ACh assay, the concentration of such drugs in the test samples were duplicated in the standard ACh solutions during the assay. The effects of drugs on the ACh release were expressed as percentage variations in respect to the control level.

Field-stimulation was used to stimulate intrinsic nerves by delivering trains of rectangular pulses of supramaximal strength, 0.5 ms duration and frequency ranging from 0.2 to 20 Hz lasting 20 s, through coaxial silver wire electrodes. The non-adrenergic, non-cholinergic (NANC) component of the response to the intrinsic nerve stimulation was revealed by applying transmural stimulation to specimens pretreated with hyoscine sulphate (3×10^{-6} M) and guanethidine sulphate (3×10^{-6} M). The sympathetic supply to the colon was stimulated by applying trains of rectangular pulses of supramaximal strength, 0.1 ms duration and frequency ranging from 0.2 to 20 Hz lasting 20 s, by means of bipolar silver wire electrodes placed around the periarterial plexus of the inferior mesenteric artery. Electrical stimulation was applied at 10 min intervals and either excitatory or inhibitory responses of the longitudinal muscle are given as percentage of the maximal responses. The maximal response was considered the highest response independent of the frequency employed. Frequency-response relationships for both excitatory and inhibitory responses to transmural nerve stimulation were obtained over the range of 0.2–8 and 0.2–3 Hz, respectively. The range of frequencies used to construct frequency-response relationships for periarterial nerve stimulation was 0.5–10 Hz. For all kinds of stimulations, the frequency able to produce half maximum effect (F_{50}) was calculated.

The ability of bicuculline to modify the efficiency of the peristaltic reflex, the ACh output and the longitudinal muscle responses to nerve stimulation was investigated both in naïve and in GABA-desensitized preparations. The effects of bicuculline were evaluated after a preincubation period of at least 20 min for each concentration of the drug. Desensitization to exogenous GABA, unless otherwise indicated, was achieved by cumulative administrations of GABA (1×10^{-4} M) at 10 min intervals (Krantis *et al.*, 1980) and assessed by the lack of any relaxation of the longitudinal muscle in response to the drug. In separate experiments, the ability of acute GABA-desensitization to modify the efficiency of the peristaltic reflex, ACh output and the longitudinal muscle responses to nerve stimulation was investigated.

The potential anticholinesterase activity of bicuculline was assayed on *Electrophorus electricus* purified acetylcholinesterase (AChE) and on horse serum butyrylcholinesterase (BuChE). The Michaelis-Menten constants for substrate transformation (K_m), the concentrations responsible for 50% cholinesterase

inhibition (IC_{50}), the equilibrium dissociation constants for drug-enzyme combination (K_i), or that for the interaction with enzyme-substrate complex K_i were calculated as previously described by Galli *et al.* (1984). The time-dependency of the drug-enzyme interaction and the reversibility of the drug's anticholinesterase activity were also evaluated. IC_{50} values were calculated from semilogarithmic plots of drug concentration against percentage inhibition of the enzyme activity. The dissociation constants were calculated from double reciprocal plots of rate against substrate concentration according to Dixon & Webb (1979).

The Tyrode solution was of the following composition (mM): NaCl 136.9, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1.04, $NaHCO_3$ 11.9, NaH_2PO_4 0.4 and glucose 5.5.

The drugs used were: acetylcholine chloride (ACh, Sigma Chemical); acetylcholinesterase from *Electrophorus electricus* (AChE, Boehringer); acetylthiocholine iodide (ATCh, Boehringer); hyoscine hydrochloride (Sigma Chemical); bicuculline methiodide (Sigma Chemical); butyrylcholinesterase from horse serum (BuChE, Sigma Chemical); butyrylthiocholine iodide (BuTCh, Boehringer); 5.5'-dithiobis-(2-nitrobenzoic acid) (DTNB, Boehringer); γ-aminobutyric acid (GABA, Sigma Chemical); guanethidine sulphate (Ciba); morphine sulphate (Carlo Erba); physostigmine sulphate (BDH), tetrodotoxin (TTX, Sankyo).

Linearization of the frequency or concentration-response relationships was obtained by log transformation and the least squares method in order to calculate the frequencies and the concentrations able to produce half maximum effect. Statistical significance of differences between groups was analysed by applying Student's *t* test for unpaired data.

Results

Effect of desensitization to exogenous GABA

The efficiency of the peristaltic reflex in 9 out of 12 preparations was not significantly modified by desensitization achieved by cumulative administration of GABA (1×10^{-4} M). In fact the mean velocity of propulsion (± s.e.mean) was similar before (2.18 ± 0.21 mm s^{-1}) and after GABA-desensitization (2.34 ± 0.19 mm s^{-1}). In the remaining three preparations after addition of GABA the peristaltic reflex could no longer be elicited. Either the frequency and amplitude of spikes and spontaneous contractions or the resting tone of longitudinal muscle were similar before and after GABA desensitization.

Similarly, desensitization to exogenous GABA did not affect the release of ACh. The mean ACh output (± s.e.mean, $n = 8$) before and after GABA desensitization was 56.50 ± 12.11 ng g^{-1} min^{-1} and 54.67 ± 10.40 ng g^{-1} min^{-1}, respectively.

The effect of GABA desensitization was tested on the hyoscine- and TTX-sensitive excitatory responses of longitudinal muscle to transmural stimulation and on the TTX-sensitive inhibitory responses to transmural or periarterial nerve stimulation. The ability of GABA-desensitization to modify the nerve-mediated responses varied according to the kind of stimulus applied. The frequency-response relationships before and after GABA-desensitization are shown in Figure 1. The excitatory responses to transmural nerve

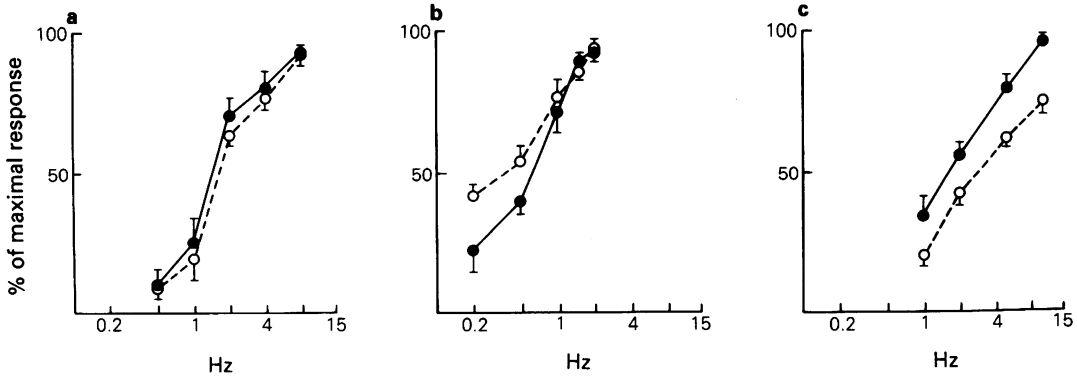

Figure 1 Effect of desensitization to exogenous GABA on the guinea-pig colon longitudinal muscle responses to nerve stimulation. Log frequency-response relationships for excitatory (a) and NANC inhibitory (b) responses to transmural stimulation and for inhibitory responses to periarterial nerve stimulation (c) before (●) and after GABA-desensitization (○). Frequency of stimulation is plotted against percentage of the maximal response. Each point represents the mean of 5 experiments. Vertical bars indicate s.e.mean.

stimulation were not modified by acute GABA desensitization at all the frequencies tested, the F_{50} values with 95% confidence limits before and after GABA desensitization being 1.68 (0.94–2.98) and 1.87 (1.12–3.10) Hz, respectively. The effect of desensitization on the NANC inhibitory responses was frequency-dependent, inhibitory responses being significantly increased only at the lower frequencies (0.2–0.5 Hz). The F_{50} values with 95% confidence limits before and after GABA desensitization were 0.41 (0.27–0.62) and 0.31 (0.17–0.59) Hz, respectively. The inhibitory responses to periarterial nerve stimulation were reduced by GABA desensitization, the F_{50} values with 95% confidence limits before and after GABA desensitization being 1.71 (1.40–2.07) and 3.13 (2.07–4.32) Hz, respectively.

Effect of bicuculline in naïve and in GABA-desensitized preparations

Peristaltic reflex The mean velocity of propulsion (± s.e.mean, $n = 8$) in naïve and in GABA-desensitized preparations was $2.03 \pm 0.19\,mm\,s^{-1}$ and $2.14 \pm 0.23\,mm\,s^{-1}$, respectively. Bicuculline at concentrations of 2×10^{-6} M, 5×10^{-6} M and 1×10^{-5} M was able to increase significantly the velocity of propulsion in naïve but not in GABA-desensitized preparations. The effect of bicuculline was concentration-dependent, the log concentration-response relationship being shown in Figure 2. The maximum average percentage increase (± s.e.mean, $n = 6$) of the velocity of propulsion was 37.14 ± 6.5 and was observed at the concentration of 1×10^{-5} M. The concentration with 95% confidence limits producing half maximum effect in increasing the velocity of propulsion was 2.21 $(1.12–4.38) \times 10^{-6}$ M. At the concentrations able to increase the efficiency of the peristaltic reflex, bicuculline did not affect either the frequency and amplitude of spikes and spontaneous contractions or the resting tone of longitudinal muscle (Figure 3). Similar concentrations were ineffective also in GABA-desensitized preparations. Bicuculline 1×10^{-4} M was able to prevent the onset of the peristaltic reflex both in naïve and in GABA-desensitized preparations.

Acetylcholine output The mean ACh output (± s.e.mean, $n = 8$) in naïve and in GABA-desensitized organs was 49.03 ± 7.20 and 53.33 ± 10.18 $ng\,g^{-1}\,min^{-1}$, respectively. Bicuculline at concentrations of 5×10^{-6} M and 1×10^{-5} M was able to increase significantly the ACh output in naïve but not in GABA-desensitized organs. The effect of bicuculline was concentration-dependent, the log concentration-response relationship being shown in Figure 2. The maximum average percentage increase (± s.e.mean, $n = 6$) of the ACh output was 19.71 ± 2.65 and was observed at a concentration of 1×10^{-5} M.

Nerve mediated excitation and inhibition of longitudinal muscle The effect of bicuculline on the frequency-

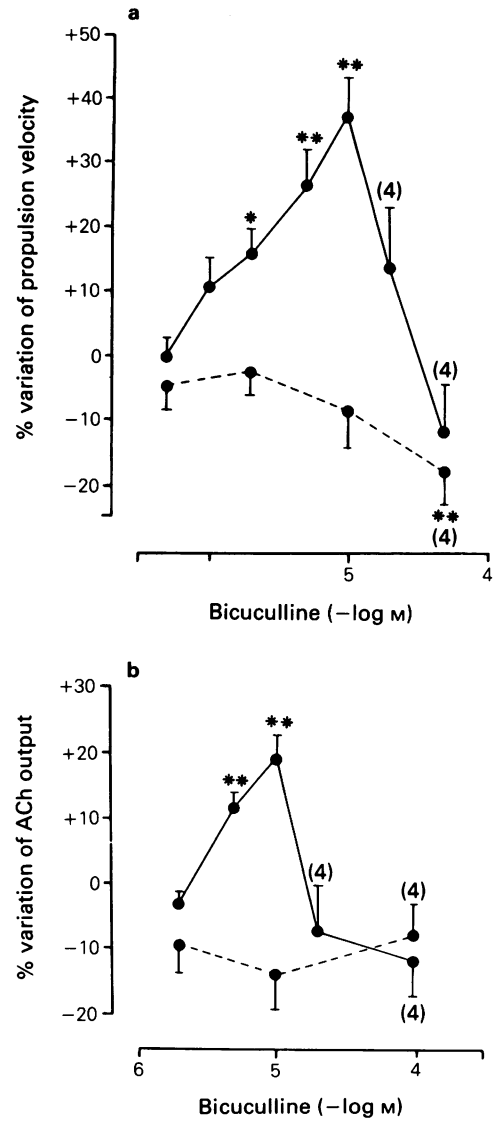

Figure 2 Effect of bicuculline on the efficiency of the peristaltic reflex and on the acetylcholine output in naïve (●—●) and in GABA-desensitized preparations (●--●) of guinea-pig colon. Log concentrations are plotted against percentage variation of velocity of propulsion (a) and of stimulation-induced acetylcholine (ACh) output (b). Unless otherwise indicated, each point represents the mean of 6 experiments. Vertical bars show s.e.mean. The asterisks indicate significant difference from the control value: ($*P < 0.05$; $**P < 0.01$).

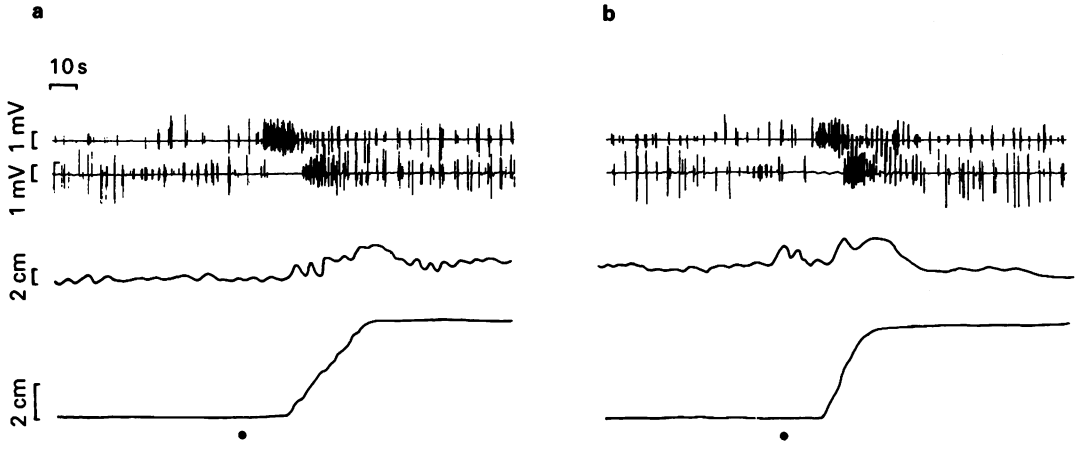

Figure 3 Effect of bicuculline on the distension-induced peristaltic reflex in the guinea-pig isolated colon. In each panel, from top to bottom: electrical activity recorded from surface electrodes placed on the oral (upper tracing) and the aboral (lower tracing) side in respect to the site of stimulation; longitudinal movements; oral-aboral displacement of the bolus. The mark (●) indicates distension of the intraluminal balloon. In (b) the reflex was elicited after 20 min incubation with bicuculline 1×10^{-5} M. Note the higher velocity of the propulsion as deduced from the steeper slope of the lower tracing in the presence of bicuculline.

response relationships of longitudinal muscle to transmural and periarterial nerve stimulation both in naïve and in GABA-desensitized preparations is shown in Figures 4 and 5. The ability of bicuculline to shift frequency-response relationships was tested at the concentration able to produce the maximum increase in both the propulsion velocity and the ACh output (1×10^{-5} M). Such a concentration was devoid of any effect on the longitudinal muscle resting tone. A potentiating effect of bicuculline 1×10^{-5} M on the excitatory responses of the longitudinal muscle to transmural stimulation was observed at the lower frequencies (0.2–2 Hz). Frequency-response relationship curve was shifted to the left, the F_{50} values with 95% confidence limits being 1.95 (1.17–3.24) Hz before and 0.84 (0.47–1.50) Hz after bicuculline administration. Bicuculline was able to increase significantly the NANC inhibitory responses at the lower frequencies (0.2–1.5 Hz), the F_{50} values with 95% confidence limits before and after bicuculline administration being 1.40 (0.49–3.94) and 0.29 (0.09–0.95) Hz, respectively. The inhibitory effect of periarterial nerve stimulation was reduced by bicuculline. The frequency-response relationship was shifted to the right, the F_{50} values with 95% confidence limits in the presence and in the absence of bicuculline being 1.04 (0.42–2.54) and 3.24 (2.50–4.21) Hz, respectively. No effect of bicuculline on either transmural or periarterial nerve stimulation could be observed in GABA-desensitized preparations.

Effect of bicuculline on cholinesterase activity

Bicuculline inhibited both electric eel AChE and horse serum BuChE in a mixed, competitive and non-competitive, manner (Figure 6). The slope inhibition constants, K_i, and the intercept inhibition constants, $K_{i'}$, are given in Table 1. The competitive nature of the inhibition, as can be deduced by comparing K_i and $K_{i'}$ values, was slightly higher for the bicuculline effect on AChE than on BuChE. Bicuculline was significantly more effective in inhibiting AChE than BuChE, the mean IC_{50} values (± s.e.mean, $n = 4$) being 130 ± 18 μM and 200 ± 21 μM, respectively. In the same conditions, physostigmine exhibited IC_{50} values as low as 0.02 and 0.06 μM on eel AChE and horse serum BuChE, respectively, proving to be about 3000–9500 times more potent than bicuculline. In the experiments carried out to evaluate the time-inhibition relationship, the mean AChE inhibition (± s.e.mean, $n = 4$) produced by 0.1 mM bicuculline (42 ± 2%) was not modified by increasing the preincubation period from 0 to 30 min. In the experiments carried out to evaluate the reversibility of the bicuculline-induced AChE inhibition, rapid dilution of bicuculline concentration from 0.3 mM to 3 μM was able to decrease the mean AChE inhibition (± s.e.mean, $n = 4$) from 69 ± 2 to 3 ± 0.4%. No further enzyme recovery was observed by allowing up to 30 min time periods to elapse before performing the assay.

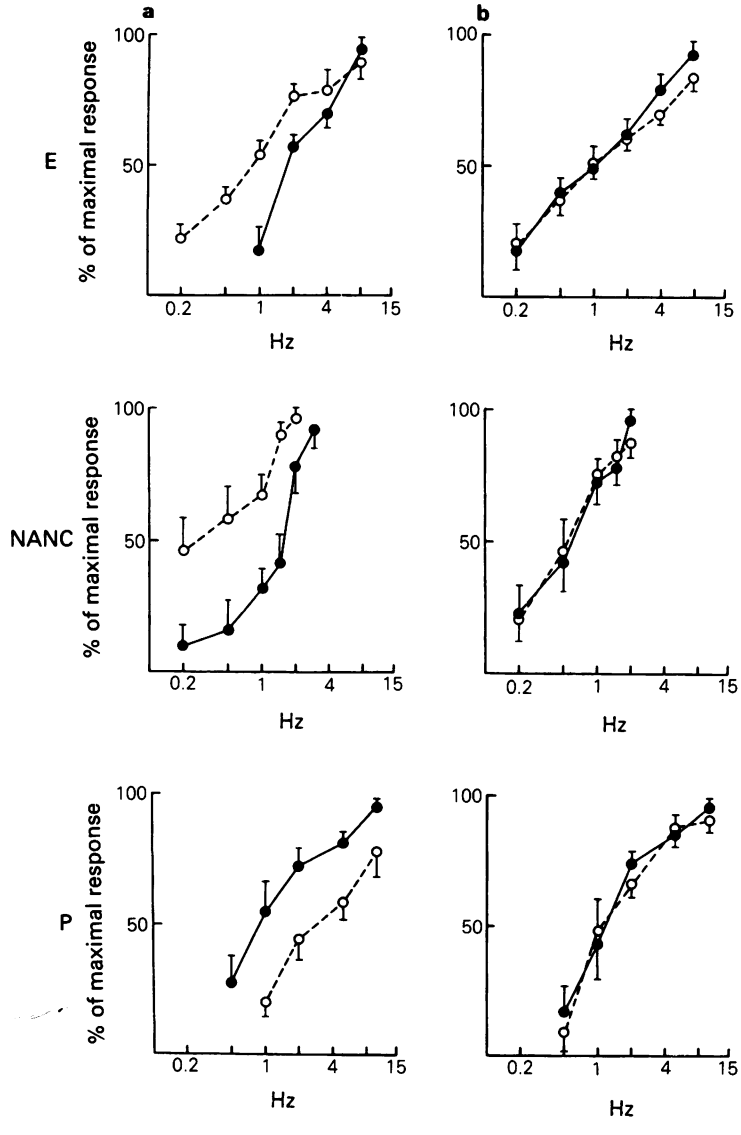

Figure 4 Effect of bicuculline on the longitudinal muscle responses to nerve stimulation in naïve and in GABA-desensitized preparations of guinea-pig isolated colon. The lines indicate log frequency-response relationships in naïve (a) and in GABA-desensitized preparations (b) for the excitatory (E) and NANC inhibitory (NANC) responses to transmural stimulation and for the inhibitory responses to periarterial nerve stimulation (P) before (●) and after (○) adding bicuculline 1×10^{-5} M. Frequency of stimulation is plotted against percentage of the maximal response. Each point represents the mean of 5 experiments. Vertical bars indicate s.e.mean.

Discussion

Measurement of the functional effects of GABA receptor blockade by competitive antagonists is considered to be a reliable pharmacological tool to assess the role of GABAergic neurotransmission in the CNS (Enna, 1983). Bicuculline, although not completely devoid of non-specific effects, is still commonly employed as the reference drug for competitive antagonism on GABA receptors (Curtis *et al.*, 1971;

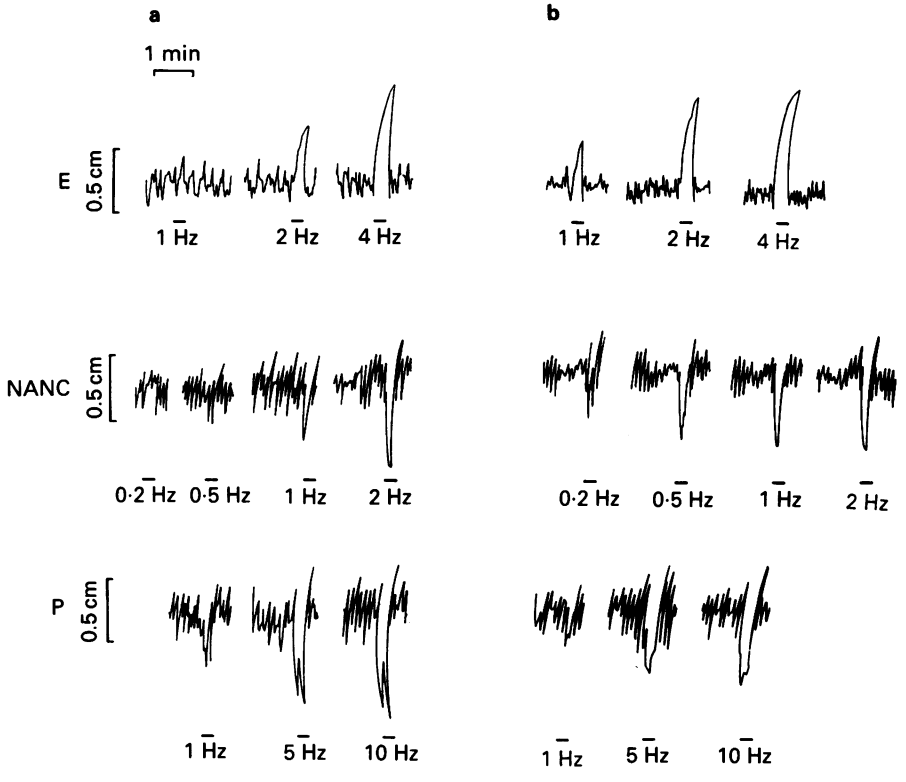

Figure 5 Effect of bicuculline on the guinea-pig colon longitudinal muscle responses to nerve stimulation. In the upper tracing (E), trains of pulses (supramaximal strength, 0.5 ms duration) at increasing frequencies delivered through coaxial electrodes were applied for 20 s periods. In the middle tracing (NANC), transmural stimulation was applied after pretreatment which hyoscine hydrochloride (3×10^{-6} M) and guanethidine sulphate (3×10^{-6} M). In the lower tracing (P), trains of pulses (supramaximal strength, 0.1 ms duration) delivered through bipolar electrodes around the periarterial plexus were applied. Between (a) and (b) bicuculline 1×10^{-5} M was added to the bath. Note that both excitatory and inhibitory responses to transmural stimulation were increased while responses to periarterial nerve stimulation were decreased in the presence of bicuculline.

Perez *et al.*, 1985). Indeed, the facilitatory effects of bicuculline on neuronal firing or neurotransmitter release has been interpreted as evidence of the involvement of GABA as a transmitter in the tonic modulation of CNS activity (Willette *et al.*, 1984). The effect of bicuculline on peripheral neurotransmission in the autonomic nervous system is less well-known although morphological and functional evidence for a GABA involvement in the neuronal modulation at autonomic synapses is accumulating (Bowery *et al.*, 1984). In particular it has been suggested that GABA plays a role as a neurotransmitter at the autonomic synapses of the gastrointestinal tract (Jessen *et al.*, 1983). However, the direct involvement of GABA in the integrated neuronal activity subserving the reflex motor responses has so far been poorly substantiated by experimental evidence.

The transient effects of exogenously applied GABA, due to the rapidly induced desensitization, does not help much in interpreting the possible role of the endogenously released GABA in the onset and propagation of peristaltic motor activity. Indeed the peristaltic reflex develops at a fairly slow rate through a highly coordinated activation of both excitatory and inhibitory pathways (Costa & Furness, 1982). Therefore, it is uncertain to what extent the GABA-induced changes in propulsive activity ought to be ascribed to the agonist effect itself or to the consequence of GABA receptor-desensitization. Moreover, it is very doubtful that high concentrations

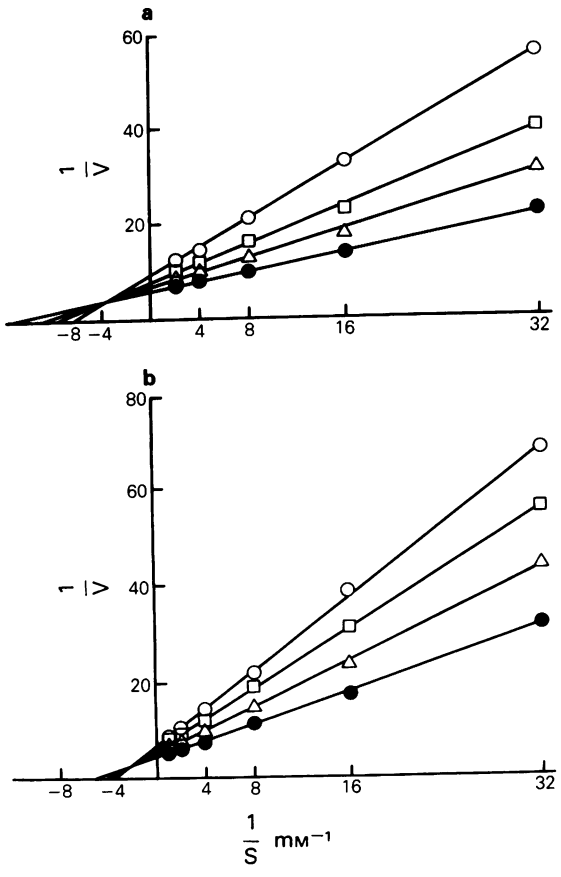

Figure 6 Anticholinesterase activity of bicuculline evaluated by the inhibition of electric eel AChE and horse serum BuChE. Double reciprocal plots of the substrate (ATCh or BuTCh) concentration (1/S) against the rate of transformation (1/V) as derived from the thiocholine related changes in the absorbance during a 30 s period. (a) The lines indicate double reciprocal plots for control (●) and in the presence of 25 μM (Δ), 50 μM (□) and 100 μM (○) bicuculline. (b) The lines indicate double reciprocal plots for control (●) and in the presence of 37.5 μM (Δ), 75 μM (□) and 150 μM (○) bicuculline. The mean K_m values (± s.e.mean) were 90 ± 17 μM for eel AChE and 240 ± 32 μM for horse serum BuChE. Each point represents the mean of 4 experiments performed in duplicate.

of exogenous GABA applied to the whole preparation could simulate the hypothetical effect of a localized activation of the postulated GABAergic pathway during the propagation of peristaltic activity.

In our experiments bicuculline seems to be able to affect the responses to the activation of the different neuronal pathways involved in the control of colonic

Table 1 Anticholinesterase activity of bicuculline: K_i and $K_{i'}$ values for the inhibition of electric eel acetylcholinesterase (AChE) and horse serum butyrylcholinesterase (BuChE)

Inhibition constants	*AChE* (μM)	*BuChE* (μM)
K_i	52 ± 7.1	140 ± 30
$K_{i'}$	186 ± 27	238 ± 37

K_i and $K_{i'}$ values (± s.e.mean) were calculated from the double reciprocal plots shown in Figure 6 by replotting the slopes and the intercepts on 1/V axis against the different bicuculline concentrations (Dixon & Webb, 1979): the intercepts on the x axis gave K_i and $K_{i'}$, respectively. Assays were performed in duplicate. Each value represents the mean of 4 assays.

motility. In preparations desensitized by cumulative administration of exogenous GABA, bicuculline failed to produce the effects observed in naïve preparations. Although the consequence of the combined effect of bicuculline and acute desensitization on the responsiveness to endogenously released GABA cannot be predicted on the grounds of the present knowledge of GABA receptor mechanisms (Enna, 1983), the failure of bicuculline to act in GABA-unresponsive preparations is highly suggestive for the effect of bicuculline being entirely dependent on the availability of functional GABA receptors.

GABA-unrelated effects of bicuculline, such as the weak and rapidly reversible inhibition of cholinesterases, as assessed *in vitro* on purified cholinesterases, were found to develop at concentrations at least 10 fold higher than those required to improve the efficiency of peristaltic reflex. This is likely to be true also in the intestinal neuromuscular preparations, whose acetyl- and butyryl-cholinesterases have been observed to behave as typical cholinesterases (Ambache *et al.*, 1971). Moreover, bicuculline has been proved to be devoid of any potentiating effect on the ileum longitudinal muscle responses to exogenous ACh (Krantis & Kerr, 1981b). Furthermore, the time courses of bicuculline-induced acetylcholinesterase inhibition as well as of enzyme activity recovery are very different from those observed for physostigmine (Wilson *et al.*, 1960; Main & Hastings, 1966).

Bicuculline exhibited opposite effects on the peristaltic reflex depending on the concentration employed. However, only the propulsion-enhancing effect appeared to be GABA-dependent, being present in naïve but not in GABA-desensitized preparations. Bicuculline was highly effective in improving the efficiency of peristalsis, being able to produce up to 40% increase of the maximal velocity of propulsion. No propulsion improvement or biphasic effect of bicuculline was described by Krantis *et al.* (1980) and

Krantis & Kerr (1981a) in the guinea-pig isolated colon. However, the range of concentrations employed and the experimental model used to elicit and to measure colonic motility were different from those used in our experiments.

An increase in the efficiency of peristaltic reflex as measured by the propulsion velocity, could result from a facilitation of either ascending excitation or descending inhibition or both, or from an improvement in their distal propagation. On the other hand, both excitatory and inhibitory responses involve cholinergic synapses (Crema *et al.*, 1970; North & Tokimasa, 1982). Bicuculline was able to increase both the hyoscine-sensitive contractile responses of longitudinal muscle to transmural stimulation and ACh output. The effect of bicuculline seemed to be frequency-dependent. Indeed an inverse relationship could be observed between the potentiation of contractile responses by bicuculline and the frequency of stimulation, the maximum effect of transmural stimulation being not significantly modified. A facilitatory effect of bicuculline on the cholinergic neurotransmission has been described both in the CNS (Bianchi *et al.*, 1982) and in the peripheral autonomic nervous system (Kusunoki *et al.*, 1984). This has been taken as evidence for a GABAergic modulation of the cholinergic neurotransmission. Such a conclusion is apparently contradicted by the observation made in the small intestine that GABA receptor activation by exogenous GABA is able to increase ACh output (Kleinrock & Kilbinger, 1983; Taniyama *et al.*, 1983b). However, it has also been shown that $GABA_A$ receptor activation is able to produce both excitatory and inhibitory effects on cholinergic neurotransmission both in the central and in the peripheral nervous system depending on the degree of the stimulation-induced neuronal activity (Bianchi *et al.*, 1982; Maggi *et al.*, 1985).

In our experiments, bicuculline has been observed to increase the NANC inhibitory responses to transmural stimulation. This at least in the colon contrasts with the postulated facilitatory role of GABA on non-adrenergic inhibitory neurones (Krantis *et al.*, 1980; Maggi *et al.*, 1984). Potentiation by bicuculline of NANC inhibitory responses to transmural stimulation was inversely related to the frequency of stimulation and could no longer be observed above 2 Hz. It is noteworthy that at the dose able to produce the maximum improvement of peristaltic reflex, bicuculline seemed to potentiate the responses to the excitation of NANC inhibitory neurones to a degree comparable to, or even higher than, that observed for the cholinergic excitatory responses. This could be relevant in interpreting the effect of bicuculline on peristalsis.

Contrary to what has been observed with direct stimulation of intramural enteric neurones, bicuculline was shown to reduce the inhibitory effect of periarterial nerve stimulation. Such an effect appeared to be frequency-independent. We cannot exclude the possibility that interference with adrenergic transmission, as suggested by Bartolini *et al.* (1985) for the vas deferens, could be responsible for the inhibitory effect of bicuculline. However the ability of both bicuculline and acute GABA-desensitization to reduce the responses to periarterial nerve stimulation together with the disappearance of effects of bicuculline in GABA-desensitized preparations does not support a bicuculline site of action other than at GABA receptors. Moreover, in the guinea-pig colon, bicuculline seems to be unable to antagonize the relaxation induced by exogenously applied noradrenaline (unpublished observation from our laboratory). It is worth noting that bicuculline-sensitive facilitatory effects of GABA on noradrenergic neurotransmission in the CNS have been observed by Bowery *et al.* (1980) and Suzdak & Gianutsos (1985).

Acute GABA-desensitization was not able to mimic the effects of bicuculline for all the kinds of responses that were examined. Comparable effects of GABA-desensitization and bicuculline could be observed for the NANC inhibitory responses to transmural stimulation and for the responses to periarterial nerve stimulation. In contrast, the contractile responses to transmural stimulation, ACh output and the efficiency of the peristaltic reflex were unaffected by acute GABA-desensitization. This could put some restrictions to the use of the combined effect of bicuculline and GABA-desensitization in order to interpret the hypothetical role of the endogenously released GABA. Discrepancies in the manner by which bicuculline and GABA-desensitization affect the responses to the stimulation of enteric neurones could rest on the different receptor specificity and on the different mechanism of receptor blockade of the two procedures. Desensitization is far less specific than bicuculline in distinguishing between GABA receptor subpopulations and the resultant effect is likely to be dependent on the existence at the intestinal level of both bicuculline-sensitive and bicuculline-insensitive GABA receptors (Giotti *et al.*, 1983; Ong & Kerr, 1983, Maggi *et al.*, 1985). The relative importance of the GABA receptor subtypes activated during nerve stimulation could differ depending on both the kind of stimulus applied and the kind of neurones involved. Indeed, activation of different GABA receptor subpopulations has been shown to produce opposite effects on cholinergic neurotransmission in the enteric nervous system (Giotti *et al.*, 1983; 1985; Kleinrock & Kilbinger, 1983).

In conclusion, if the effects of bicuculline are assumed to be entirely dependent on a selective blockade of GABA receptors, the following hypotheses could be advanced: (1) GABA receptors do not

seem to be involved to any significant extent in the local neuronal control of tone and spontaneous motility. The postulated intrinsic GABAergic pathways are unlikely to be tonically active, at least as can be inferred by the lack of any significant effect of bicuculline and GABA-desensitization either on resting tone or spontaneous electrical and mechanical activity.

(2) A bicuculline-sensitive modulation of both excitatory cholinergic and NANC inhibitory neurones through GABA receptors seems to operate during electrical activation of intrinsic enteric neurones. This is highly suggestive for a simultaneous activation of a subpopulation of enteric neurones using GABA as a transmitter to modulate the activity of both excitatory and inhibitory motor neurones. The effect of bicuculline on frequency-response relationships seems to indicate that the efficiency of GABAergic modulation is maximal at low levels of neuronal excitation.

(3) The viability of the postulated GABAergic pathways seems not to be a prerequisite for the triggering and development of peristaltic activity. Indeed, a peristaltic reflex can be elicited even after GABA receptor blockade or GABA receptor desensitization, so that GABAergic synapses seem not to be directly involved in the activation of motor neurones responsible for the reflex responses. However, an intrinsic neural mechanism able to modulate the speed of propagation and to regulate the efficiency of propulsive activity through bicuculline-sensitive GABA receptors seems to be activated during the reflex excitation of peristalsis.

(4) In the regulation of integrated intestinal motor activity, it could be suggested that a GABAergic mechanism may be involved in the feed-back modulation of the reflex motor responses responsible for the propulsion of the intraluminal content. Beside the other tonically active modulatory systems responsible for the long-lasting control of motor neurone excitability (i.e. adrenergic modulation) a phasic feed-back modulation carried out through a GABAergic pathway could operate during activation of the peristaltic reflex. The enhancing effect exhibited by bicuculline on both excitatory and inhibitory responses to enteric neurone stimulation and its suppressive effect on the adrenergically-mediated inhibition are all compatible with such a hypothetical role.

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Metabolic requirements for release of endogenous noradrenaline during myocardial ischaemia and anoxia

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1 The metabolic conditions required for noradrenaline (NA) release from ischaemic and anoxic perfused hearts of the rat were studied.

2 Forty minutes of flow reduction to approximately 0.25 ml g^{-1} min^{-1} did not elicit enhanced noradrenaline overflow from the isolated heart perfused with normoxic perfusate even in the absence of added substrate. Enhanced overflow did occur when substrate-free ischaemia was induced after a 60 min period of substrate-free perfusion.

3 Noradrenaline overflow was enhanced by perfusion at normal flow rates with an anoxic ($Po_2 < 1$ mmHg) perfusate containing no substrate. Such enhanced overflow occurred in the absence of calcium in the perfusate and was almost completely abolished by the addition of 11 mM glucose.

4 Enhanced noradrenaline overflow occurring either during low flow ischaemia after substrate deprivation or during anoxic substrate-free perfusion at normal flow rates was markedly suppressed by desipramine.

5 Exocytotic noradrenaline overflow induced by electrical stimulation of the left cervico-thoracic ganglion continued unchanged during 60 min of anoxia if the perfusate contained 11 mM glucose. In the absence of added substrate there was a decline in the overflow induced by such stimulation which was more rapid with anoxic than normoxic perfusate.

6 Re-introduction of calcium, oxygen and substrate after 10, 20 or 30 min of calcium-free, substrate-free, anoxic perfusion was associated with a massive overflow of the intracellular enzyme lactate dehydrogenase. At 10 min there was an associated transient minor increase in NA overflow but at 20 and 30 min the overflow of NA, elevated as a result of anoxic perfusion, returned to pre-anoxic levels on the re-introduction of substrate and oxygen.

7 These studies demonstrate a central role for the metabolic status of the sympathetic nerve terminal in determining the magnitude of exocytotic and nerve-impulse independent noradrenaline release from the heart. During the course of myocardial ischaemia *in vivo* nerve-impulse independent release would be expected to occur only in regions of severe flow reduction. This may produce heterogeneous stimulation of the myocardium.

Introduction

Catecholamine release within the heart is thought to play an important role in modulating the course of myocardial ischaemia, particularly with regard to the development of serious arrhythmias. Catecholamines could be released within the heart following reflex neural activity in the cardiac sympathetic nerves and enhanced sympathetic activity has been detected during early myocardial ischaemia (Malliani *et al.*, 1969). However, early in severe ischaemia there is a depression of the noradrenaline release produced by stimulation of cardiac sympathetic nerves (Dart *et al.*, 1984). Catecholamine release may also occur independently of reflex activity since catecholamine overflow has been found to occur from isolated perfused hearts made ischaemic (Schömig *et al.*, 1984; Abrahamsson *et al.*, 1985). The mechanism of this release is different from neurally mediated exocytotic release and its behaviour is compatible with a carrier-mediated efflux (Paton, 1976; Schömig *et al.*, 1984). The aim of the present studies was to define more precisely the

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metabolic conditions necessary for nerve-impulse independent release to occur. In order to distinguish reperfusion from ischaemic processes, a distinction not possible in earlier studies (Schömig *et al.*, 1984), experimental models not dependent on total ischaemia with subsequent reperfusion have been used. Furthermore, anoxia at unchanged flow rate has also been used to study the effect of changes in oxygen and substrate supply in the absence of the secondary effects of low flow ischaemia: changes in external ion concentration, acidosis and metabolite accumulation. Study of anoxia at unchanged flow also circumvents the difficulty that, under conditions of low flow, neuronal reuptake of exocytotically released noradrenaline (NA) becomes an increasingly important clearance mechanism (Dart & Riemersma, 1985). The effects of subsequent re-introduction of oxygen and substrate were studied, in combination with the re-introduction of calcium, to determine whether the sympathetic nerve terminals underwent damage analogous to the calcium/oxygen paradox (Hearse *et al.*, 1978). In complementary experiments the metabolic requirements of release in response to nerve stimulation were also studied.

These studies have been presented, in part, at the Winter Meeting (1985) of the British Pharmacological Society.

Methods

All experiments were performed on male Wistar rats (150–200 g) anaesthetized with thiopentone (50 mg kg^{-1} i.p).

Non-innervated hearts

Following anaesthesia heparin (500 u) was injected into the inferior vena cava. The thorax was then opened and the heart excised and rapidly mounted for perfusion by the Langendorff method. All experiments were performed with constant flow perfusion using a Watson Marlow multichannel peristaltic pump (202U). In all experiments an initial perfusion with a modified Krebs-Henseleit buffer (composition (mM): Na^+ 141.5, K^+ 4.05, Ca^{2+} 1.85, Mg^{2+} 1.05, Cl^- 128.0, HCO_3^- 25.0, PO_4^{3-} 0.38, EDTA 0.0269) containing 5.5 mM glucose and 1.8 mM pyruvate was performed for at least 20 min before any intervention. Hearts were mounted in chambers (4–8 simultaneously) kept at 37.5°C and perfusate was 37.5°C at the point of entry into the heart and had a pH of 7.4 (produced by gassing with 95% O_2 plus 5% CO_2). Po_2 was >500 mmHg. Hearts were weighed at the end of each experiment.

Ischaemic experiments

Ischaemia was induced by reducing the flow rate from 5.50 ± 0.15 ml g^{-1} min^{-1} to 0.246 ± 0.006 ml g^{-1} min^{-1}. The following experiments were performed: (1) Ischaemia with perfusate containing 5.5 mM glucose and 1.8 mM pyruvate ($n = 11$). (2) As (1), but with 100 nM desipramine (Ciba-Geigy) begun 20 min before the ischaemic episode and continued throughout the remainder of the experiment ($n = 11$). (3) Ischaemia with perfusate containing no substrate (substrate-free series, $n = 7$). (4) Substrate depletion was induced by a 60 min substrate-free, normoxic, perfusion. Ischaemia, also substrate-free, was then induced (substrate-depleted series, $n = 8$). (5) As (4) but with 100 nM desipramine begun 20 min before the ischaemic period and continued for the remainder of the experiment ($n = 8$).

Anoxia experiments

Anoxic experiments were all performed at unchanged flow rates (4.88 ± 0.15 ml g^{-1} min^{-1}). Anoxic perfusate was produced by gassing the perfusate with 95% N_2 and 5% CO_2. Remaining traces of oxygen were removed by the addition of the reducing agent sodium dithionite (final concentration 0.5 mM). Anoxia ($Po_2 < 1$ mmHg) was confirmed by Po_2 measurement (Instrumentation Laboratory System 1302 pH/blood gas analyser).

The following experiments were performed: (1) Perfusion with anoxic perfusate containing no substrate ($n = 15$). (2) Perfusion with anoxic perfusate containing no substrate and no calcium ($n = 7$). (3) Substrate depletion was induced by 60 min perfusion with a normoxic perfusate containing no substrate. Anoxic perfusion, also without substrate, was then instituted ($n = 7$). (4) Perfusion with an anoxic perfusate containing 11 mM glucose ($n = 7$). (5) As (1) but with the perfusate containing 100 nM desipramine, started 20 min before anoxia and continued for the remainder of the experiment ($n = 8$).

In all experiments (ischaemia and anoxia) investigating the action of desipramine an equal number of control and desipramine treated hearts were perfused simultaneously.

Re-oxygenation experiments

In these experiments re-oxygenation was combined with re-introduction of calcium in order to determine whether the calcium/oxygen paradox (Hearse *et al.*, 1978) would cause noradrenaline release from the nerve terminals, or whether the re-introduction of oxygen and substrate would allow rapid restoration of nerve terminal function. In these experiments a low pH was used during anoxia to simulate more closely an

'ischaemic' environment. Flow rates were unchanged (4.69 ± 0.11 ml g^{-1} min^{-1}) throughout the experiment. A period of 10, 20 or 30 min ($n = 7$ for each group) perfusion with an anoxic perfusate containing no substrate or calcium and at pH 6.6 (produced by addition of HCl) was followed by re-introduction of normal perfusate (pH 7.4, $Po_2 > 500$ mmHg, Ca^{2+} 1.85 mM, glucose 5.5 mM and pyruvate 1.8 mM). A pH of 6.6 was chosen since during ischaemia with added substrate the pH of the venous effluent at 10 min was 6.60 ± 0.05 ($n = 5$).

Innervated hearts

Details of this preparation have been given previously (Dart *et al.*, 1983). In these experiments stimulation was performed on each occasion for 30 s at 5 pulses s^{-1}, 0.8 mA. Effluent was collected before and for 60 s after the start of each stimulation. NA overflow is given as overflow in excess of basal, prestimulation, overflow. The sympathetic ganglion and cardiac sympathetic nerves were superfused with oxygenated buffer containing glucose and hearts were superfused with paraffin warmed to 37.5°C. Apex displacement was recorded (Devices M4) for assessment of heart rate increases. To overcome problems caused by atrioventricular block during stimulation the post-stimulation heart rates were determined by measuring the peak rates sustained for 10 s or more during the period of stimulation. Anoxia was produced as above. Flow rates were 4.24 ± 0.12 ml g^{-1} min^{-1} and were unchanged throughout each experiment.

The following experiments were performed: (1) Control series: perfusion was performed with normoxic perfusate containing 5.5 mM glucose, 1.8 mM pyruvate and stimulation performed at 0, +15, +30, +45 and +60 min ($n = 6$). (2) Stimulation was performed before and 10 min after change to an anoxic perfusate containing no substrate ($n = 6$). (3) Stimulation was performed before and after 15, 30, 45 and 60 min perfusion with normoxic perfusate containing no substrate ($n = 6$). (4) Stimulation was performed before and after 15, 30, 45 and 60 min perfusion with an anoxic perfusate containing 11 mM glucose ($n = 6$).

In (2)–(4) the initial stimulation was performed during perfusion with normoxic perfusate containing 5.5 mM glucose, 1.8 mM pyruvate.

Biochemical and statistical analyses

Samples for catecholamine estimation were collected on ice and immediately stabilized by 1:1 addition of 0.6 N perchloric acid. Samples were stored at −40°C until assayed, in duplicate, by the radio-enzymatic method of Da Prada & Zürcher (1976). Assay blanks were typically in the range 30–50 c.p.m. with an inter-assay coefficient of variation of 17% whilst 600 fmol standards yielded 800–1000 c.p.m. with a coefficient of variation of 7%. All samples from each heart (up to a maximum of 4 hearts) were determined in the same assay and, in experiments investigating the action of desipramine, samples from equal numbers of treated and untreated hearts were assayed together. Sodium dithionite (0.5 mM) and desipramine (100 nM) were without effect on the noradrenaline assay (blanks and standards). Samples for lactate and lactate dehydrogenase (LDH) estimation were immediately assayed, in duplicate, using an enzymatic method (Boehringer Mannheim) on a Cobas Bio centrifugal analyser. Potassium concentrations in the effluent were measured with a Corning 435 flame photometer. In calculations of biochemical data the means of the duplicate measurements were used.

Statistical analyses between groups were performed using analysis of variance on a Minitab statistical package (CLE.COM Ltd). Significance is assumed if $P < 0.05$. Values given are mean ± s.e.mean.

Results

The effect of reducing flow from approximately 5 ml g^{-1} min^{-1} to approximately 0.25 ml g^{-1} min^{-1} on noradrenaline overflow from the non-innervated heart is shown in Figure 1. In the presence of 5.5 mM glucose and 1.8 mM pyruvate such ischaemia did not increase

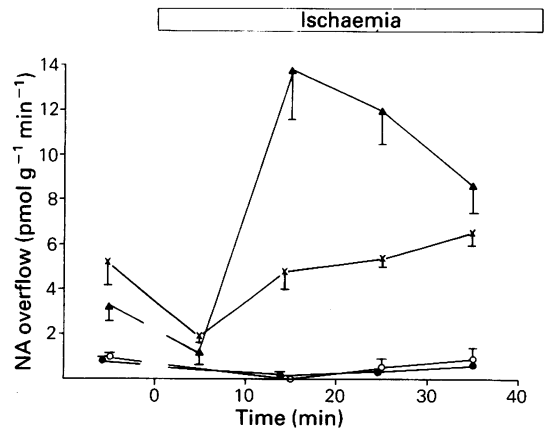

Figure 1 Noradrenaline (NA) overflow (pmol g^{-1} min^{-1}) from hearts made ischaemic for 40 min (open bar) in the presence of 5.5 mM glucose and 1.8 mM pyruvate (○) ($n = 11$), in the absence of substrate (substrate-free) (●) ($n = 7$), in the absence of substrate after a 60 min substrate-free pre-perfusion (substrate-depleted) with (×) ($n = 8$) and without (▲) ($n = 8$) desipramine (100 nM). Each point represents the mean with vertical lines indicating s.e.mean.

NA overflow with respect to the pre-ischaemic values although during the course of ischaemia there was a significant rise in NA overflow from 0.10 ± 0.02 pmol g^{-1} min^{-1} at 15 min to 1.00 ± 0.42 pmol g^{-1} min^{-1} at 37 min. When ischaemia was produced in the absence of added substrate there was again no increase in NA overflow above pre-ischaemic values but a significant rise during ischaemia from 0.24 ± 0.09 pmol g^{-1} min^{-1} at 15 min to 0.80 ± 0.23 pmol g^{-1} min^{-1} at 37 min. However, when substrate-free ischaemia was produced following a period of substrate depletion there was an enhanced overflow, with respect to pre-ischaemic values, at 15, 25 and 37 but not at 5 min. Cumulative lactate overflows (μmol g^{-1} 40 min^{-1}) during ischaemia were 20.3 ± 2.9 in the substrate-free and 20.5 ± 2.6 in the substrate-depleted series.

Desipramine did not significantly affect the overflow of NA during low flow ischaemia in the presence of glucose and pyruvate (not shown). At 15 min overflows (pmol g^{-1} min^{-1}) were 0.19 ± 0.04 in the desipramine treated series, in comparison to 0.10 ± 0.02 in the control series, whereas at 25 and 37 min desipramine was associated with lower overflows of 0.43 ± 0.11 and 0.60 ± 0.16 in comparison to respective control values of 0.67 ± 0.19 and 1.00 ± 0.42. Pre-ischaemic NA overflow in the desipramine-treated series was 1.19 ± 0.36 pmol g^{-1} min^{-1}. Desipramine was also without effect on lactate overflow during ischaemia. Lactate overflows (μmol g^{-1} min^{-1}) were 0.98 ± 0.16 (15 min), 1.09 ± 0.14 (25 min) and 0.99 ± 0.13 (37 min) in the absence of desipramine, 1.04 ± 0.18, 1.02 ± 0.18 and 0.99 ± 0.19, respectively, in the presence of desipramine. Desipramine, however, did suppress the enhanced NA overflow produced by 60 min substrate depletion followed by substrate-free ischaemia (Figure 1). There was no difference in the control, pre-ischaemic overflows, between the two groups. During such ischaemia, desipramine significantly suppressed the NA overflow at 15 and 25 min but not at 5 and 37 min. Cumulative lactate overflow during substrate-free ischaemia after a period of substrate deprivation was not affected by desipramine at 19.6 ± 3.1 μmol g^{-1} 40 min^{-1}.

Perfusion with an anoxic perfusate containing no substrate resulted in enhanced NA overflow in the presence and absence of extracellular calcium (Figure 2). In the presence of calcium enhanced overflow, in comparison to pre-anoxic values, occurred after 15 (but not 5 or 10) min. However following substrate depletion, induced by 60 min perfusion with normoxic perfusate containing no substrate, substantially increased overflow was already apparent by 10 min. In the absence of calcium, overflow of NA at 20 min (73.90 ± 5.25 pmol g^{-1} min^{-1}) was significantly higher than during normal calcium, substrate-free perfusion (27.0 ± 3.4 pmol g^{-1} min^{-1}). Perfusion

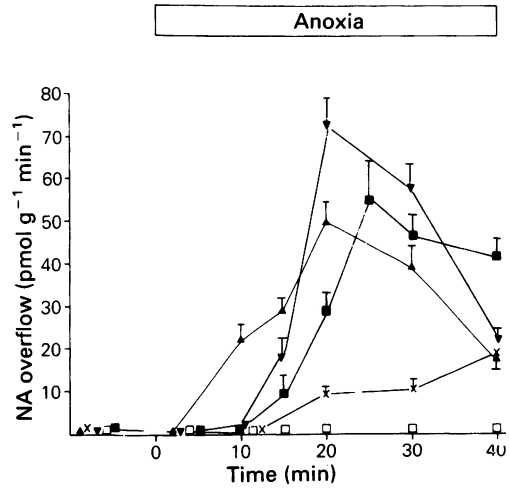

Figure 2 Noradrenaline (NA) overflow (pmol g^{-1} min^{-1}) from hearts made anoxic ($Po_2 < 1$ mmHg) in the presence of 11 mM glucose (□), in the absence of substrate (with (×) and without (■) 100 nM desipramine), in the absence of substrate following a 60 min substrate-free, normoxic, pre-perfusion (▲) and in the absence of substrate and calcium (▼). Flow was unchanged throughout (4.88 ± 0.15 ml g^{-1} min^{-1}). Each point is the mean of 7–15 observations and vertical lines indicate s.e.mean.

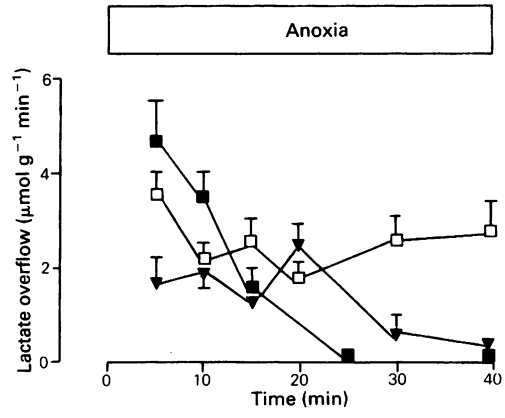

Figure 3 Lactate overflow (μmol g^{-1} min^{-1}) from hearts made anoxic ($Po_2 < 1$ mmHg) in the absence of glucose and calcium (▼), in the absence of glucose (■) and in the presence of 11 mM glucose (□). Flow rates were unchanged throughout. Each point represents the mean ($n = 7$ for each series) and vertical lines indicate s.e.mean.

with an anoxic buffer containing 11 mM glucose almost completely suppressed the enhanced NA overflow (Figure 2). The lactate overflows (μmol g^{-1} min^{-1}) with and without added glucose are shown in Figure 3. In the absence of added substrate but with normal calcium, lactate overflow fell to low levels by 25 min and was undetectable at 40 min. In contrast in the presence of 11 mM glucose, lactate overflow was sustained throughout the experiment. The total lactate overflows during anoxia were 70.8 ± 4.5 μmol g^{-1} 40 min^{-1} in the absence of substrate and 103.7 ± 9.3 μmol g^{-1} 40 min^{-1} in the 11 mM glucose group ($P < 0.02$). Lactate overflow was significantly reduced with a calcium-free perfusate at 52.2 ± 2.9 μmol g^{-1} 40 min^{-1} ($P = 0.0063$ vs glucose free, normal calcium values); following a substrate-free pre-perfusion, lactate overflow was

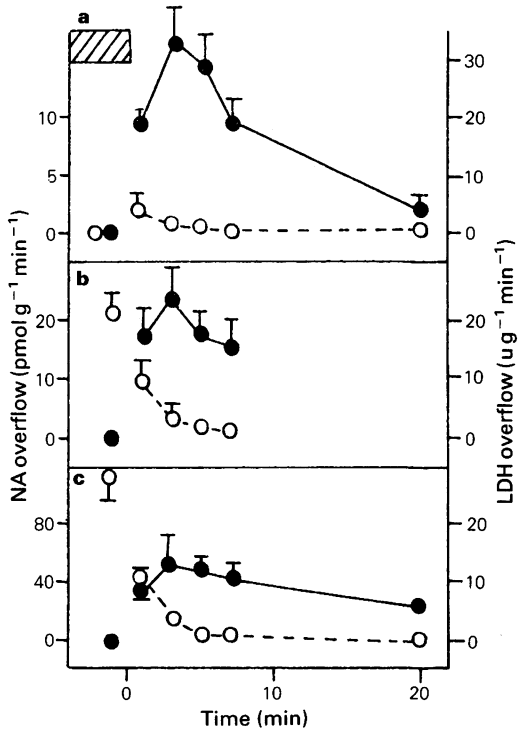

Figure 4 The effect of re-introduction of calcium, oxygen and substrate after 10 (a), 20 (b) and 30 (c) min of calcium-free, substrate-free, acidic (pH 6.6), anoxic perfusion ($n = 7$ for each group) on (○) noradrenaline (NA) overflow (pmol g^{-1} min^{-1}) (note different scales), and (●) lactate dehydrogenase (LDH) overflow (u g^{-1} min^{-1}). Error bars not shown fall within the limits of the symbols. Flow rates were unchanged throughout these experiments (4.69 ± 0.11 ml g^{-1} min^{-1}).

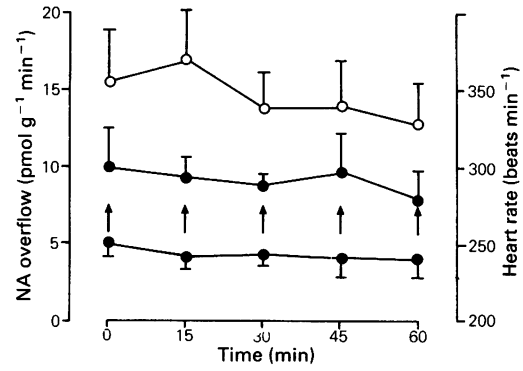

Figure 5 Noradrenaline (NA) overflow (pmol g^{-1} min^{-1}) and heart rate increase produced by repetitive stimulation of the left cervico-thoracic ganglion at 5 pulses s^{-1}, 0.8 mA for 30 s. Perfusate contained 5.5 mM glucose, 1.8 mM pyruvate and had $Po_2 > 500$ mmHg. (○) indicate stimulation-induced NA overflow, (●) indicate heart rate and respective pre- and post-stimulation values are indicated by arrows. Pre-stimulation overflows (pmol g^{-1} min^{-1}) were unchanged with values of 0.75 ± 0.13 (0 min), 1.16 ± 0.24 (15 min), 0.80 ± 0.09 (30 min), 0.66 ± 0.17 (45 min) and 0.90 ± 0.21 (60 min). Each point represents the mean and vertical lines indicate s.e.mean.

59.6 ± 6.4 μmol g^{-1} 40 min^{-1}. The enhanced NA overflow produced by perfusion with an anoxic perfusate containing no substrate was suppressed by 100 nM desipramine (Figure 2). At later times, however, NA overflows were higher in the desipramine series than in the control experiments. After 50 min anoxia NA overflows (pmol g^{-1} min^{-1}) were 22.5 ± 2.0 (control) and 27.3 ± 4.3 (desipramine), and at 60 min they were 15.7 ± 1.6 (control) and 22.4 ± 2.6 (desipramine). Desipramine was without effect on lactate production during substrate-free anoxia. Lactate overflows (μmol g^{-1} min^{-1}) were 2.57 ± 0.41 (10 min) and 1.02 ± 0.31 (20 min) in the absence of desipramine and 2.66 ± 0.54 (10 min) and 0.59 ± 0.35 (20 min) in the presence of desipramine. At 30 min mean overflows were < 0.25 μmol g^{-1} min^{-1} for both groups.

During anoxic, substrate-free perfusion, potassium concentrations in the effluent rose significantly by 0.1–0.2 mM (mean values 4.10 ± 0.03 and 4.22 ± 0.03 mM, $n = 15$) between 10 and 20 min of anoxia. No significant change in external Na^+ concentration was observed.

Restoration of perfusate to normal after a 10 min period of anoxic, calcium-free perfusion resulted in a transient rise in NA overflow to 2.40 ± 0.80 pmol g^{-1} min^{-1} (Figure 4a) in the first 2 min. Subsequent overflows were not greater than the pre-anoxic values.

In contrast LDH overflow rose from less than $0.1 ug^{-1} min^{-1}$ at the end of the anoxic perfusion to a peak value of more than $30 u g^{-1} min^{-1}$ following re-introduction of calcium and oxygen. After 20 and 30 min anoxia, NA overflows had already risen and on restoration of perfusate to normal there was a return of these elevated levels to pre-anoxic values despite the marked myocyte damage (LDH release) produced (Figure 4b, c).

The effect of repetitive stimulation of the left cervico-thoracic sympathetic ganglion (5 pulses s^{-1}, 0.8 mA) on NA overflow and heart rate increase for hearts perfused with oxygenated buffer containing glucose and pyruvate is shown in Figure 5. There was no significant decline in nerve stimulation-induced NA overflow or heart rate increase over the period tested. In the absence of substrate and oxygen there was a significant fall in NA overflow by 10 min; in the absence of substrate, but with oxygen, a significant decline in NA overflow had occurred by 30 min (Figure 6). In the presence of glucose, however, anoxia was without effect on stimulation-induced NA release which was maintained over the period tested (Figure 6).

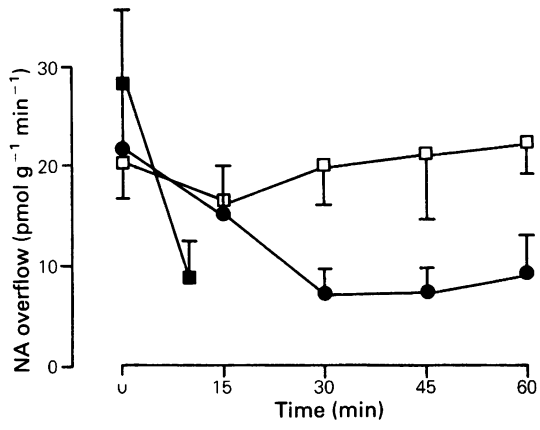

Figure 6 Noradrenaline (NA) overflow (pmol g^{-1} min^{-1}) produced by repetitive stimulation of the left cervico-thoracic ganglion at 5 pulses s^{-1}, 0.8 mA for 30 s. Perfusion was with anoxic perfusate ($Po_2 < 1$ mmHg) containing no substrate (■) ($n = 6$), anoxic perfusate containing 11 mM glucose (□) ($n = 6$) or normoxic ($Po_2 > 500$ mmHg) perfusate containing no substrate (●) ($n = 6$). In all experiments the initial stimulation was performed during perfusion with normoxic perfusate containing 5.5 mM glucose and 1.8 mM pyruvate. Perfusate was then changed as indicated. Pre-stimulation overflows (pmol g^{-1} min^{-1}) were 2.09 ± 0.89 (control), 2.37 ± 1.02 (15 min), 2.23 ± 0.84 (30 min), 2.40 ± 0.92 (45 min) and 2.77 ± 1.00 (60 min) in the anoxia with glucose series; 0.70 ± 0.07 (control), 0.83 ± 0.34 (15 min), 1.21 ± 0.50 (30 min), 0.62 ± 0.36 (45 min) and 0.57 ± 0.23 (60 min) in the glucose-free, normoxia series; 0.80 ± 0.16 (control) and 1.21 ± 0.11 (10 min) in the glucose-free, anoxia series. Each point represents the mean and vertical lines indicate s.e.mean.

Discussion

The experiments described demonstrate that the energy status of the sympathetic nerve terminal is the major determinant of the occurrence of nerve-impulse independent (non-exocytotic) noradrenaline release during myocardial ischaemia.

It is not possible to obtain information relating directly to the metabolic status of the sympathetic nerve terminals within the heart. However, during substrate-free anoxia the hearts rapidly become quiescent so that differences in energy utilization between neurones and myoctyes, as a consequence of mechanical activity, will be reduced. In addition, the major source of adenosine 5'-triphosphate (ATP) generation will be by anaerobic glycolysis of endogenous glycogen with the production of lactate which, in the absence of oxygen, cannot be utilized and can thus serve as an index of ATP generation. Under these conditions, over 80% of total lactate overflow had occurred by 15 min and, interestingly, it was at 15 min that anoxic release of NA became apparent. Provision of glucose almost completely suppressed NA overflow and allowed glycolytic activity to continue throughout the period studied. However, differences between myocyte and nerve terminal metabolism were also evident from the observation that, although pre-perfusion with a normoxic perfusate containing no substrate shortened the latent period before anoxic NA overflow, there was only a minor and non-significant reduction in anoxic lactate overflow. This implies that myocytes are better able to conserve glycogen during perfusion with normoxic, but substrate-free, perfusate than are the nerve terminals, probably by utilization of non-glucose endogenous substrate. Lactate overflow during anoxia should therefore be seen as a guide to events in the nerve terminal rather than as providing precise information of nerve terminal metabolism. Although following substrate depletion anoxic NA release was apparent earlier, the peak NA overflow achieved was not increased. This can be interpreted as indicating that although NA efflux occurs when ATP levels have fallen below a critical level the rate of efflux is then determined by other factors. The effects of calcium-free perfusion may also be partly due to changes in ATP generating capacity, since total lactate overflow was lower under these conditions, although the tendency to a higher peak NA overflow during calcium-free perfusion may indicate an additional mechanism.

In contrast to anoxia, low flow ischaemia, even in the absence of added substrate, did not lead to enhanced noradrenaline overflow in comparison to pre-ischaemic values. This can be explained by the much greater efficiency of oxidative metabolism in ATP generation in comparison to anaerobic glycolysis. Although global 90% ischaemia, following pre-ischaemic loading with [^{3}H]-noradrenaline, leads to an increased ^{3}H concentration in the venous effluent (Carlsson *et al.*, 1986) calculation of the data as overflow g^{-1} min^{-1} showed no increase above pre-ischaemic levels at 10 and 20 min, despite the use of lactate as the only exogenous substrate during ischaemia. After 60 min ischaemia, however, ^{3}H overflow g^{-1} min^{-1} was approximately twice the pre-ischaemic value (calculated from Table 1, Carlsson *et al.*, 1986). The initial fall in endogenous NA overflow during ischaemia can be partly explained by neuronal re-uptake of NA (Dart & Riemersma, 1985). Enhanced noradrenaline overflow does become apparent when substrate-free ischaemia is produced after a period of substrate depletion. It should, however, be noted that the peak NA overflow occurring under these conditions is several-fold lower than the peak overflow found with anoxia. This may be due to the much higher extracellular NA concentration found during ischaemia, reducing the intracellular/extracellular NA gradient and thereby limiting the rate of NA efflux from the neurones. Despite the striking difference in NA overflow between substrate-free and substrate-depleted ischaemia, there were no differences in lactate overflow, again implying that glycogen comprises the major energy reserve of the nerve terminals, whereas myocytes, in the presence of oxygen, are able to utilize alternative substrate reserves. The resistance of the nerve terminals to ischaemia, and the fact that the flow used is far less than the normal *in vivo* resting coronary flow of 3.8 ml g^{-1} min^{-1} (Wicker & Tarazi, 1982), implies that *in vivo* such nerve-impulse independent release will be restricted to regions of profound ischaemia, perhaps leading to heterogeneous stimulation of the ischaemic myocardium. NADH surface fluorescence data from ischaemic rat hearts have suggested that localized areas of severe ischaemia could exist (Steenberger *et al.*, 1977). Nerve-impulse independent noradrenaline release during anoxia and ischaemia was suppressed by desipramine, in keeping with the hypothesis that such release is due to carrier-mediated efflux (Schömig *et al.*, 1984). Desipramine did not alter the production of lactate during anoxia and is thus unlikely to act by increasing nerve terminal ATP generation. In addition, severity of ischaemia, as indicated by lactate production, was also unchanged by desipramine. Changes in myocardial contractility were not monitored but the hearts rapidly became quiescent and it is unlikely that, even if present, changes in the energy balance of the myocyte could have influenced the energy balance of the nerve terminal. Such a mechanism is conceivable *in vivo* if desipramine were associated with a reduced level of sympathetic activity, leading to less ATP utilization by the nerve terminal, but such a mechanism could not operate in the experiments described here. Even in the presence of desipramine, however, NA overflow was not completely suppressed and this desipramine-resistant release became more prominent at later stages of anoxia. The mechanism of this release has not been elucidated. Interpretation of NA overflow at later stages of anoxia was complicated by the inevitable depletion of NA which occurs following high early overflows and peak NA overflow may be a better indicator of NA efflux under different conditions.

Restoration of oxygen and substrate after a period of anoxia led to a fall in elevated NA overflow, even when combined with re-introduction of calcium. Re-introduction of calcium and oxygen did, however, lead to a massive release of the intracellular enzyme LDH ('calcium/oxygen paradox', Hearse *et al.*, 1978). This suggests that at least as far as non-exocytotic release is concerned, the processes are rapidly reversible as soon as the capacity for ATP generation is restored. There was also a marked difference between the myocyte and nerve terminal in response to the calcium/oxygen paradox.

It has been previously found that neurally mediated (exocytotic) NA release in the rat heart was depressed early during the course of severe ischaemia (Dart *et al.*, 1984). The experiments described here suggest that this is largely due to a failure of energy production by the nerve terminal since in these experiments other consequences of severe ischaemia (e.g. acidosis, hyperkalemia, metabolite accumulation) would not pertain, as the studies were all performed at relatively high flow rates. Glycolytic activity from exogenously supplied glucose is capable of supporting this release even in the absence of supplied oxygen.

In conclusion, our results show that nerve-impulse independent noradrenaline release can be expected to occur during severe myocardial ischaemia and is not dependent on the process of reperfusion. Desipramine suppresses this release and this may contribute to the anti-arrhythmic effect of desipramine and related drugs (Bril & Rochette, 1985; Daugherty *et al.*, 1986; Riemersma *et al.*, 1986). The metabolic status of the nerve terminal is the major determinant of whether nerve-impulse independent or dependent NA release will occur.

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Lack of involvement of α-adrenoceptors in sympathetic neural vasoconstriction in the hindquarters of the rabbit

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1 The hypothesis that sympathetic nerves in arterial blood vessels activate excitatory receptors distinct from α-adrenoceptors was investigated *in vivo* in the rabbit.

2 In anaesthetized, ganglion-blocked rabbits, graded stimulation of the lumbar sympathetic nerve chains caused graded hind limb vasoconstriction. The responses to single pulses and short trains of stimuli were unaffected by benextramine ($10\,mg\,kg^{-1}$) and the longer trains were enhanced. Phenoxybenzamine ($5\,mg\,kg^{-1}$) slightly reduced the responses to short trains of stimuli and did not affect the responses to long trains. The dose-response curve to intra-arterial noradrenaline (after β-adrenoceptor blockade) was shifted rightwards about ten fold by benextramine ($10\,mg\,kg^{-1}$) and by phenoxybenzamine ($5\,mg\,kg^{-1}$).

3 In conscious rabbits the vasoconstriction caused by the nasopharyngeal reflex initiated by smoke inhalation was unaffected by benextramine ($10\,mg\,kg^{-1}$).

4 Small mesenteric arteries (<250 μm) taken from untreated rabbits responded to noradrenaline with a threshold concentration of about 1 μM. Similar tissues from benextramine ($10\,mg\,kg^{-1}$)-treated rabbits were unresponsive to noradrenaline at concentrations up to 300 μM. However, these tissues were able to respond to potassium and angiotensin II. Aortic ring segments taken from the same rabbits were only about ten fold less sensitive to noradrenaline than segments from control rabbits.

5 These results are in accord with the hypothesis that sympathetic nerves activate non-α-receptors in the vasculature of the rabbit.

Introduction

The vascular responses to sympathetic nerve stimulation are generally considered to result from the activation of α-adrenoceptors by noradrenaline (e.g. Langer *et al.*, 1981; McGrath *et al.*, 1982; Elsner *et al.*, 1984; Alabaster & Davey, 1984). However, data from electrophysiological studies in isolated tissues suggest that the receptor activated by the sympathetic transmitter is not an α-adrenoceptor (Holman & Surprenant, 1979; 1980; Surprenant, 1980; Hirst & Neild, 1980; 1981; Itoh *et al.*, 1983; Hirst *et al.*, 1985). Hirst & Neild (1980) suggested that during neurotransmission, noradrenaline released from sympathetic nerves activates a receptor in the synaptic junction that is different from the α-adrenoceptor, called a γ-receptor. An alternative proposal is that the sympathetic transmitter is adenosine 5'-triphosphate (ATP) rather than noradrenaline and the junctional receptor is an ATP receptor (Sneddon & Burnstock, 1984). Both of these hypotheses imply either a lack of involvement or a secondary role of α-adrenoceptors in sympathetic neurotransmission. Although the results of some *in vivo* studies can be interpreted as supporting the role of non-α-receptors in vasoconstriction (e.g. Glick *et al.*, 1967), a study designed specifically to investigate the role of non-α-receptors in sympathetic neural vasoconstriction *in vivo* (Bell, 1985) led to the conclusion that sympathetic nerve-mediated vasoconstriction involved the activation of α-adrenoceptors; no evidence for a role of other receptors was found. Flavahan *et al.* (1985) on the other hand decided that 50% of the response of the pithed rat to sympathetic nerve stimulation was mediated by non-α-adrenoceptors.

The experiments reported here were designed to examine the role of α-receptors in mediating vasoconstriction *in vivo*. Both direct electrical and reflex stimulation of the sympathetic vasoconstrictor nerves were used. In the direct electrical stimulation ex-

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periments, unlike previous studies, the autonomic ganglia were blocked so that the stimulation was exclusively postganglionic in nature. Moreover, as well as two more commonly used α-adrenoceptor blocking drugs, we used benextramine. Benextramine is a covalent, irreversible inhibitor of α-adrenoceptors *in vitro* (Melchiorre *et al.*, 1978; Lew & Angus, 1984) and *in vivo* (Furnace *et al.*, 1985). This allowed us to examine the extent of α-receptor blockade that was achieved in the experiments both *in vivo* (by noting the effect of benextramine on the responses to injected noradrenaline dose-response curves) and *in vitro* (by removing blood vessels from benextramine treated animals and comparing the responsiveness of these tissues with that of tissues from untreated animals). The data suggest that α-adrenoceptors play little or no role in sympathetic neurotransmission in the rabbit hind limb vascular bed, and that circulating catecholamines may affect the circulation through different receptors and at different regions of the vascular bed from those activated by the sympathetic nerves.

Methods

Rabbits of either sex (1.9–2.6 kg) were anaesthetized with Saffan (induction 20–30 mg kg^{-1} i.v. followed by 6 mg kg^{-1} min^{-1}), intubated, and ventilated with room air (60 strokes min^{-1}, 18 ml per stroke). The right femoral artery was cannulated for blood pressure measurement and the right femoral vein cannulated for injections. The lower abdominal aorta was then exposed using a retroperitoneal flank incision on the left side, and a pulsed Doppler type flow probe placed around the aorta. A Barger type indwelling catheter was installed below the renal arteries and proximal to the flow probe. The lumbar sympathetic chains were located and a thread tied around both nerves distal to L5. The nerves were then cut between the thread and the ganglion and the distal portion placed on a bipolar stimulating electrode. Blood pressure, hind limb flow, heart rate and hind limb conductance (calculated from blood pressure and hind limb flow by a dividing circuit) were recorded on a Grass polygraph model 7D. After the surgical procedures the anaesthetic infusion was reduced to about 3 mg kg^{-1} min^{-1} and 30 ml of blood from a donor rabbit was injected. The rabbit was then injected with mecamylamine (10 mg kg^{-1}) to block transmission through autonomic ganglia. If the mecamylamine caused the blood pressure to fall to below 55 mmHg, 20 ml of plasma expander (Rheomacrodex 10% in dextrose) was injected. Additional plasma expander in 5 ml aliquots was given as required during the experiment. The nerves were stimulated with 1–100 pulses at 5 Hz, 1 ms pulse width and supramaximal voltage (4–25 V). Noradrenaline 0.01–0.2 μg kg^{-1} was injected into the aorta through the indwelling catheter with injection volumes of less than 0.4 ml. Propranolol (1 mg kg^{-1}, i.v.) was given every hour. The stimuli and noradrenaline injections were always performed in ascending order, and the nerve stimuli were delivered before the noradrenaline. The tests were performed before and 60 min after either no drug, benextramine (10 mg kg^{-1}), or phenoxybenzamine (5 mg kg^{-1}).

In these experiments there was a decrease in the resting hind limb vascular conductance between the first and second periods of stimulation. In an attempt to minimize the distortion of the patterns of responses caused by this, the results were calculated both as absolute conductance at peak response and as % change from resting conductance. The data from each group of animals was analysed in three way analysis of variance, and the tests were performed with the data expressed both as absolute peak conductance and as % change of conductance. The vertical lines in the the figures represent s.e.mean from the analysis of variance. The differences between the first and second stimulus-response curves were tested by subtracting the first curve from the second within groups and comparing these differences in a three way analysis of variance.

In another group of rabbits, catheters were inserted under local anaesthetic (prilocaine 1%) into the central ear artery and marginal ear vein. Blood pressure was recorded through the arterial catheter. The nasopharyngeal reflex was elicited by blowing cigarette smoke into the nose of the rabbits (White *et al.*, 1974). This reflex causes a profound bradycardia and vasoconstriction. After the cardiac musarinic receptors were blocked with methscopolamine (50 μg kg^{-1}) the vasoconstriction was expressed as an increase in the blood pressure. The pressor response was measured before and after benextramine (10 mg kg^{-1}).

In order to test *in vitro* the effectiveness of the blockade of α-adrenoceptors by the benextramine in large and small vessels, three rabbits were injected with benextramine (10 mg kg^{-1}) and left for 60 min before they were killed by pentobarbitone overdose. The thoracic aorta and a piece of the ileal mesentery were excised and placed in Krebs solution. The aorta was cut into ring segments and set up in organ baths using standard techniques. Segments of small (250 μm diameter) mesenteric arteries were set up in a myography apparatus according to the technique of Mulvany & Halpern (1976). The data from these experiments were compared with the results obtained using tissues from 5 untreated rabbits.

The drugs used were: Saffan (alphaxalone and alphadalone acetatone, Glaxo); benextramine tetrahydrochloride (Sigma); guanethidine sulphate (Ciba); mecamylamine hydrochloride (Merck); methscopolamine bromide (Sigma); noradrenaline bitartrate (Sigma); phenoxybenzamine hydrochloride

(Dibenzyline ampoules, Smith, Kline and French); phentolamine mesylate (Regitine ampoules, Ciba); prazosin hydrochloride (Pfizer); propranolol hydrochloride (ICI); yohimbine hydrochloride (Sigma). All drug doses are expressed as free base.

Results

Lumbar nerve stimulation

Stimulation of the lumbar sympathetic nerve chains caused vasoconstriction in the hind limb bed, with only a small change in the arterial blood pressure (<5 mmHg). The responses were graded with the number of stimuli applied and were all reduced to about half by ganglion blockade (mecamylamine 10 mg kg^{-1}). The response to continuous stimulation at 5 Hz was only marginally larger than the response to 100 pulses. Larger responses were obtained by using 10 and 20 Hz stimulation, so the response to 100 pulses at 5 Hz does not represent the maximum response of the vascular bed to sympathetic nerve stimulation. The responses to nerve stimulation were not affected by propranolol or methscopolamine. Intra-arterial injection of noradrenaline caused a dose-related decrease in vascular conductance, the maximum decrease in conductance was larger than that produced by continuous nerve stimulation at 5 Hz. In 3 initial experiments it was found that the responses to small doses of noradrenaline were not changed by propranolol, but the responses to large doses were enhanced. After benextramine treatment small doses of noradrenaline failed to cause vasoconstriction and larger doses caused vasodilatation in the absence of propranolol. After β-adrenoceptor blockade the larger doses of noradrenaline produced vasoconstriction. Propranolol (1 mg kg^{-1}) was therefore administered before the construction of each noradrenaline dose-response curve in subsequent experiments in order that an estimate of α-adrenoceptor function could be obtained.

In time control experiments the responses to lumbar sympathetic nerve stimulation were found to be reasonably stable. The second stimulus-response curve was significantly different from the first when the data were expressed as absolute conductance, but not when the data was transformed to % change (Figure 1). This difference was more likely to be due to the altered resting conductance than to altered response sizes. The responses to intra-arterial noradrenaline were similarly stable with a significant difference between the curves when the data were expressed as % change but not absolute conductance. The rightwards shift of the noradrenaline curve with time was less than 2 fold (Figure 1).

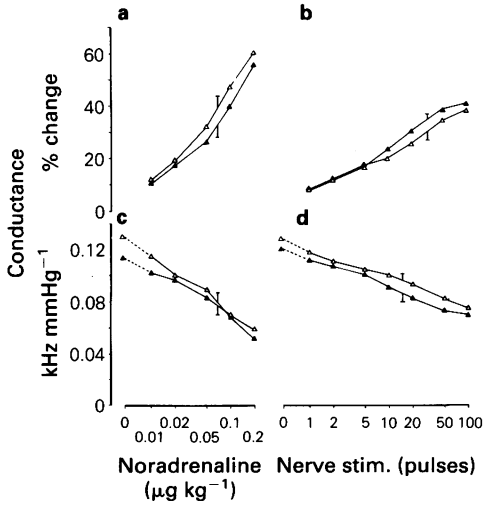

Figure 1 Time control experiments for the nerve stimulation and noradrenaline injection protocol. Hind limb vascular conductance responses to intra-arterial noradrenaline (a and c) and to lumbar sympathetic nerve stimulation (b and d), expressed as absolute hind limb vascular conductance (c and d) and as % change from resting (a and b). Vertical lines show s.e.mean, analysis of variance ($n = 4$).

Benextramine

Benextramine (10 mg kg^{-1}) inhibited the responses to injected noradrenaline, causing about a 10 fold rightward shift of the noradrenaline dose-response curve (Figures 2 and 3). Benextramine (10 mg kg^{-1}) also initially depressed the lumbar sympathetic nerve stimulus-response curve slightly. The depression of the responses to nerve stimulation was reversed over about 60 min to give a net enhancement of the stimulus-response curve (when tested by analysis of variance). Inspection of the curves showed that the responses to long trains of stimulation (20, 50 and 100 pulses) were enhanced ($P < 0.05$, paired t test), while the responses to shorter trains were unaffected (Figure 3).

Phenoxybenzamine

Phenoxybenzamine (5 mg kg^{-1}) caused a slightly larger rightward shift of the noradrenaline dose-response curve than did benextramine, with some depression of the maximum response (Figure 4). The responses to lumbar sympathetic nerve stimulation were initially greatly depressed by phenoxybenzamine and recovered to some extent over 60 min (Figure 4). The

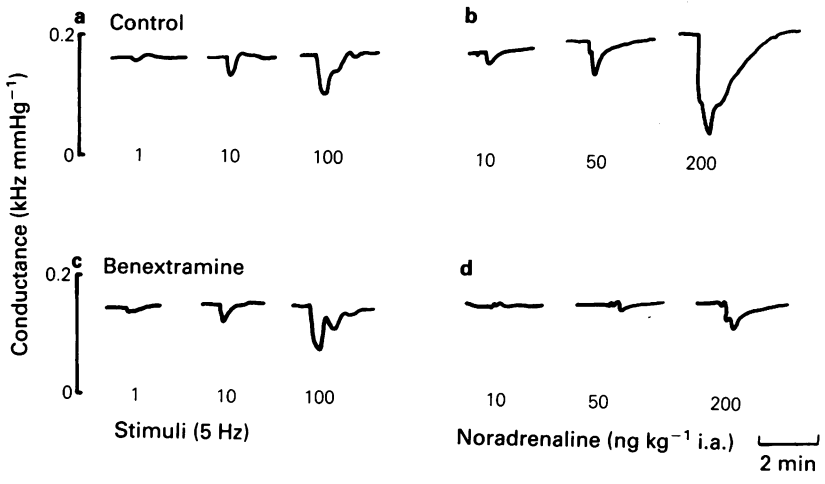

Figure 2 Traces showing the effect of benextramine (10 mg kg^{-1}) on the hind limb vascular conductance responses to lumbar sympathetic nerve stimulation (a and c) and to intra-arterial noradrenaline (b and d).

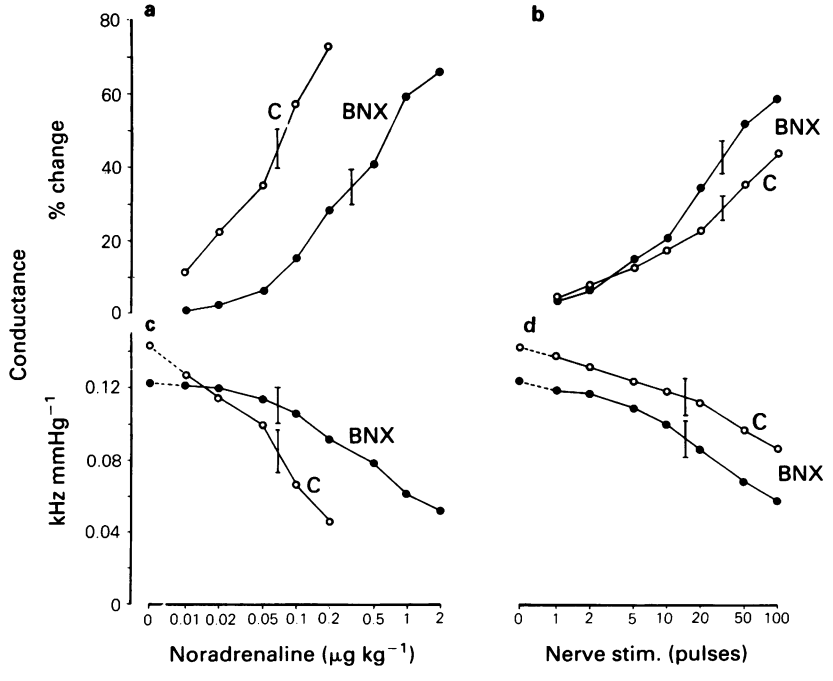

Figure 3 Effect of benextramine (BNX) on the responses to intra-arterial noradrenaline (a and c) and lumbar sympathetic nerve stimulation (b and d). Responses expressed as absolute hind limb vascular conductance (c and d) and converted to % change from resting (a and b). Responses before (O, C) and 60 min after benextramine (10 mg kg^{-1}) (●) are shown. Vertical lines indicate s.e.mean, analysis of variance (n = 4).

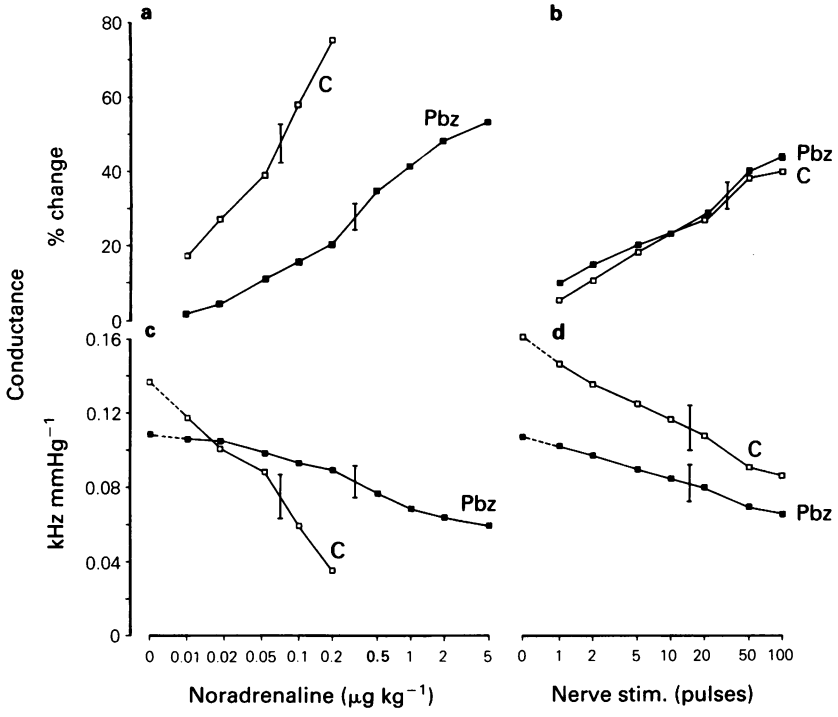

Figure 4 Effect of phenoxybenzamine (Pbz) on the responses to intra-arterial noradrenaline (a and c) and lumbar sympathetic nerve stimulation (b and d). Responses expressed as absolute hind limb vascular conductance (c and d) and converted to % change from resting (a and b). Responses before (□, C) and 60 min after phenoxybenzamine (5 mg kg^{-1}) (■) are shown. Vertical lines indicate s.e.mean, analysis of variance ($n = 4$).

responses to 1 and 2 stimuli 60 min after phenoxybenzamine were significantly less than the first responses (paired t test) but the other responses were not different. The overall difference between the first and second curves in these animals was significantly less than the difference in the benextramine-treated group but not than that in the time control group, indicating that phenoxybenzamine induced a slight depression of the responses to nerve stimulation.

Phentolamine

Phentolamine (0.5 mg kg^{-1}) caused an increase in the resting blood pressure and a decrease in the hind limb vascular conductance. The responses to lumbar sympathetic nerve stimulation and to injected noradrenaline were greatly reduced (Figure 5). Both the vasoconstrictor effect and the depression of the responses to nerve stimulation by phentolamine were partly reversed by yohimbine (1 mg kg^{-1}), but the inhibition of the responses to injected noradrenaline was not reversed.

Reflex stimulation

In conscious rabbits the vasoconstriction elicited by the nasopharyngeal reflex was very reproducible. Ganglionic blockade with mecamylamine (10 mg kg^{-1}) abolished the responses. Benextramine (10 mg kg^{-1}) caused no significant change in the blood pressure of these rabbits and did not depress the reflex responses (Figure 6).

In three experiments it was found that blowing ammonia-saturated air into the nostrils of the anaesthetized rabbits elicited the nasopharyngeal reflex. The cardiovascular responses were accompanied by attempted sneezing. Phenoxybenzamine (5 mg kg^{-1}), but not benextramine (10 mg kg^{-1}) abolished both the cardiovascular and sneezing responses to ammonia. Phenoxybenzamine (5 mg kg^{-1}) also abolished the vasoconstriction elicited by the nasopharyngeal reflex in the conscious rabbit.

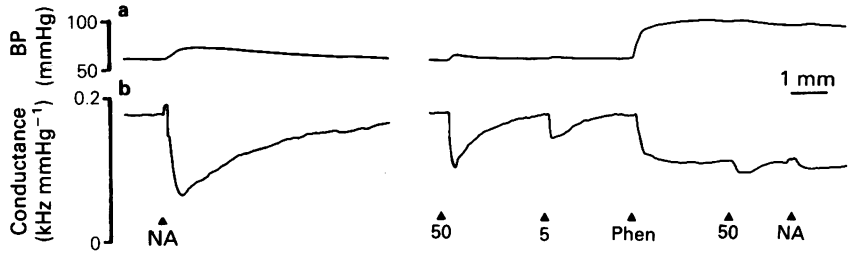

Figure 5 Traces showing the effect of phentolamine (Phen, 0.5 mg kg^{-1}) on (a) blood pressure (BP), (b) hind limb vascular conductance and the responses to noradrenaline (NA, 0.1 μg kg^{-1}, i.a.) and lumbar sympathetic nerve stimulation (50 pulses).

In vitro

Large and small blood vessels appear to have different receptor reserves, resulting in the potential for differential inhibition by an irreversible antagonist such as benextramine (See Discussion). This possibility was examined by treating 3 rabbits with benextramine (10 mg kg^{-1}) 60 min before death, and comparing the effect of this treatment on the α-adrenoceptor responses in the isolated aorta and small mesenteric artery with the responses of tissues from untreated animals. The control pieces of small mesenteric artery required a large concentration of noradrenaline for response, but the tissues from the benextramine- treated animals were completely unresponsive to noradrenaline (up to 300 μM), even in the presence of propranolol (1 μM) (Figure 7). These tissues were all responsive to potassium and angiotensin II. The aorta was much more sensitive to noradrenaline than the mesenteric vessel and the benextramine treatment only caused a rightward shift of the concentration-response curve (Figure

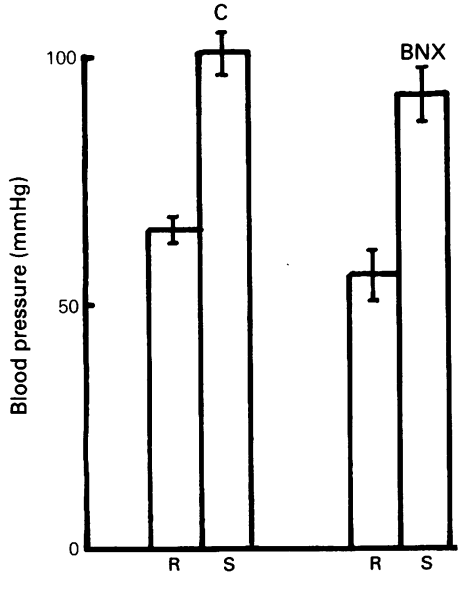

Figure 6 Effect of benextramine (BNX, 10 mg kg^{-1}) on the nasopharyngeal reflex elicited by smoke in conscious rabbits. Blood pressure at rest (R) and at the peak of the response (S) before (C) and after benextramine is shown. Vertical lines indicate s.e.mean, $n = 3$.

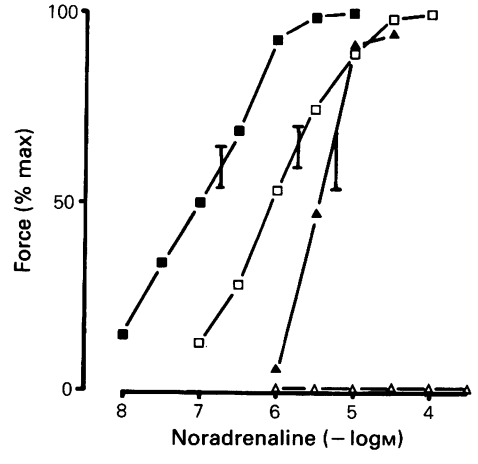

Figure 7 Noradrenaline concentration-response curves in aortae (■, □) and small mesenteric arteries (▲, △) from untreated rabbits (■, ▲) and benextramine (10 mg kg^{-1})-treated rabits (□, △). Vertical lines indicate s.e.mean, analysis of variance ($n = 4$).

7). The responses to noradrenaline of the aorta from the benextramine-treated animals were abolished by *in vitro* treatment with benextramine (10 μM, 30 min, 30 min washout).

Discussion

In these experiments the vascular response to sympathetic nerve stimulation persisted after a substantial reduction by benextramine of the responses of the vascular bed to injected noradrenaline. The persistence of the responses to sympathetic nerve stimulation after treatment with α-adrenoceptor blocking agents is consistent with a role for non-α-adrenoceptors in sympathetic vasoconstriction. If the sympathetic neurotransmitter is noradrenaline, then these non-α-adrenoceptors are likely to be the same receptors as those demonstrated in electrophysiological studies (Holman & Surprenant, 1980; Hirst & Neild, 1980; 1981; Hirst *et al.*, 1982; Itoh *et al.*, 1983) which have been named 'γ-receptors'. Alternately, if the neurotransmitter is a purine or related compound, then noradrenaline would appear to have little role in vasoconstriction in the rabbit hind limb bed.

Lumbar sympathetic chain stimulation

Benextramine caused no diminution of the vasoconstriction response to sympathetic nerve stimulation. It might be argued that this result is because of increased transmitter release after benextramine treatment through blockade of presynaptic α-adrenoceptors. This effect, if it occurred, would cancel a part-reduction of the responses. However, this was not the case in these experiments because in such a situation the response to one pulse (where no autoinhibitory feedback can occur) would have been reduced by the benextramine. This did not happen. Interruption of the autoinhibitory feedback system was, however, probably responsible for the significant enhancement by benextramine of the responses to longer trains of stimuli.

Phenoxybenzamine did cause a significant reduction of the responses to lumbar sympathetic chain stimulation. This difference between the drugs may have been due to a non-specific depression of the vascular responsiveness by phenoxybenzamine, as alluded to by Glick *et al.* (1967). Bevan *et al.* (1963) showed that phenoxybenzamine can inhibit the vasoconstrictor effects of potassium on rabbit aortic strips and McPherson *et al.* (1985) suggested that phenoxybenzamine is able to block receptor-operated calcium channels. These actions may be the reason for the depression of the responses to lumbar sympathetic chain stimulation by phenoxybenzamine. Whatever the mechanism is, it does not appear to involve blockade of α-adrenoceptors because in two experiments where stimulus-response curves were constructed at 120 min as well as 60 min after the injection of phenoxybenzamine, we found that the responses had recovered considerably after the extra hour. This recovery can not have been due to a reversal of α-adrenoceptor blockade as Hamilton *et al.* (1984) found that the recovery of α-adrenoceptor function in rabbits had a half-time of between 0.9 and 1.4 days.

It might be argued that the amount of inhibition of the α-adrenoceptors caused by the antagonist was insufficient to inhibit neurotransmission through these receptors. This cannot be answered directly as the doses of antagonists used in this study are about as large as can be used without concern for the toxic and non-specific effects of the drugs. However, there are some reasons to expect that activation of α-receptors by the neurotransmitter should be readily prevented by α-receptor blockers. In the case of exogenous agonist, the agonist concentration can be increased as necessary to achieve a response, but where the agonist is released by sympathetic nerves, there is a definite limit to the concentration of agonist that can be obtained. Black *et al.* (1980) described the kinetics of antagonism of tyramine responses in rat heart by β-adrenoceptor antagonists and showed that the maximum response is readily depressed, even by competitive antagonists. The kinetics of the antagonism of nerve released noradrenaline should be more like those of indirectly acting agonist, as described by Black *et al.* (1980), than like the classical competitive kinetics of antagonism of exogenous noradrenaline. Another reason for expecting the responses to sympathetic nerve stimulation to be more readily blocked than the responses to exogenous noradrenaline is relevant to competitive antagonists. Noradrenaline released from sympathetic nerves is present at the receptors for only a very short time, certainly insufficient time for equilibrium at the receptors of the noradrenaline and the antagonist to occur because the half dissociation time of a potent antagonist can be tens of minutes (Rang, 1966; Ginsborg & Stephenson, 1974; Ruffolo & Patil, 1978). Exogenous noradrenaline is present longer and thus can at least partially equilibrate with the antagonist, exposing receptors with which nerve released noradrenaline cannot interact. This effect is most marked in *in vitro* experiments where full equilibration between agonist and antagonist is attempted.

The experiments using blood vessels removed from animals treated with benextramine are also relevant to this question. The responses to noradrenaline of the small mesenteric arteries were abolished, but the responses of the aorta were only moderately inhibited. This difference is almost certainly a result of the relative receptor reserves of the two tissues. The threshold concentration of noradrenaline for contraction of the mesenteric vessel is much higher than that

of the aorta. A low sensitivity to noradrenaline is also evident with small (150–250 μm) arteries of the rabbit hind limb skeletal muscle and skin (threshold 0.1–0.3 μM in the presence of propranolol, J.A. Angus personal communication). It appears that a relatively low sensitivity to noradrenaline is a common property of small arteries (Edvinsson & Owman, 1974; Duckles & Bevan, 1979; Duling *et al.*, 1981; Mulvany *et al.*, 1982). This should make these vessels more sensitive to covalent α-adrenoceptor blockers than vessels which have larger receptor reserves. This being the case, it is likely that the 10 fold shift of the noradrenaline dose-response curve by benextramine in these experiments is a vast underestimation of the effect of benextramine on the arterioles. This may increase confidence in the adequacy of α-adrenoceptor blockade in these experiments, and it also raises the interesting possibility that changes of tone in large (>250 μm) vessels mediate changes in vascular resistance, at least to intraluminal noradrenaline.

Flavahan *et al.* (1985) investigated sympathetic nerve mediated vasoconstriction in the pithed rat. These investigators found that half of the pressor effect of the nerve stimulation was resistant to blockade by prazosin. The residual responses were also resistant to blockade by the α_2-adrenoceptor blocker rauwolscine. The authors concluded that only half of the pressor effect of sympathetic nerve stimulation is due to the activation of α-adrenoceptors. This may be an underestimation of the role played by non-α-receptors. The conclusions of Flavahan *et al.* (1985) rest on the effect of prazosin. In addition to its well known α_1-adrenoceptor blocking action, this drug has the ability to dissociate the smooth muscle cell contraction from the membrane potential (Hirst *et al.*, 1985). This effect of prazosin could be expected to inhibit the responses to sympathetic nerve stimulation if these are mediated by non-α-receptors which rely on membrane depolarization for initiating contraction, but not the responses to exogenous noradrenaline which rely more on pharmacomechanical coupling for contraction. This situation allows prazosin to inhibit the sympathetic nerve responses through a non-receptor action and the responses to noradrenaline through blockade of α-adrenoceptors. This possibility is discounted by Flavahan *et al.*, on the grounds that they used 'doses of prazosin that are sufficiently low' that the receptor action of the drug can account for all of the observed effects. However, the dose used (1 mg kg^{-1}) was 10 times more than Timmermans & van Zwieten (1980) needed to cause a 53 fold rightward shift of the dose-response curves of phenylephrine in the pithed rat. We have found that prazosin (1 mg kg^{-1}) reduced to about half the pressor response to the nasopharyngeal reflex in the rabbit. Given the inability of benextramine to reduce this response, this effect of prazosin seems unlikely to be due to an action of α-receptors. Prazosin (1 mg kg^{-1}) also causes a large transient hypotension in rabbits, through an action not at α-adrenoceptors (Hamilton *et al.*, 1985), and in ganglion blocked rabbits causes a smaller but sustained fall in blood pressure (C.E. Wright, personal communication). Tanz & Fairfax (1985) found that prazosin inhibited the vasoconstrictor effect of ouabain in the pig coronary artery at a concentration of less than 1 pM. It seems that prazosin is more potent at inducing depression through an unknown mechanism than it is at blocking α_1-adrenoceptors. It can be seen from this that the relative roles of α- and non-α-receptors in sympathetic vasoconstriction cannot be decided by the use of prazosin. The finding that the responses of rat mesenteric arteries to field stimulation of the sympathetic nerves are entirely mediated by α-adrenoceptors confirms that at least a part of the half reduction of responses by prazosin in the experiments of Flavahan *et al.* (1985) was due to α-receptor blockade. This points to an inter-species difference between the sympathetic vasoconstrictor mechanisms of the rat and rabbit.

Bell (1985) performed similar experiments using lumbar sympathetic chain stimulation in non-ganglion blocked dogs. In these experiments the responses to lumbar nerve stimulation were depressed by phentolamine (0.5 mg kg^{-1}) to the same extent as were the responses to noradrenaline. These results led Bell to conclude that there was no evidence for neurotransmission via non-α-receptors *in vivo*. There are some criticisms of the experiments of Bell (1985) that can be made. Phentolamine is a partial agonist at α_2-adrenoceptors in the rabbit (Angus & Lew, 1985) and by activating these receptors causes both a large increase in blood pressure and inhibits the release of noradrenaline from sympathetic nerves. Vatner *et al.* (1985) demonstrated that in ganglion blocked dogs the total peripheral resistance was doubled by phentolamine, showing that agonist effects of this drug are manifest in the dog as well as the rabbit. The pressor effect of phentolamine is not seen in rabbits with an intact autonomic nervous system because the pressor effects of postsynaptic α_2-adrenoceptor stimulation are opposed by the depressor effects of inhibiting both the sympathetic nerves and the pressor action of any circulating catecholamines. Any imbalance between the pressor and depressor effects can be compensated for by baroreflexes. This is also likely to be the case in dogs. Bell (1985) found no sustained change in the resting blood pressure and hind limb flow on administration of phentolamine. However, the dogs were autonomically intact. The experiments of Bell (1985) are therefore complicated by the large but undetected changes in the resting state of the cardiovascular system of the dogs following the administration of phentolamine and by the potential presynaptic inhibitory action of phentolamine. However, the possibility of a difference

between the junctional receptor type of the rabbit and other species cannot be discounted.

Reflex sympathetic nerve stimulation

Stimulation of the sympathetic nervous system via reflexes has a major drawback for quantitative analysis of drug effects on the vascular neuroeffector junction. There is a large number of sites at which the drug may decrease the final response without actually interfering with the synapse on the smooth muscle. The function of the sensory receptors may be altered by the drug, either by changing the tone of the smooth muscle of the carotid sinus (Munch & Brown, 1985) or by some other mechanism. The drug may act in the brain to block a pathway of cardiovascular regulation, or to alter an input that is normally integrated with the baroreceptor signal. Transmission through the efferent pathway may be affected, and of course the functional state of the end organ may be altered.

The inhibition of the nasopharyngeal reflex responses by phenoxybenzamine does not appear to have been due to blockade of peripheral α-adrenoceptors, as α-adrenoceptor blockade by benextramine did not also cause the inhibition. It is likely that the abolition by phenoxybenzamine of the sneezing response to ammonia is a central nervous system action of the drug and, as a corollary to this, the effect of phenoxybenzamine on the cardiovascular component of this reflex is also probably mediated in the central nervous system. Furnace *et al.* (1985) found that phenoxybenzamine (5 mg kg^{-1}, i.v.) enters the rabbit forebrain in a sufficient concentration to interfere with prazosin and clonidine binding. Central nervous system α-adrenoceptors are considered to be involved in both the resting level of sympathetic tone and in cardiovascular reflexes (Kobinger, 1978; McCall & Humphrey, 1981; Korner & Angus, 1981). Benextramine enters the brain less well than phenoxybenzamine (Furnace *et al.*, 1985).

Glick *et al.* (1967) performed experiments in dogs in which they investigated the vasoconstrictor response of the hind limbs to carotid occlusion with and without α-adrenoceptor blockade. These authors treated the dogs with phenoxybenzamine (5 mg kg^{-1}) 3 days, 1 day and several hours before the experiment, giving a total of 15 mg kg^{-1} phenoxybenzamine. Even after this large total dose of phenoxybenzamine the responses to carotid occlusion were not completely blocked.

Thus the data presented show that, at least in the rabbit, sympathetic neural vasoconstriction is largely independent of α-adrenoceptors. Further work is necessary to determine the relevance of this finding to other species and to determine whether all vascular beds within an animal have the same or different mechanisms of neural vasoconstriction.

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Effects of α-adrenoceptor agonists on cardiac output and its regional distribution in the pithed rat

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1 Cardiac output, its distribution and tissue blood flows were determined with tracer microspheres in pithed rats during pressor responses elicited by either α_1-adrenoceptor agonists (cirazoline, phenylephrine) or α_2-adrenoceptor agonists (xylaxine, B-HT 933). Two doses were used for each of cirazoline and B-HT 933 and phenylephrine was investigated in the presence of propranolol (3 mg kg^{-1}). The rats were pithed under halothane anaesthesia.

2 Cardiac output was increased by xylazine, the higher dose of B-HT 933 and phenylephrine. Heart rate was increased by phenylephrine and the higher doses of both cirazoline and B-HT 933. Stroke volume was greater in those groups given xylazine, phenylephrine and the higher dose of B-HT 933 but was decreased in those animals given the higher dose of cirazoline.

3 Both α_2-adrenoceptor agonists increased the number of microspheres trapped in the lungs and the proportion of the cardiac output passing through the hepatic artery but decreased that flowing through the spleen and gastrointestinal tract. The higher dose of B-HT 933 also decreased the fraction of cardiac output flowing to the kidneys but kidney blood flow was maintained as a result of the increased cardiac output. Also, this treatment reduced blood flow in the epididimal fat pads.

4 Both α_1-adrenoceptor agonists increased the fraction of cardiac output received by the coronary vasculature but the only other effect on distribution common to these agents was an increase in the percentage of the cardiac output passing to the hepatic artery. Cirazoline decreased the proportion of cardiac output distributed to the gastrointestinal tract and spleen but the total fraction of cardiac output passing to the hepatosplanchnic region was maintained as a result of the increase to the hepatic artery.

5 Cirazoline markedly reduced the proportion of the cardiac output received by the kidneys and absolute flow in these organs was only 1.4% of control after the higher dose of this agonist but flow at the lower dose was maintained by the higher cardiac output.

6 It is concluded that there is a significant contribution to the pressor responses elicited by α-agonists resulting from an α-adrenoceptor-mediated increase in cardiac output that may result from greater heart rates or stroke volumes. Also, there is a differential distribution of α-receptor subtypes throughout the vasculature which is especially noticeable in the kidneys.

Introduction

It is now well established that α-adrenoceptors of both α_1- and α_2-subtypes exist postjunctionally in the vasculature of the rat and other species (McGrath, 1982). It is also accepted that stimulation of either population of receptors can bring about vasoconstriction (Digges & Summers, 1983; Yamamoto *et al.*, 1984). The systemic pressor responses mediated by these receptors have been widely described (Drew & Whiting, 1979; Docherty & McGrath, 1980; Flavahan *et al.*, 1985) but few investigations have attempted to elucidate the sites at which these agents cause the individual vasoconstrictions which produce the increased peripheral resistance leading to the development of the pressor response.

However, studies carried out on isolated organ preparations have shown that activation of α_1- and α_2-adrenoceptor subtypes summates to mediate vasoconstriction in a variety of organs including the

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hind limbs of the rat (Yamamoto *et al.*, 1984), cat and dog (Gardiner & Peters, 1982); the cat lung (Hyman & Kadowitz, 1985) and rabbit kidney (Hesse & Johns, 1984; see also Ruffolo *et al.*, 1981; Timmermans & Van Zwieten, 1981). Meanwhile, other investigators have demonstrated a vasoconstriction primarily mediated by α_1-adrenoceptors in the kidneys of the rat (Schmitz *et al.*, 1981), dog (Horn *et al.*, 1982; Wolff *et al.*, 1984) and cat (Drew & Whiting, 1979) while vasoconstriction of the canine coronary vessels appears to be mediated selectively by α_2-adrenoceptors (Kakihana *et al.*, 1985).

Of the range of vascular beds thus studied, uncertainty still exists about the precise nature of the receptors mediating vasoconstriction of the rat superior mesenteric arterial bed. Considerable evidence from studies of the isolated vasculature suggests that the vasoconstriction seen here is mediated entirely by means of α_1-adrenoceptors (Eikenburg, 1984; Yamamoto *et al.*, 1984; Hogestatt & Andersson, 1984; Agrawal & Daniel, 1985: Nichols & Hiley, 1985). Despite this, there is evidence for a mixed population of pharmacologically functional α_1- and α_2-adrenoceptors in this tissue; decreases in cardiac output distribution to this bed occur after administration of either methoxamine (an α_1-adrenoceptor agonist) or UK 14,304 (an α_2-adrenoceptor agonist) to the pithed rat (Hicks & Waldron, 1983; Waldron & Hicks, 1985).

Thus, there is much evidence for the existence of post-junctional adrenoceptors of both subtypes in several vascular beds but little is known about the relative contributions of these discrete vasoconstrictions or changes in cardiac output to the establishment of a pressor response. In view of this lack of knowledge of the co-ordinated haemodynamic responses resulting from the vasoconstrictor and other cardiovascular actions of α-adrenoceptor agonists and the uncertainty that surrounds the role of α_2-adrenoceptors in the rat superior mesenteric arterial vascular bed, we have carried out a study, using radioactive tracer microspheres in the pithed rat, of the haemodynamic changes brought about by the administration of selective α_1- and α_2-adrenoceptor agonists.

Methods

Determination of cardiac output and its distribution

Male Wistar rats weighing 220–250 g (Bantin & Kingman Ltd, Hull) were pithed under halothane anaesthesia by passing a 16 gauge steel needle through the orbit, through the foramen magnum and down into the spinal canal. Immediately after pithing, the rats were respired with air through a tracheal cannula by means of a respiratory pump (BioScience, Sheerness, U.K.) operating at 54 cycles min^{-1} with a volume of 20 ml kg^{-1}.

The right femoral artery was cannulated and connected to a Bell & Howell type 4-422-0001 transducer to measure systemic arterial blood pressure which was recorded on a Grass 79D polygraph. The left femoral artery was also cannulated and connected to a Braun Perfusor IV pump for the withdrawal of blood. With the aid of pressure monitoring, a cannula was passed down the right common carotid artery into the left ventricle. Drugs were administered through a cannula placed in the left external jugular vein and, when a sustained response had been obtained, 60,000–80,000 ^{113}Sn labelled microspheres (15 ± 3 μm; NEN, Boston, MA), suspended by ultrasonication in 0.3 ml saline containing 0.01% Tween 80, were injected into the ventricle over 20 s. Blood was withdrawn from the left femoral artery at a rate of 0.5 ml min^{-1} during and for 70 s after the microsphere injection. The circulation was stopped with an air embolism and the organs dissected out, weighed and placed in scintillation vials for counting in a Packard Autogamma 500 γ-counter. The number of counts in the blood sample was also determined and cardiac output and tissue blood flow were determined as described by McDevitt & Nies (1976).

Blood gases and pH were sampled in 19 animals (including 3 from each experimental group) by removing a sample of 125 μl from a femoral artery cannula 5 min before the administration of agonist or saline and 3 min after the microsphere injection. These samples were then placed into a Corning 166 micro blood gas analyser. The values obtained before and after the microsphere injection respectively were pH 7.41 ± 0.02 and 7.42 ± 0.02; PO_2 78 ± 1 and 78 ± 2 mmHg; PCO_2 33 ± 2 and 32 ± 2 mmHg. There were no significant differences between any of these values when assessed with Student's paired *t* test.

Drugs

All drugs were administered in saline in the form of a bolus injection of 0.5 ml followed by an infusion of 0.1 ml min^{-1}; the details are as follows. Cirazoline (Synthelabo, Paris): lower dose, 0.25 μg bolus, infusion 1 μg min^{-1}; higher dose, 10 μg bolus followed by 2 μg min^{-1}. Phenylephrine (Sigma, Poole, Dorset): 5 μg bolus, 0.4 μg min^{-1} infusion. Xylazine (Bayer U.K., Newbury, Berkshire): 0.5 mg bolus, 100 μg min^{-1} infusion. B-HT 933 (2-amino-6-ethyl-5,6,7,8-tetrahydro-4H-oxazolo-[4,5-d]-azepine dihydrochloride) Boehringer Ingelheim U.K., Bracknell, Berkshire): lower dose, 0.25 mg bolus, 100 μg min^{-1} infusion; higher dose, 1 mg bolus, 100 μg min^{-1} infusion. Control animals received a bolus injection and infusion of physiological saline. Animals which were given phenylephrine were pretreated with

Table 1 Effects of α-adrenoceptor agonists on blood pressure, cardiac index and heart rate in pithed rats

Group	*Diastolic pressure* (mmHg)	*Mean arterial pressure* (mmHg)	*Heart rate* (beats min^{-1})	*Cardiac index* (ml min^{-1} 100g b wt^{-1})	*Stroke volume* (ml)	*TPR* (mmHg ml^{-1} min 100g b wt)
Saline	46 ± 3	52 ± 4	234 ± 8			
	−1 ± 1	−1 ± 2	−4 ± 1	10.6 ± 1.4	0.121 ± 0.012	5.2 ± 0.5
Xylazine	47 ± 2	53 ± 2	249 ± 6			
	47 ± 5***	56 ± 6***	−5 ± 3	14.2 ± 0.7	0.156 ± 0.007*	7.0 ± 0.3
B-HT 933	44 ± 2	50 ± 3	234 ± 6			
(low dose)	52 ± 3***	59 ± 3***	0 ± 0	13.6 ± 1.7	0.162 ± 0.022	8.7 ± 0.9**
B-HT933	44 ± 2	50 ± 3	254 ± 9			
(high dose)	56 ± 4***	63 ± 5***	19 ± 6***	18.5 ± 0.9***	0.165 ± 0.015*	6.2 ± 0.5
Cirazoline	43 ± 2	48 ± 2	251 ± 12			
(low dose)	38 ± 3***	43 ± 3***	3 ± 3	12.0 ± 0.9	0.134 ± 0.009	8.0 ± 0.5*
Cirazoline	48 ± 2	55 ± 3	222 ± 8			
(high dose)	85 ± 5***	103 ± 5***	19 ± 6***	8.6 ± 1.1	0.082 ± 0.011*	21.7 ± 4.1***
Phenylephrine	48 ± 2	57 ± 2	237 ± 15			
and	60 ± 4†††	71 ± 5†††	34 ± 8†††	16.8 ± 1.9†††	0.166 ± 0.012††	8.8 ± 1.6†††
propranolol						
Saline and	44 ± 2	51 ± 3	246 ± 11			
propranolol	−1 ± 1	−2 ± 1	−4 ± 3	10.3 ± 0.6	0.120 ± 0.008	4.9 ± 0.2

Where two values are given in a column, the upper values are those immediately before administration of the agonist or saline. The lower value is the change between the basal value and that at the midpoint of the microsphere injection. Cardiac index is the absolute value determined by the tracer microsphere method. TPR is the total peripheral resistance calculated from cardiac index and mean arterial pressure during the microsphere injection, assuming central venous pressure to be zero. Significant differences between the saline control and the experimental groups were determined by analysis of variance: $*P<0.05$; $**P<0.01$; $***P<0.001$. Significant differences between the group given saline after propranolol and those given phenylephrine after propranolol were also determined by analysis of variance: $††P<0.01$; $†††P<0.001$. There were no significant differences between the two groups given saline. For all groups, $n = 8$.

propranolol (ICI Pharmaceuticals, Macclesfield, Cheshire), at a dose of 3 mg kg^{-1} i.v., 5 min before starting the administration of phenylephrine; these animals were compared with a control group similarly pretreated with propranolol.

The specificity of the agonists was assessed by administering to groups of 3 rats either (for α_1-adrenoceptor agonists) prazosin (50 μg kg^{-1}; Pfizer, Sandwich, Kent) or (for α_2-adrenoceptor agonists) yohimbine (0.75 mg kg^{-1}; Sigma) 5 min before the start of one of the above infusions. When an equilibrium response had been obtained, the second antagonist was administered in order to determine the extent to which the residual response was dependent on cross-stimulation of receptors.

Statistical comparison

All results are given as the mean ± s.e.mean and the statistical significance between groups was assessed by one way, random block, analysis of variance followed by the least significant difference procedure (Snedecor & Cochran, 1980).

Table 2 Percentage of the cardiac output distributed to the various organs after administration of α-adrenoceptor agonists

Group	*Heart*	*Lungs*	*Kidneys*	*Testes*	*Epididimides*
Saline	6.1 ± 1.8	3.7 ± 0.8	14.6 ± 1.5	1.7 ± 0.1	0.23 ± 0.03
Xylazine	7.8 ± 0.6	7.4 ± 1.4**	12.2 ± 0.8	1.3 ± 0.2	0.24 ± 0.02
B-HT 933 (low dose)	6.6 ± 0.9	8.7 ± 1.0**	12.0 ± 0.6	1.0 ± 0.1	0.25 ± 0.03
B-HT 933 (high dose)	7.2 ± 0.6	10.2 ± 1.0***	10.1 ± 0.4*	1.1 ± 0.2	0.23 ± 0.02
Cirazoline (low dose)	9.9 ± 1.5*	2.5 ± 0.3	9.9 ± 1.4***	1.7 ± 0.2	0.24 ± 0.02
Cirazoline (high dose)	31.6 ± 2.9***	4.5 ± 1.0	0.3 ± 0.1***	2.0 ± 0.4	0.24 ± 0.06
Phenylephrine and propranolol	7.4 ± 0.6†	7.1 ± 0.6††	11.8 ± 0.9	1.3 ± 0.1	0.26 ± 0.02
Saline and propranolol	3.9 ± 0.4	3.1 ± 0.6	11.3 ± 0.7*	1.5 ± 0.1	0.24 ± 0.02

	Liver	*Spleen*	*G.I.T.*	*Total hepatosplanchnic*
Saline	4.8 ± 0.5	0.96 ± 0.11	20.0 ± 1.0	26.8 ± 1.1
Xylazine	11.6 ± 0.6***	0.51 ± 0.05***	16.1 ± 0.6**	28.2 ± 0.5
B-HT 933 (low dose)	8.0 ± 0.8*	0.59 ± 0.07***	16.9 ± 0.6**	25.5 ± 0.8
B-HT 933 (high dose)	10.2 ± 0.6***	0.60 ± 0.06***	17.3 ± 0.9*	26.9 ± 1.4
Cirazoline (low dose)	12.6 ± 1.4***	0.45 ± 0.05***	17.1 ± 0.9*	30.8 ± 1.4
Cirazoline (high dose)	6.3 ± 2.0	0.09 ± 0.02***	11.0 ± 1.1***	17.4 ± 0.3***
Phenylephrine and propranolol	8.6 ± 1.1††	0.72 ± 0.06	20.3 ± 0.8	29.8 ± 1.4
Saline and propranolol	4.1 ± 0.3	0.80 ± 0.04	21.7 ± 0.9	26.6 ± 1.0

G.I.T. = gastrointestinal tract

Significant differences between the saline control and the experimental groups were determined by analysis of variance: *$P<0.05$; **$P<0.01$; ***$P<0.001$.

Significant differences between the group given saline after propranolol and those given phenylephrine after propranolol were also determined by analysis of variance: †$P<0.05$; ††$P<0.01$. There were no significant differences between the two groups given saline. For all groups, $n = 8$.

Results

There were no significant differences between the groups in the pre-infusion values of diastolic blood pressure, mean arterial pressure or heart rate. Table 1 shows that all the agonists produced significant increases in both diastolic and mean arterial pressure. The changes brought about by the two doses of B-HT 933 were not significantly different. However, it may be seen from the heart rate data that the higher doses of both B-HT 933 and cirazoline increased heart rate but not to the same extent as the single dose of phenylephrine used. In the experiments to confirm agonist specifity it was found that after 50 $\mu g\,kg^{-1}$ prazosin the increases in diastolic pressure and heart rate were, for phenylephrine, 3.3 ± 0.3 mmHg and 5 ± 3 beats min^{-1}; for the lower dose of cirazoline, 4.3 ± 5.5 mmHg and 3 ± 8 beats min^{-1}; and, for the higher dose of cirazoline, 27.6 ± 2.4 mmHg and 2 ± 13 beats min^{-1} ($n = 3$ for each measurement). Yohimbine at 0.75 $mg\,kg^{-1}$ had no effect on the residual response with the higher dose of cirazoline. After 0.75 $mg\,kg^{-1}$ yohimbine, the changes in diastolic blood pressure and heart rate were, for the lower dose of B-HT 933, 8.0 ± 0.6 mmHg and -3 ± 3 beats min^{-1}; for the higher dose of B-HT 933, 13.0 ± 0.6 mmHg and -7 ± 9 beats min^{-1}; and, for xylazine, 23.8 ± 4.4 mmHg and -6 ± 6 beats min^{-1}. Subsequent administration of prazosin had no effect on the responses to B-HT 933 but reduced the residual response to xylazine by 3.7 ± 2.6 mmHg with no effect on heart rate ($n = 3$ for each group).

It may be seen from Table 1 that, in the absence of antagonists, cardiac index was significantly greater in three groups of animals, that is those given either xylazine (in which it was 34% higher than in the control animals), the higher dose of B-HT 933 (74% higher than control) or phenylephrine (58% greater than control). The mean cardiac index in those animals given the lower dose of B-HT 933 was greater than the control by 28% but the difference was not significant. Table 1 also shows that the stroke volume was significantly greater than control in those animals given xylazine, the higher dose of B-HT 933 and phenylephrine. It is noteworthy that the high dose of cirazoline produced a significant reduction in stroke volume.

Table 2 shows that the two α_2-adrenoceptor agonists had no effect on the percentage of cardiac output passing to the heart itself. However, both compounds apparently increased the fraction of cardiac output passing to the lungs. Both xylazine and B-HT 933 increased the proportion of the cardiac output flowing directly to the liver through the hepatic artery but decreased that passing to the spleen and gastrointestinal tract.

It can also be seen from Table 2 that, apart from a decrease in the fraction passing to the kidneys, propranolol (3 $mg\,kg^{-1}$, i.v.) had no significant effect on the pattern of cardiac output distribution. Both α_1-adrenoceptor agonists, cirazoline and phenylephrine, significantly increased the percentage of the cardiac output received by the heart; in the case of the higher dose of cirazoline, nearly one-third of the microspheres were trapped in the heart and this organ experienced a five fold increase in the fraction of cardiac output it received. Apart from their effects on distribution to the cardiac vascular bed, the only other change brought about in common by the two α_1-adrenoceptor agonists was an increase in the fraction of the cardiac output passing through the hepatic artery; however, it must be pointed out that the increase with cirazoline was not dose-related and did not occur with the higher dose. Cirazoline at both doses reduced the fraction of cardiac output received by the gastrointestinal tract and the spleen in a dose-dependent manner, with the changes in the spleen being the most pronounced. However, because of the increase in hepatic arterial supply the total hepato-splanchnic share of the cardiac output was not significantly different from control in the group treated with the lower dose of cirazoline. The most notable effect of cirazoline was the reduction in the distribution of the cardiac output to the kidneys; with the lower dose there was a reduction to 68% of the control value but with the higher dose the decrease was to 2%.

Table 3 gives details of tissue blood flows and it can be seen that, despite the lower fraction of cardiac output going to the kidneys with the higher dose of B-HT 933, the greater cardiac index resulted in renal blood flow being unchanged. The increased cardiac output also resulted in gastrointestinal blood flow being significantly greater than the control despite the reduction of 2.7% in the proportion of cardiac output passing to this vascular bed. The 74% greater cardiac index also accounts for the increased flow through the epididimides (increased by 69%) and the pectoral skeletal muscle (increased by 72%). The higher dose of B-HT 933 significantly reduced blood flow in the epididimal fat pad and, although it did not reach significance, the mean flow with the lower dose was nearly 50% less than that in the saline control.

Although both doses of cirazoline reduced the fraction of cardiac output passing to the kidneys, it may be seen in Table 3 that renal blood flow was not significantly affected by the lower dose of cirazoline (because the cardiac index was elevated) but the higher dose reduced flow to 1.4% of that observed in the saline control group. Splenic blood flow was only 10% of the control during the pressor response induced by this higher dose of cirazoline and flow in the gastrointestinal tract was decreased by 50%. The only significant decrease in organ blood flow resulting from

Table 3 Organ blood flow (ml min^{-1} g^{-1} organ wt) after administration of α-adrenoceptor agonists

Group	*Heart*	*Lungs*	*Kidneys*	*Testes*	*Epididimides*
Saline	2.31 ± 0.98	0.96 ± 0.23	1.99 ± 0.38	0.16 ± 0.01	0.084 ± 0.008
Xylazine	3.29 ± 0.34	2.29 ± 0.52**	2.15 ± 0.09	0.19 ± 0.01	0.122 ± 0.016
B-HT 933 (low dose)	2.62 ± 0.33	2.51 ± 0.39**	2.06 ± 0.18	0.14 ± 0.02	0.090 ± 0.017
B-HT 933 (high dose)	3.72 ± 0.40	3.90 ± 0.45***	2.18 ± 0.10	0.21 ± 0.05	0.142 ± 0.021**
Cirazoline (low dose)	3.46 ± 0.50	0.66 ± 0.09	1.65 ± 0.25	0.19 ± 0.03	0.088 ± 0.008
Cirazoline (high dose)	7.36 ± 0.73***	0.88 ± 0.30	0.03 ± 0.013***	0.16 ± 0.03	0.06 ± 0.017
Phenylephrine and propranolol	3.98 ± 0.51†††	2.93 ± 0.36†††	2.68 ± 0.23††	0.22 ± 0.02	0.124 ± 0.007†
Saline and propranolol	1.16 ± 0.14	0.69 ± 0.14	1.68 ± 0.10	0.16 ± 0.01	0.075 ± 0.005

	Fat	*Muscle*	*Skin*	*Liver*	*Spleen*	*G.I.T.*
Saline	0.026 ± 0.003	0.053 ± 0.007	0.062 ± 0.006	0.19 ± 0.006	0.31 ± 0.06	0.38 ± 0.06
Xylazine	0.019 ± 0.003	0.079 ± 0.009	0.056 ± 0.004	0.41 ± 0.03*	0.22 ± 0.02	0.46 ± 0.01
B-HT 933 (low dose)	0.014 ± 0.004	0.075 ± 0.020	0.057 ± 0.014	0.39 ± 0.12*	0.27 ± 0.05	0.48 ± 0.07
B-HT 933 (high dose)	0.009 ± 0.002*	0.091 ± 0.010*	0.109 ± 0.017*	0.43 ± 0.04**	0.38 ± 0.06	0.64 ± 0.06***
Cirazoline (low dose)	0.018 ± 0.002	0.063 ± 0.007	0.68 ± 0.008	0.39 ± 0.05*	0.18 ± 0.03*	0.46 ± 0.03
Cirazoline (high dose)	0.015 ± 0.011	0.032 ± 0.006	0.024 ± 0.006	0.14 ± 0.05	0.03 ± 0.01***	0.19 ± 0.03***
Phenylephrine and propranolol	0.017 ± 0.002	0.075 ± 0.009	0.124 ± 0.038†	0.39 ± 0.06†	0.52 ± 0.06†††	0.77 ± 0.04††
Saline and propranolol	0.017 ± 0.003	0.069 ± 0.008	0.068 ± 0.007	0.10 ± 0.01	0.27 ± 0.03	0.47 ± 0.03

G.I.T. = gastrointestinal tract.
Significant differences between the saline control and the experimental groups were determined by analysis of variance: *$P<0.05$; **$P<0.01$; ***$P<0.001$. Significant differences between the group given saline after propranolol and those given phenylephrine after propranolol were also determined by analysis of variance: †$P<0.05$; ††$P<0.01$; †††$P<0.001$. There were no significant differences between the two groups given saline. For all groups, $n = 8$.

the administration of the lower dose of cirazoline was of 42% occurring in the spleen. This dose of cirazoline increased hepatic arterial blood flow by 105%. Although neither dose of cirazoline significantly affected skin blood flow, phenylephrine increased it by 82% and much of this can be accounted for by the 63% increase in cardiac output. Phenylephrine also increased blood flow in the heart (43%), kidneys (60%), epididimides (65%), hepatic artery (290%), spleen (93%) and the gastrointestinal tract (64%). As with xylazine and B-HT 933, phenylephrine significantly increased the fraction of the injected microspheres trapped in the lungs, in this case by 325%.

Discussion

The α_2-adrenoceptor agonists, xylazine and B-HT 933, both increased cardiac output as did one of the α_1-adrenoceptor agonists, phenylephrine. Gerold & Haeusler (1983) have also found increases in cardiac output in pithed rats with both α-$_1$ and α_2-adrenoceptor agonists. We found that with xylazine, the increase in the stroke volume (29%) was very similar to the increase in cardiac index (34%) as would be expected since heart rate was unchanged by this agent. For the other two compounds, the 36% increases in stroke volume accounted for just under half the 74% increase in cardiac index with the high dose of B-HT 933 and approximately two-thirds of 58% increase in cardiac index given by phenylephrine. Both α_2-adrenoceptor agonists also apparently increased the fraction of cardiac output distributed to the lungs, although it should be noted that the microspheres trapped here include not only those passing through the bronchial arteries but also those passing through peripheral arteriovenous shunts and becoming trapped after returning through the venous system and the right heart. Hence the increased proportion of the injected microspheres trapped in the lungs may represent an increase in shunting. Xylazine and B-HT 933 also increased the fraction of cardiac output flowing through the hepatic artery. These haemodynamic changes, common to both α_2-adrenoceptor agonists, appear to be dose-dependent for B-HT 933.

The two α_2-adrenoceptor agonists also reduced the proportion of the cardiac output delivered to the spleen and gastrointestinal tract. However, the increased hepatic arterial supply compensated for the reduction in the proportion of the cardiac output passing through the hepatic portal vein such that the total fraction of the cardiac output received by the liver was unchanged. These observations are in good agreement with those of Waldron & Hicks (1985) who showed that the α_2-adrenoceptor agonist, UK 14,304, reduced the fractions of the cardiac output reaching only the mesenteric vascular bed and the tail.

It is not easy to reconcile these observations with *in situ* and *in vitro* studies on the perfused mesenteric bed in which α_2-adrenoceptor agonists have been found to be without vasoconstrictor action, even at very high doses. Fiotakis & Pipili (1983) reported that UK 14,304 had no vasoconstrictor effect in isolated tissue perfused with physiological salt solution. Also, we have found only a very small and inconsistent vasoconstriction with xylazine (in doses up to 1 mg), clonidine or B-HT 933 in the *in situ* blood perfused mesenteric bed in intact anaesthetized or pithed rats (Nichols, 1985; Nichols & Hiley, 1985). Redistribution of cardiac output away from a bed without vasoconstriction in that region could only occur if other parts of the vasculature experienced vasodilatation. This does not seem a likely explanation with the agents studied here since calculation of organ resistances, using the mean arterial pressure as inflow pressure and zero as venous outflow pressure (which might not be justified in view of the changes occurring in stroke volume), showed that all the significant changes took the form of increases in resistance with the exception of the increase in coronary flow with the higher dose of cirazoline (see below) and the increases in hepatic arterial flow with xylazine and the lower dose of cirazoline where vascular resistance fell from 397 ± 66 (control) to 247 ± 12 (xylazine) and 260 ± 26 (cirazoline) mmHg min ml^{-1}g tissue. Thus, there do appear to be α_2-adrenoceptors mediating an increase in resistance to flow in the mesenteric vascular bed and it would seem that these are not detectable under the perfusion conditions hitherto used on this bed.

The α_1-adrenoceptor agonists used in this study, cirazoline and phenylephrine, did not produce parallel responses. The kidneys, spleen, gastrointestinal tract and the total hepatosplanchnic arcade were sensitive to vasoconstriction mediated only by cirazoline. With the exception that Hicks & Waldron (1983) also found that methoxamine constricted the skeletal muscle vascular beds, there is general agreement between the studies with these two agonists. It was also apparent that all of the changes in blood flow observed here in response to cirazoline administration were dose-dependent. However, it must be noted that the higher dose of cirazoline caused a generalized vasoconstriction in most of the tissues studied and particularly to the kidneys, where blood flow was reduced to 2% of that in saline-treated controls. In the same group of animals it was also found that 33% of the injected microspheres were trapped in the heart. This may represent sparing of cardiac vasculature by α_1-adrenoceptors and, indeed, calculated cardiac resistance fell from 44.3 (control) to 23.3 mmHg min ml^{-1} g tissue in these rats. In view of the large increase in total peripheral resistance underlying the pressor response seen with the larger dose of cirazoline it is interesting to note that the stroke volume was significantly lower

than in the control animals. However, the trapping of so great a proportion of the injected microspheres in the heart coupled with a massive increase in peripheral resistance is likely to distort the haemodynamic effects and caution must be observed in interpreting these results.

In contrast to the widespread vasoconstriction caused by cirazoline, phenylephrine failed to bring about reductions in the fraction of the cardiac output distributed to any of the tissues studied. The only similarities in the actions of phenylephrine and cirazoline appear to be increases in the proportions of the cardiac output received by the heart and the liver. In their study, Waldron & Hicks (1985) found methoxamine, at a dose giving a mean pressor response of 68 mmHg, caused an increase in the proportion of the cardiac output going to the heart and a decrease in that received by the kidneys, mesentery, skeletal muscle and the spleen. The responses to this agonist thus resemble more closely those we found for cirazoline than for phenylephrine.

It is not clear why there is this lack of parallelism between the effects of the two α_1-adrenoceptor agonists studied here, especially since the lower dose of cirazoline gave rise to a pressor response lower than that given by the single dose of phenylephrine used. Part of the difference might be due to β-adrenoceptor stimulation by phenylephrine underlying the increase in cardiac output observed with the phenethylamine agonist. However, propranolol was present in those animals to which phenylephrine was given at a dose of $3\,mg\,kg^{-1}$ and we found no change in heart rate in animals given phenylephrine after both propranolol and prazosin. Flavahan & McGrath (1981a) reported that $1\,mg\,kg^{-1}$ propranolol abolished the depressor response unmasked by the administration of prazosin to pithed rats and antagonized the increase in heart rate. In a later paper they reported that $1\,mg\,kg^{-1}$ propranolol had no effect on the heart rate responses to phenylephrine at doses equal to, or less than, $10\,\mu g\,kg^{-1}$. Thus it is likely that the responses we obtained to phenylephrine were independent of its potential for activating β-adrenoceptors.

Not only phenylephrine but also cirazoline produced tachycardia in our experiments and α_1-adrenoceptors mediating such effects have been reported (Flavahan & McGrath, 1981b; McGrath *et al.*, 1982). However, α_2-mediated increases in heart rate have not been reported previously and it is possible that the chronotropic responses we observed to the higher dose of B-HT 933 were the consequence of it activating both types of α-receptors with only α_1-adrenoceptors participating in this response. Against this, it should be noted that the higher doses of both B-HT 933 and cirazoline gave identical increases in heart rate, but changes in stroke volume were opposite for the two drugs and the α_2-adrenoceptor agonist gave a much smaller pressor effect. Also, in our pilot experiments to verify agonist specificity, there was no change in heart rate with B-HT 933 in rats previously given yohimbine. Thus, in view of this specificity of B-HT 933 for α_2-adrenoceptors (van Meel *et al.*, 1981), it is unlikely that the chronotropic response is due to activation of α_1-adrenoceptors on the myocardium.

When considering blood flow to various organs it is noticeable that the most widespread changes are seen in response to phenylephrine administration. Except for the heart, the hepatic artery and the spleen, the changes were similar in magnitude to the 58% in cardiac output and thus it is likely that, apart from these flows, most of these increases may be simply attributed to the enhanced cardiac output. Similar considerations apply to the blood flow responses to the higher dose of B-HT 933, a treatment which resulted in an increase in cardiac index (of 74%) somewhat greater than that produced by phenylephrine. There were increases in flow through the epididimides (69%), skeletal muscle (72%), skin (76%), liver (126%) and gastrointestinal tract (68%) and a decrease in flow, of 65%, to the epididimal fat pad. Hence, most of the increases in organ blood flow are a product of the increased cardiac output seen with these drugs. For both, this is a product of a positive chronotropic effect and an increase in stroke volume and, although α_1-adrenoceptors mediating an increase in contractility have been described (Wagner & Brodde, 1978; Schumann, 1980), no such effect has been described for α_2-adrenoceptors. The increases in stroke volume with xylazine and B-HT 933 could result from increased venous return to the heart due to venoconstriction and this explanation has been advanced previously for the increase in cardiac output observed in pithed rat and cats by Kalkman *et al.* (1974) after administration of the α_2-adrenoceptor agonist, B-HT 920. It is interesting to note that these authors did not find any changes in heart rate with either α_1- or α_2-adrenoceptor agonists; their animals were pithed under hexobarbitone anaesthesia and we have found that pithing rats under pentobarbitone anaesthesia blunts the cardiovascular responses obtained relative to those seen in animals pithed under halothane anaesthesia (Hiley, Reid & Thomas, unpublished observations).

In conclusion, we have shown that the patterns of vasoconstriction brought about by α_1- and α_2-adrenoceptor agonists differ in the pithed rat although we found marked differences between the two α_1-adrenoceptor agonists studied. We have also shown that the pressor effects of these compounds are, at least in part, mediated by increases in cardiac output, be it a consequence of chronotropic effects, increases in stroke volume or a combination of both. Thus stimulation of α-adrenoceptor subtypes by systemic administration brings about a complex series of

changes affecting both vascular smooth and cardiac muscle. Further study of this area is essential before a comprehensive profile of the haemodynamic effects of these drugs can be presented.

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The action of pentobarbitone on stimulus-secretion coupling in adrenal chromaffin cells

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1 The action of pentobarbitone on stimulus-secretion coupling was studied in bovine isolated adrenal medullary cells.

2 Pentobarbitone inhibited catecholamine release evoked by 500 μM carbachol with half maximal inhibition (IC_{50}) around 50 μM. It also inhibited catecholamine release induced by depolarization with 77 mM potassium (IC_{50} 100 μM). These effects of pentobarbitone were observed with concentrations that lie within the range encountered during general anaesthesia.

3 Evoked secretion required the presence of calcium in the extracellular medium and was associated with an influx of Ca^{2+} through voltage-sensitive channels. Pentobarbitone inhibited ^{45}Ca influx in response to both carbachol (IC_{50} 50 μM) and K^+-depolarization (IC_{50} 150 μM).

4 The action of pentobarbitone on the relationship between intracellular free Ca and exocytosis was examined using electropermeabilised cells which were suspended in solutions containing a range of concentrations of ionised calcium between 10^{-8} and 10^{-4} M. Catecholamine secretion was measured in the presence of 0, 50, 200 or 500 μM pentobarbitone. The anaesthetic had no effect on the activation of exocytosis by intracellular free calcium.

5 When catecholamine secretion in response to potassium or carbachol was modulated by varying extracellular calcium or by adding pentobarbitone to the incubation medium, the amount of catecholamine secretion for a given Ca^{2+} entry was the same.

6 Pentobarbitone inhibited the secretion and ^{45}Ca uptake induced by carbachol in a non-competitive manner.

7 The secretion evoked by nicotinic agonists was associated with an increase in ^{22}Na influx. Pentobarbitone inhibited this influx with an IC_{50} of 100 μM.

8 We concluded that: (a) Pentobarbitone inhibits the catecholamine secretion from bovine adrenal chromaffin cells induced by nicotinic agonists by non-competitive inhibition of the nicotinic receptor. (b) The decrease in Ca influx caused by pentobarbitone accounts fully for the decrease in secretion in response to depolarization with potassium.

Introduction

Barbiturates are known to depress excitatory synaptic transmission in the central nervous system at concentrations similar to those required for the induction and maintenance of anaesthesia. Detailed electrophysiological analysis of their mode of action has shown that the process of chemical transmission itself is affected rather than impulse conduction in the afferent fibres or the electrical properties of the postsynaptic cells (Weakly, 1969; Richards, 1972; 1982; Richards & Strupinski, 1986). This implies that their action is mediated by a disturbance of neurosecretion, a reduction in the sensitivity of the postsynaptic receptors, or both. Weakly (1969) found that pentobarbitone and thiopentone decrease the quantal content of excitatory postsynaptic potentials (e.p.s.ps) recorded from spinal motoneurones and Potashner *et al.* (1980) have shown that the calcium-dependent stimulus-evoked release of the putative excitatory transmitter glutamate is depressed by pentobarbitone. Although barbiturates have been shown to depress calcium conductance in neurones from mouse dorsal root ganglia (Werz & MacDonald, 1985), their effects on the relationship between calcium movement and secretion are not known.

Within the CNS it has proved difficult to assign particular transmitters to specific pathways which can be studied readily *in vitro*. However, clarification of this kind is an essential prerequisite for any detailed

analysis of the action of anaesthetics on the mechanisms of transmitter release.

The adrenal chromaffin cell offers a suitable experimental model for such analysis as the mechanisms of transmitter secretion are well characterized (Baker & Knight, 1984). In addition, it is already known from studies on whole glands that pentobarbitone inhibits the secretion of catecholamine that is induced by nicotinic agonists and high K^+ (Holmes & Schneider, 1973). Chromaffin cells are derived from embryonic neural crest tissue and are homologous with sympathetic postganglionic neurones. The cells may be isolated in large numbers from bovine adrenal glands and form a relatively homogeneous population which secretes adrenaline and noradrenaline. It is also possible to manipulate their intracellular environment using the technique of electropermeabilisation (Knight & Baker, 1982). They therefore offer considerable advantages over other possible model systems such as synaptosomes.

Activation of the nicotinic receptors on the surface of the chromaffin cell causes inward movement of Na and possibly Ca ions. The resultant depolarization opens voltage sensitive channels to bring about a rise in free Ca^{2+} which triggers granule exocytosis (Knight & Kesteven, 1983). Exposure of cells to high K^+ bypasses the acetylcholine receptor and elicits secretion by opening the voltage sensitive Ca^{2+} channels directly (Baker & Knight, 1981). We made a detailed examination of the action of pentobarbitone on secretion induced by both pathways and on the relationship between intracellular free Ca^{2+} and exocytosis. We concluded that the inhibitory effect of pentobarbitone on secretion results from inhibition of Ca^{2+} movement through the voltage sensitive Ca^{2+} channel and from inhibition of ion movements through the channels gated by the nicotinic receptor.

Methods

Isolation of chromaffin cells

Bovine adrenal glands were obtained from a local abattoir. They were removed from the animals within 30 min of death and perfused via the adrenal vein with 5–10 ml of ice-cold Locke's solution containing 0.2% (w/v) bovine serum albumin. The glands were then transported to the laboratory on ice.

Chromaffin cells were isolated by enzymatic digestion of thin slices of the adrenal medulla as described previously (Pocock, 1983a). Care was taken to remove any obvious islands of cortical tissue. Approximately 5×10^7 cells could be obtained from each gland and 6–10 glands were used in most experiments. The viability of the cells was assessed on the basis of their ability to exclude trypan blue and on this criterion our preparations contained at least 95% live cells. Furthermore, more than 95% of the total catecholamine present in suspensions was associated with the cells. The cell suspensions contained up to 40% non-chromaffin cells estimated by neutral red and electron microscopy. This raised the possibility that Ca^{2+} fluxes induced by depolarization with high K^+ (but not by carbachol) might have been due in part to influx into cortical cells (see Results and Discussion).

Measurement of catecholamine secretion and ion fluxes

Basal and evoked secretion of adrenaline and noradrenaline and the associated ion fluxes were measured by centrifuging cell suspensions through a layer of oil (see below). Catecholamine was assayed by the method of Von Euler & Floding (1955) using a Perkin-Elmer fluorescence spectrophotometer. At the concentrations used in this study, pentobarbitone did not interfere with this assay.

To examine the effect of pentobarbitone on ion fluxes and secretion, cell suspensions were incubated at 37°C with varying concentrations of the anaesthetic for 5–10 min. Isotope (^{22}Na or ^{45}Ca) was added and the suspensions were then mixed with equal volumes of either Na-Locke, Na-Locke containing carbachol, or Locke solution in which varying amounts of the Na^+ had been replaced with K^+. Each solution contained an appropriate concentration of anaesthetic. After various time intervals (see Results), triplicate samples from the cell suspensions were centrifuged (12,000 *g* for 20–30 s) through a layer of oil consisting of di-n-butyl pthalate and light liquid paraffin (10:1 v/v). Sodium influx was measured in the presence of tetrodotoxin (TTX; 10^{-5} M) to prevent Na^+ movement through the voltage sensitive channel and ouabain (2×10^{-5} M added one minute before the addition of ^{22}Na) to inhibit Na^+ efflux via the sodium pump.

The catecholamine in the supernatant was assayed and expressed as a percentage of that present in the total cell suspension after the cells had been lysed with 0.1% Triton X–100. To determine the amount of isotope present in the cells, the centrifuge tubes were frozen and the end containing the pellet was cut off with a razor blade. This technique permits good separation of cells from extracellular fluid and reduces contamination of the pellet with extracellular isotope to very low levels. In experiments with ^{22}Na, the radioactivity associated with the cells was counted directly in a γ-counter. For ^{45}Ca, the pellets were solubilized in 0.5ml of 0.1% Triton X–100 (v/v in distilled water) before liquid scintillation counting.

The uptake of ^{22}Na or ^{45}Ca was expressed as mM or $\mu mol\ l^{-1}$ of cells. The pellet volume was estimated as follows. The relationship between total catecholamine content and cell number was determined in one sample

for each experiment. Total catecholamine content of all subsequent samples was measured and the pellet volume calculated from this relationship. The volume of a single cell was assumed to be 3.054×10^{-12} l. (see Pocock, 1983b).

Electropermeabilised cells

A suspension of cells was washed in Ca^{2+}-free Locke solution containing 0.4 mM EGTA (pCa < 8) and resuspended in a medium similar to the intracellular fluid (see below). The cells were then subjected to 10 high-voltage electric discharges ($2 kV cm^{-1}$). This treatment renders the cells permeable to small molecules (Knight & Baker, 1982) without damaging intracellular organelles, and the free Ca is determined by that of the extracellular medium. The cells were then added to media containing pentobarbitone (0–500 μM) and $CaCl_2$ and EGTA in varying ratios to define free Ca^{2+} at the desired levels (pCa was between 4 and 8). The amount of catecholamine released into the medium after 10 min was measured and expressed as a percentage of the total.

Physiological media

Normal Locke solution (Na-Locke) contained (mM): NaCl 140, KCl 5, $MgCl_2$ 1.8, $CaCl_2$ 1.8, HEPES 15, glucose 5.5. The pH was adjusted to 7.4 with NaOH. For K-Locke the NaCl was replaced on an equimolar basis with KCl.

The composition of the electropermeabilisation medium was (mM): potassium glutamate 140, sodium glutamate 5, Mg–ATP 5.0, $MgCl_2$ 1.8, PIPES 15, EGTA 0.4, glucose 5.5 and the pH adjusted to 7.0 with KOH. For experimental measurements, the level of free Ca^{2+} was adjusted by adding varying amounts of $CaCl_2$ and EGTA and the final pCa determined by an Orion 93–20 Ca electrode.

Results

The action of pentobarbitone on catecholamine secretion

The catecholamine content of our cell suspensions varied from 44 to 133 nmol per 10^6 cells with a median value around 70 nmol per 10^6 cells. In the absence of pentobarbitone, the amount of catecholamine present in the supernatant was 2–5% of the total and it increased slowly with time (approximately 3% of total catecholamine per hour). Addition of carbachol (10–1000 μM) to the medium resulted in a very rapid increase in the rate of secretion of catecholamine which reached a plateau after 3–5 min. A similar increase in secretion was observed when the cell suspension was added to Locke solution containing elevated levels of K^+. Stimulation reached a plateau when carbachol was 200–500 μM and K^+ was 50–77 mM. We therefore chose to stimulate the cells with 500 μM carbachol or 77 mM K^+ in most experiments. The quantity of catecholamine released by the cells in response to either method of stimulation was similar. Evoked secretion was generally 4–8% of total which corresponds to 1.8–5.6 nmol of catecholamine secreted per 10^6 cells and a basal secretion of 3% h^{-1} corresponds to 2.1 nmol per 10^6 cells. These

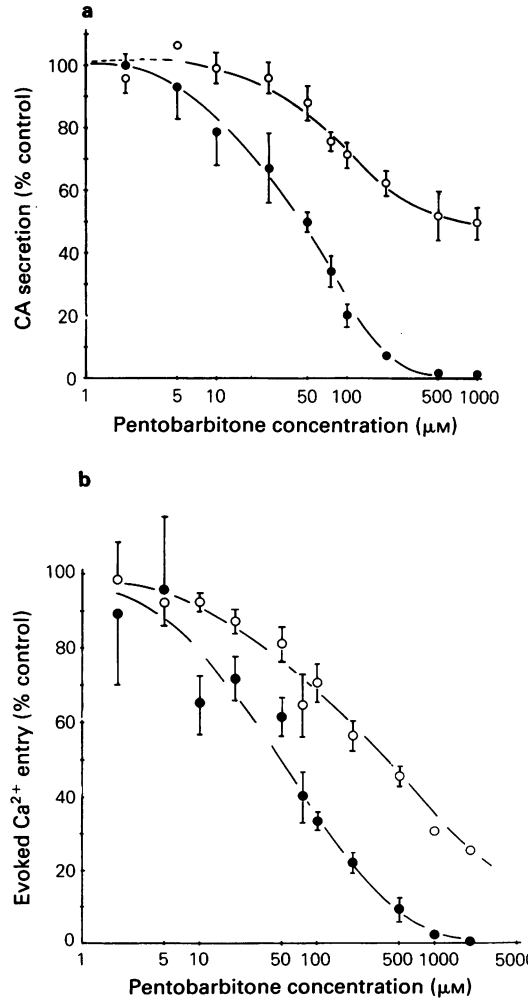

Figure 1 The dose-response relationship for the effect of pentobarbitone on catecholamine (CA) secretion (a) and ^{45}Ca uptake (b) induced by 500 μM carbachol (●) and 77 mM potassium (○). The points shown are the mean of 2–7 determinations and the vertical lines represent s.e.mean

values compare favourably with previously published values for freshly isolated chromaffin cells (Pocock, 1980; Derome *et al.*, 1981).

Addition of pentobarbitone (5–500 μM) to the cell suspensions had no significant effect on basal catecholamine secretion but inhibited secretion stimulated by either 500 μM carbachol or 77 mM K^+-Locke. The extent of the inhibition was dependent on the concentration of the anaesthetic (Figure 1a). Of the evoked catecholamine secretion induced by 77 mM K^+ about 50–60% could be inhibited by pentobarbitone. The IC_{50} for the anaesthetic-sensitive component was approximately 100 μM. Secretion stimulated by carbachol was more readily inhibited (IC_{50} 50 μM). The inhibition for a given concentration was approximately constant throughout the time course of stimulation (Figure 2) and was independent of the time for which the cells were incubated in the presence of pentobarbitone before stimulation (2–20 min; not shown). Consequently, the duration of the pre-incubation period and the time at which the cell suspensions were sampled were not critical.

Under the conditions of our experiments, isolated chromaffin cells did not show consistent responses to repeated stimulation. However, we were able to demonstrate that cells exposed to pentobarbitone showed normal levels of secretion after the drug had been washed from the cells (see Figure 3 for details).

The action of pentobarbitone on ^{45}Ca influx

The increase in secretion of catecholamine that results from stimulation of the cells with carbachol or high K^+ is associated with an increase in Ca^{2+} influx (Holz *et al.*, 1982). The inhibition of secretion by pentobarbitone was accompanied by a proportionate decrease in ^{45}Ca influx (Figure 1b). This inhibition of ^{45}Ca influx was evident throughout the time course of stimulation (Figure 2). However, pentobarbitone did not affect the resting ^{45}Ca influx.

Pentobarbitone (50 μM) inhibited both the secretion and ^{45}Ca uptake induced by carbachol in a non-competitive manner (Figure 4). A similar displacement of the dose-response curve by 50 μM pentobarbitone was observed for the secretion induced by acetylcholine (not shown), and for the effects of 200 μM pentobarbitone on the catecholamine secretion and ^{45}Ca influx induced by elevated concentrations of K^+ (Figure 5).

Ca influx and catecholamine secretion in response to stimulation are dependent on the extracellular Ca^{2+} concentration. Pentobarbitone inhibited secretion at concentrations of extracellular Ca^{2+} between 0.1 and 5.0 mM. The percentage decrease was similar in all cases (Figure 6), suggesting that pentobarbitone is not a competitive inhibitor of Ca^{2+} influx.

We examined the relationship between ^{45}Ca entry

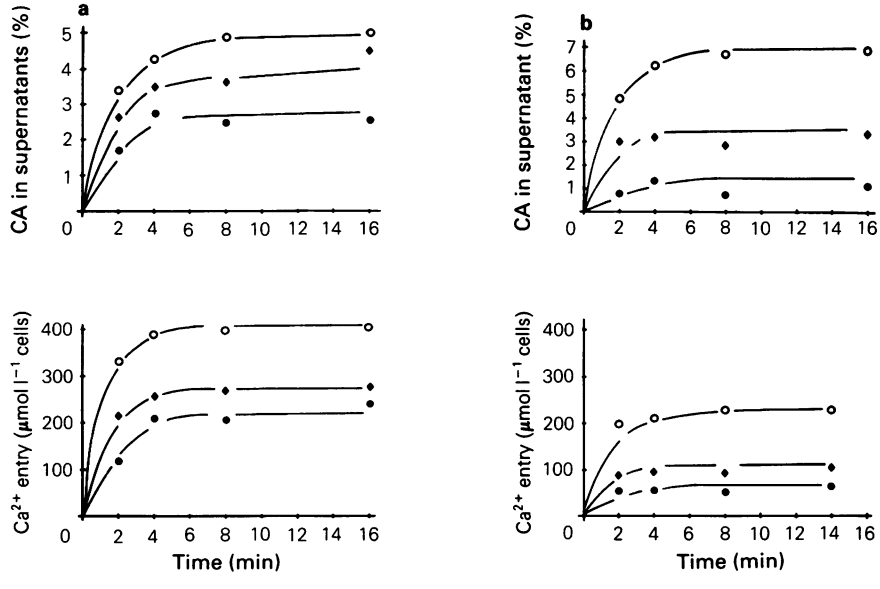

Figure 2 The action of 50 μM and 200 μM pentobarbitone on the time course of catecholamine (CA) secretion and ^{45}Ca uptake induced by (a) 77 mM potassium and (b) 500 μM carbachol: (○) control responses; responses in presence of 50 μM (◆) and 200 μM (●) pentobarbitone.

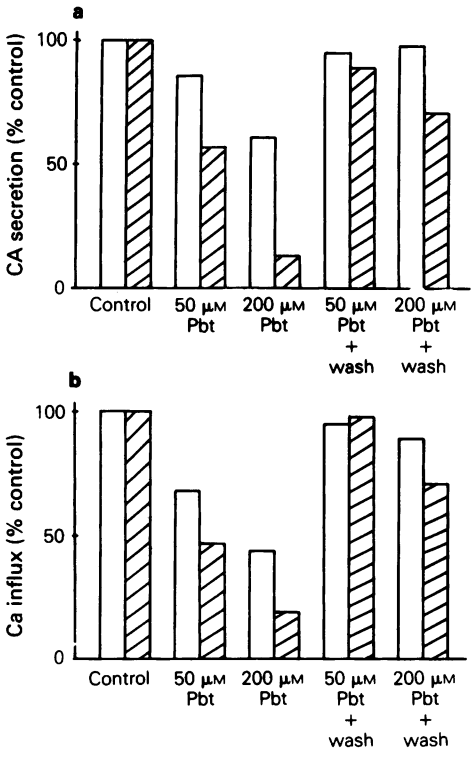

Figure 3 Demonstration of the reversibility of the effect of pentobarbitone on catecholamine (CA) secretion and ^{45}Ca uptake. A cell suspension was divided into three and treated with 0, 50 or 200 μM pentobarbitone (Pbt) respectively. The cells exposed to pentobarbitone were then further subdivided, half being washed free of anaesthetic and half resuspended in fresh Locke solution containing the same concentration of anaesthetic. The resulting five groups of cells were then stimulated with either 77 mM K^+ (open columns) or 500 μM carbachol (hatched columns). The three pairs of columns on the left show the effect of 0, 50 and 200 μM pentobarbitone on catecholamine secretion (a) and ^{45}Ca influx (b). The two pairs of columns on the right show that removal of pentobarbitone before stimulation results in near normal responses.

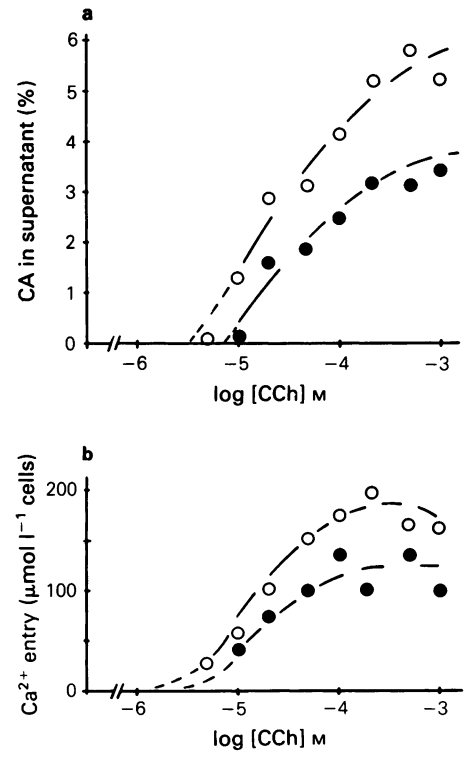

Figure 4 The effect of 50 μM pentobarbitone on the dose-response curve for the action of carbachol (CCh) on catecholamine (CA) secretion (a) and ^{45}Ca uptake (b). (○) Control responses, (●) responses in presence of pentobarbitone.

and catecholamine secretion under the following conditions: (1) in the presence of varying concentrations of pentobarbitone (20–500 μM) at constant extracellular calcium (1 mM). (2) In the absence of pentobarbitone, with varying extracellular calcium (0.1–5.0 mM).

For cells stimulated by either potassium or carbachol, the amount of catecholamine secretion for a given Ca^{2+} entry was the same under both sets of conditions. This suggests that pentobarbitone inhibits catecholamine secretion by inhibiting Ca^{2+} influx (Figure 7).

Pentobarbitone does not interfere with exocytosis

We also examined the action of pentobarbitone on the relationship between intracellular free Ca^{2+} and exocytosis in electropermeabilised cells. These cells were suspended in solutions containing a range of concentrations of Ca^{2+} between 10^{-8} and 10^{-4} M; The amount of catecholamine secreted by the cells in the presence of 0, 50, 200 or 500 μM pentobarbitone was the same. The data for 200 μM pentobarbitone are shown in Figure 8. The anaesthetic therefore had no effect on the process of exocytosis itself.

Effect of pentobarbitone on ^{22}Na influx

The catecholamine secretion induced by carbachol was inhibited by 80% in the presence of 100 μM pentobarbitone. In contast, the secretion induced by high K^+ was only partially inhibited even by 500 μM pentobarbitone. There is, therefore, an additional effect of the anaesthetic on the nicotinic receptor or on

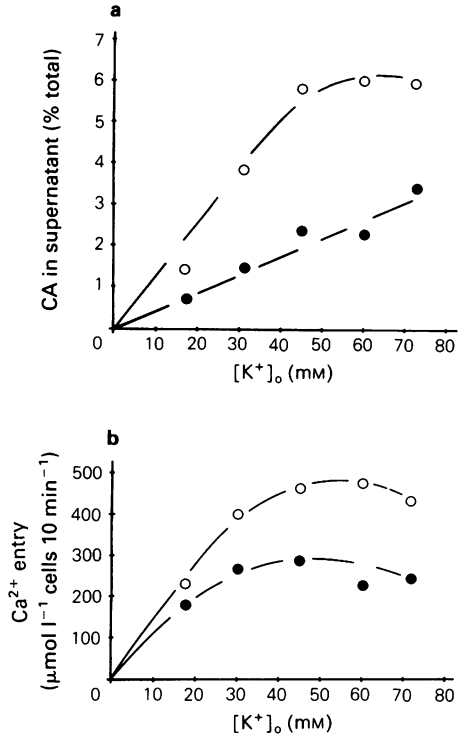

Figure 5 The effect of 200 μM pentobarbitone on the catecholamine (CA) secretion (a) and ^{45}Ca uptake (b) induced by elevated concentrations of potassium. (○) Control responses, (●) responses in presence of pentobarbitone.

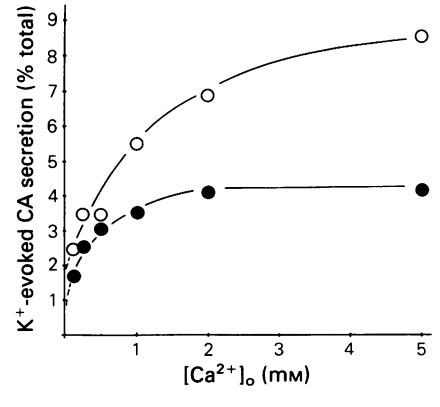

Figure 6 The effect of 200 μM pentobarbitone on catecholamine (CA) secretion induced by 77 mM potassium in the presence of varying amounts of calcium in the bathing fluid. (○) Control responses, (●) responses in presence of pentobarbitone.

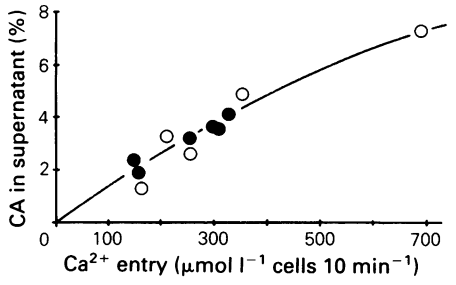

Figure 7 The relationship between catecholamine (CA) secretion and calcium uptake during stimulation by 77 mM potassium. (○) show the relationship when external calcium is varied in the absence of pentobarbitone and (●) show the relationship in cell suspensions treated with varying concentrations of pentobarbitone and 1 mM Ca. (The pentobarbitone concentrations used were: 20, 50, 100, 200 and 500 μM and the calcium concentrations were: 0.1, 0.25, 0.5, 1.0, 2.0 and 5.0 mM).

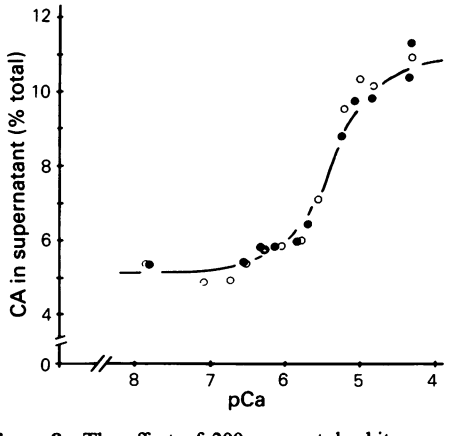

Figure 8 The effect of 200 μM pentobarbitone on the activation of catecholamine (CA) secretion in electropermeabilised chromaffin cells by ionised calcium. At low external calcium concentrations (pCa < 6) the secretion was about 5% over the 15 min period. Raising ionised calcium levels from 10^{-6} to 10^{-4} (pCa 6–4) caused an increase in secretion. This pattern was not affected by 200 μM pentobarbitone. (○) Control responses, (●) responses in presence of pentobarbitone.

the events associated with its activation. We measured the effect of pentobarbitone on the influx of ^{22}Na and correlated these measurements with the secretion of catecholamine. The cells were pre-incubated as before in the absence and presence of 50 or 200 μM pentobarbitone.

The cells steadily accumulated ^{22}Na in the absence of stimulation. However, when they were stimulated by carbachol the influx of ^{22}Na was increased for 1–2 min. Pentobarbitone did not affect the basal ^{22}Na influx but inhibited the increase in ^{22}Na influx elicited by carbachol by approximately 40% at 50 μM and by 75% at 200 μM. The IC_{50} for the inhibition of ^{22}Na influx was around 100 μM (Figure 9).

We examined the relationship between ^{22}Na entry and catecholamine secretion evoked by carbachol under the following conditions: (1) in the presence of varying concentrations of pentobarbitone (10–200 μM) with 147 mM sodium in the extracellular medium. (2) In the absence of pentobarbitone with progressive replacement of extracellular sodium by N-methyl glucamine (meglumine).

During stimulation with carbachol, the amount of catecholamine secreted following a given entry of ^{22}Na was lower when secretion was inhibited by pentobarbitone than when secretion was inhibited by reducing extracellular Na (see Figure 10).

Ca^{2+} fluxes in cortical cells

Our suspensions contained 30–40% cells that did not stain with neutral red. These non-staining cells are thought to be non-chromaffin cells (Livett, 1985)

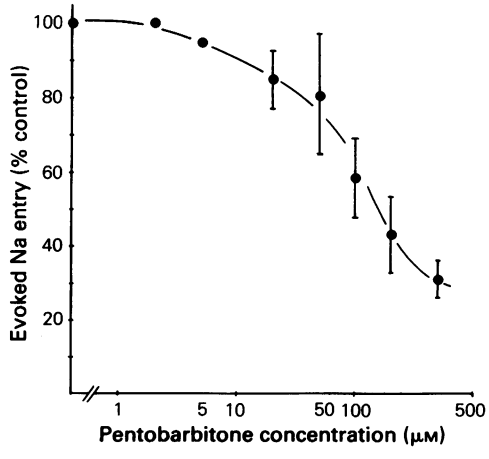

Figure 9 The dose-response curve for the action of pentobarbitone on the uptake of ^{22}Na stimulated by 500 μM carbachol. The points are the mean of 2–5 determinations and the vertical lines represent s.d.

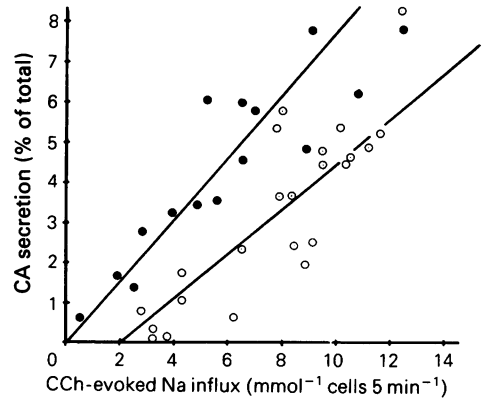

Figure 10 The relationship between catecholamine (CA) secretion and ^{22}Na uptake during stimulation by 500 μM carbachol (CCh). (●) Show the relationship when secretion was inhibited by varying external Na^+ and (○) show the relationship in cell suspensions treated with varying concentrations of pentobarbitone (5–500 μM). The data are from three experiments.

which may be derived from islands of cortical cells present in the adrenal medulla. To determine the extent to which this contamination affected our results and conclusions we measured ^{45}Ca influx in suspensions of cortical cells treated with 500 μM carbachol or 77 mM K^+. Carbachol had no effect on Ca^{2+} uptake whereas depolarization with 77 mM K^+ increased Ca^{2+} uptake with a time course similar to that seen for stimulated chromaffin cells. The uptake of ^{45}Ca was, however, lower than that for medullary cells (232 ± 15 μM per 10^6 cells per 10 min compared to 462 ± 37 μM per 10^6 cells per 10 min) and this uptake was inhibited by 10% by 200 μM pentobarbitone. The ^{45}Ca uptake of medullary cells was inhibited by about 45% at 200 μM pentobarbitone (see Figure 1).

Discussion

The principal object of our experiments was to determine the mechanisms by which pentobarbitone inhibits neurosecretion using the chomaffin cell as a model. We showed that this anaesthetic decreases the secretion induced by carbachol and elevated concentrations of K^+. This inhibition occurred at concentrations of pentobarbitone within the range required for the induction and maintenance of general anaesthesia (50–300 μM; see Richards, 1972) and, at these concentrations, pentobarbitone had no significant effect on the basal secretion of catecholamine.

As the choice of the chromaffin cell as a model for neurosecretion was based on the homology between the adrenal medulla and sympathetic ganglia, it was

important to compare the action of pentobarbitone on the chromaffin cell with its action on synaptic transmission in sympathetic ganglia. The early work of Larrabee & Posternak (1952) showed that this anaesthetic, amongst others, selectively depressed synaptic transmission through the superior cervical ganglion. This effect occurred at concentrations of pentobarbitone comparable to those required for the maintenance of general anaesthesia. Later Matthews & Quilliam (1964) showed that the closely related barbiturate, amylobarbitone, depressed the release of acetylcholine in response to nerve stimulation. Barbiturates also depress the sensitivity of sympathetic ganglion cells to exogenously applied nicotinic agonists (Nicoll & Iwamoto, 1976). So in these important respects, the action of pentobarbitone on chromaffin cells parallels its action on sympathetic ganglia.

Depolarization of the cells with elevated concentrations of K^+ bypasses the acetylcholine receptor and directly activates the voltage-dependent Ca^{2+} channel. Under these conditions the influence of pentobarbitone on the process of transmitter secretion can be studied in isolation. The inhibition of catecholamine secretion induced by high-K^+ was accompanied by a decrease in Ca^{2+} influx. Moreover, the relationship between catecholamine secretion and Ca^{2+} influx during inhibition by pentobarbitone was the same as that observed between catecholamine secretion and Ca^{2+} influx determined by varying external Ca^{2+}. As pentobarbitone had no effect on the Ca^{2+}-activated secretion of catecholamine in electropermeabilised cells (see Figure 8), we concluded that it does not affect those steps in exocytosis that occur after Ca^{2+} entry. The inhibition of Ca^{2+} entry by pentobarbitone appears to be sufficient to account in full for the inhibition of catecholamine secretion induced by direct depolarization and activation of the voltage-gated Ca^{2+} channel.

Our cell suspensions appeared to contain up to 40% non-chromaffin cells. These cells were probably adrenal cortical cells which showed a smaller increase in Ca^{2+} entry when they were depolarized by 77 mM K^+ than chromaffin cells. This contamination would, therefore, result in an underestimate of the Ca^{2+} entry for a given level of catecholamine secretion. Moreover, pentobarbitone was less effective in inhibiting Ca^{2+} entry in adrenal cortical cells. These two factors would result in an underestimate of the IC_{50} for the action of pentobarbitone on Ca^{2+} entry. Nevertheless, as our determination of the relationship between Ca^{2+} entry and catecholamine secretion was made under the same conditions, the conclusion that pentobarbitone inhibits catecholamine secretion by decreasing Ca^{2+} entry through the voltage gated channels remains valid.

Secretion of catecholamine from isolated chromaffin cells stimulated by carbachol was more sensitive to the action of pentobarbitone than that stimulated by high K^+. This confirms the observations of Holmes & Schneider (1973) on the effect of pentobarbitone on catecholamine secretion from intact glands. This difference in sensitivity indicated that, in addition to the effects occuring at the voltage-gated Ca^{2+} channel, there was a direct action of anaesthetic on the nicotinic receptor or on the processes associated with its activation.

We showed that pentobarbitone inhibited catecholamine secretion induced by acetylcholine and carbachol in a non-competitive manner and that it inhibited both ^{45}Ca and ^{22}Na influx. While the inhibition of Na^+ movement does not appear to be sufficient to account for the decrease in catecholamine secretion, the inhibition of ^{45}Ca influx paralleled the inhibition of catecholamine secretion. This suggests that pentobarbitone may inhibit Ca movements gated by the acetylcholine receptor as well as those of the voltage gated channel. However, the detailed mechanisms responsible for this effect remain to be worked out. It is known that pentobarbitone decreases the binding of acetylcholine to the acetylcholine receptor of the *Torpedo* electroplax (Sauter *et al.*, 1980) and this may also be true of its action on the nicotinic receptor of the chromaffin cell. However, it may act directly on the Na^+ channel associated with the nicotinic receptor as Adams (1976) has shown that, at the neuromuscular junction, barbiturates preferentially block ion channels in the open conformation possibly by rendering the open state less stable (Gage & McKinnon, 1985). The resolution of this issue in our preparation will depend on a detailed analysis of channel properties and receptor binding.

Although bovine chromaffin cells possess both nicotinic and muscarinic cholinoceptors catecholamine secretion can only be induced by nicotinic agonists (Schneider *et al.*, 1977; Derome *et al.*, 1981). The muscarinic receptors may, however, modulate catecholamine secretion induced by nicotinic agonists (Derome *et al.*, 1981). As we used carbachol as our nicotinic agonist and as it possesses muscarinic properties it is possible that the decrease in catecholamine secretion caused by pentobarbitone may have resulted from modulation of cyclic GMP levels. This possibility was discounted as atropine (10^{-7}–10^{-9} M) does not affect the potency of pentobarbitone (Pocock & Richards, unpublished observations). The possibility that the action of pentobarbitone might be mediated via activation of γ-aminobutyric acid (GABA) receptors (Kataoka *et al.*, 1984) was likewise rejected as catecholamine secretion is the same in the presence and absence of GABA (5–100 μM) and the potency of pentobarbitone is the same in the absence and presence of GABA (Pocock & Richards, unpublished observations).

Implications for anaesthetic action in the CNS

In the CNS the nerve impulse depolarizes the nerve terminal and this opens voltage-sensitive Ca^{2+} channels to bring about a rise in free Ca^{2+} within the terminal which initiates transmitter release (Ashley *et al.*, 1984; Richards *et al.*, 1984). Electrophysiological analysis has demonstrated that concentrations of barbiturates within the anaesthetic range do not interfere significantly with the propagation of the nerve impulse, but they inhibit the release of putative neurotransmitters such as glutamate from synaptosomes and slices of brain tissue (see Collins, 1981; Minchin, 1981; Kendall & Minchin, 1982). Moreover, Blaustein & Ector (1974) have shown that concentrations of barbiturates within the anaesthetic range depress the K^+-stimulated Ca^{2+} influx into synaptosomes, but these authors did not correlate this decrease in Ca^{2+} flux with changes in transmitter release. In the present study we showed that the inhibition of Ca^{2+} influx was sufficient to account for the decrease in catecholamine secretion. As the mechanism of catecholamine secretion from chromaffin cells is thought to be similar to that of transmitter release from the nerve terminals of the CNS (see above), it is probable that the inhibition of Ca^{2+} influx is sufficient to account for the inhibitory action of pentobarbitone on transmitter release in the CNS.

Pentobarbitone has been shown to affect the function of various receptors in the CNS. In the olfactory cortex, it depresses the sensitivity of pyramidal cells to iontophoretically-applied L-glutamate (Richards & Smaje, 1976), a putative transmitter in this pathway. It also depresses the sensitivity of hippocampal cells to glutamate (Sawada & Yamamoto, 1985). These results with glutamate are consistent with the known depressant effect of pentobarbitone on excitatory synaptic transmission in the hippocampus and olfactory cortex (Bliss & Richards, 1971; Richards, 1972).

In conclusion, we have shown that pentobarbitone inhibits the catecholamine secretion from bovine isolated adrenal chromaffin cells evoked by carbachol or by high K^+. This results from non-competitive inhibition of the nicotinic receptor and from inhibition of the voltage-gated Ca^{2+} entry. The decrease in Ca^{2+} influx accounts fully for the decrease in secretion in response to depolarization. A similar inhibition of the voltage sensitive Ca^{2+} channels could account for the depressant action of pentobarbitone on synaptic transmitter release in the CNS.

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Muscarinic receptors mediate negative and positive inotropic effects in mammalian ventricular myocardium: differentiation by agonists

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1 The concentration-dependence of the negative and positive inotropic effect of choline esters and of oxotremorine was studied in isometrically contracting papillary muscles of the guinea-pig. The preparations were obtained from reserpine-pretreated animals and were electrically driven at a frequency of 0.2 Hz.

2 In the presence of the phosphodiesterase inhibitor 3-isobutyl-1-methyl xanthine (IBMX, 100 μmol l^{-1}), choline esters and oxotremorine produced concentration-dependent negative inotropic effects. Oxotremorine exhibited the highest negative inotropic potency (with a half-maximal effective concentration, EC_{50}, of 20 nmol l^{-1}) followed by carbachol (139 nmol l^{-1}), methacholine (490 nmol l^{-1}), acetylcholine in the presence of 10 μmol l^{-1} physostigmine (1.36 μmol l^{-1} and bethanechol (10 μmol l^{-1})). Atropine was a competitive antagonist of the negative inotropic effects. Carbachol and oxotremorine decreased $\dot{V}_{max}$, overshoot and duration of slow Ca^{2+}-dependent action potentials which had been elicited in the presence of 100 μmol l^{-1} IBMX.

3 Choline esters produced a concentration-dependent positive inotropic effect. With an EC_{50} of 32 μmol l^{-1}, carbachol was the most potent compound, followed by methacholine (35 μmol l^{-1}), acetylcholine in the presence of 10 μmol l^{-1} physostigmine (46 μmol l^{-1}) and bethanechol (142 μmol l^{-1}). Compared to carbachol and methacholine which increased force by 100% of control, the increase induced by acetylcholine and bethanechol was only 64 and 58%, respectively. Atropine shifted the concentration-effect curves of all choline esters to higher concentrations. Choline esters caused intracellular Na^+ activity to increase in the quiescent papillary muscle. This effect was reversed by atropine.

4 Oxotremorine produced a small concentration-dependent positive inotropic effect (about 30% of the maximal effect of carbachol) which was resistant to atropine. Oxotremorine was a potent inhibitor of the positive inotropic effect of choline esters, and did not cause an increase in intracellular Na^+ activity in the quiescent papillary muscle.

5 The results show that muscarinic receptors of the ventricular myocardium mediate two inotropic effects, which are opposite in direction and differ in their concentration-dependence by a factor of 100. Although agonists differentiate between both inotropic effects, it is unknown whether the receptors involved represent receptor states or separate receptor subpopulations. The negative inotropic effect of choline esters and of oxotremorine can be best explained by adenylate cyclase inhibition. While stimulation of phosphoinositide hydrolysis might have been responsible for the positive inotropic effect of choline esters via modulation of cation-fluxes across the cell membrane, such a mechanism was not involved in the positive inotropic effect of oxotremorine.

Introduction

Activation of muscarinic receptors in heart muscle results in various biochemical effects, including increased synthesis of cyclic GMP, decreased synthesis of cyclic AMP (George *et al.*, 1973), rise in intracellular Na^+ activity (Korth & Kühlkamp, 1985) and enhanced turnover of membrane phosphoinositides (Brown & Brown, 1983). While most of the biochemical events have been linked with the inotropic

state of the heart, either via modulation of voltage-controlled ionic channels (Trautwein *et al.*, 1982), Na^+-Ca^{2+} exchange (Korth & Kühlkamp, 1985) or by regulation of the sensitivity of cardiac myofibrils to Ca^{2+} (Horowits & Winegrad, 1983), a possible role of phosphoinositide turnover in the control of force has not yet been established. In a recent study utilizing embryonic chick heart cells, Brown & Brown (1984) have demonstrated that activation of phosphoinositide metabolism by carbachol requires full receptor occupancy, while only a fraction of muscarinic receptors need to be occupied to cause inhibition of adenosine 3' : 5'-cyclic monophosphate (cyclic AMP) synthesis. As a consequence, the concentration-effect relationships differed 100 fold for both effects. Interestingly, the muscarinic receptor agonist oxotremorine has been found, in the same study, to be an extremely weak agonist for phosphoinositide turnover, but to be a full agonist for inhibition of cyclic AMP synthesis.

The present study was undertaken to test the hypothesis that the positive inotropic effect of high concentrations of carbachol (Korth & Kühlkamp, 1985) could be related to enhanced phosphoinositide metabolism. For this purpose, concentration-effect relationships for the negative inotropic action, due to inhibition of cyclic AMP synthesis, and for the positive inotropic action of choline esters and of oxotremorine were determined on guinea-pig papillary muscles. The accordance of the present results with those of Brown & Brown (1984), together with recent findings of elevated intracellular Ca^{2+} levels in response to phosphoinositide hydrolysis (Berridge, 1984), makes a causal relationship between enhanced phospholipid metabolism and positive inotropic effect an attractive hypothesis.

Methods

Preparations

Guinea-pigs of either sex weighing 250–350 g were killed by cervical dislocation. The animals were pretreated with reserpine (5 mg kg^{-1} body weight, injected intraperitoneally 24 h before the experiment) to avoid the release of endogenous noradrenaline. Right ventricular papillary muscles with a diameter of <1 mm were rapidly excised from the isolated heart and mounted in a two-chambered organ bath with an internal circulation of the bath solution (volume 50 ml) as described by Reiter (1967). The incubation medium was constantly gassed and kept in circulation by 5% CO_2 and 95% O_2; the temperature was 35°C, pH 7.5. The composition of the medium was (in mmol $^{-1}$): NaCl 115, KCl 4.7, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2, glucose 10, $CaCl_2$ 3.2 or 6.2.

Force measurement

The muscles were stimulated electrically at their mural end through two punctate platinum electrodes with square wave pulses of 1 ms in duration and at an intensity slightly above stimulation threshold. Force of contraction was recorded isometrically by means of an inductive force transducer (Q11, 10p; Hottinger Baldwin Messtechnik, Darmstadt, FRG) connected to an oscilloscope and a pen recorder. The resting force was kept constant at 3.96 mN throughout the experiment. After dissection, the muscles were allowed to equilibrate in the bath solution for 1 h at a stimulation frequency of 1 Hz. Thereafter, the stimulation frequency was lowered to 0.2 Hz and the drug intervention was started as soon as force of contraction had reached a steady state.

Electrophysiological measurements

In order to measure force and transmembrane electrical activity, the papillary muscle was mounted horizontally in a perfusion chamber (volume 2 ml) perfused at a constant rate of 10 ml min^{-1}. The voltage recording electrodes had tip resistances of 15–20 MΩ and small tip potentials when filled with 3 mol l^{-1} KCl acidified to a pH of 2 with HCl. The construction and calibration of Na^+-sensitive microelectrodes using the neutral ion exchange resin ETH 227 (Steiner *et al.*, 1979) has been described in detail elsewhere (Sheu & Fozzard, 1982). The muscles were impaled with a conventional electrode and a Na^+-sensitive microelectrode so that the impalements were as close as possible. From the potentials measured with the two microelectrodes, the a^i_{Na} of the cell was calculated by means of the following equation

$$E^i_{Na} - E_m = E_o + S \log (a^i_{Na} + k_{Na,K}\, a^i_K), \qquad (1)$$

where E^i_{Na} is the transmembrane potential measured with the Na^+-sensitive microelectrode with respect to the reference electrode in the bath, E_m the transmembrane potential measured with the conventional microelectrode, E_o a constant potential of the Na^+-sensitive microelectrode, and S the slope of the Na^+-sensitive microelectrode (ranging from 50 to 61 mV per decade) as determined in NaCl solutions containing 0.1 mmol l^{-1} EGTA. The $k_{Na,K}$ is the selectivity coefficient of the Na^+-sensitive microelectrode which ranged from 0.01 to 0.02 for $K^+ : Na^+$ in a mixture of 10 mmol l^{-1} NaCl/140 mmol l^{-1} KCl. The a^i_K is the intracellular K^+ activity which was 110 mmol l^{-1}, as experimentally determined with K^+-sensitive microelectrodes in 3 papillary muscles. Before and after each experiment the electrodes were calibrated at 35°C with pure solutions of NaCl and with mixtures of NaCl and KCl with constant total ionic strength of 300 mmol l^{-1}. Any change in calibration meant that

the experiments were discarded. Pure NaCl activity coefficients were calculated using the equations derived by Pitzer & Mayorga (1973). For the mixed solutions the activity coefficients were calculated by Guggenheim-Scatchard-Robinson generalized equations (Harned & Robinson, 1968). Ion activity coefficients were calculated using the McInnes convention (1961). Na^+-sensitive microelectrodes are affected by Ca^{2+} (Steiner *et al.*, 1979). The average selectivity coefficient, $k_{Na,Ca}$, of the Na^+-sensitive microelectrode was 2.8 in a mixture of 10 mmol l^{-1} NaCl/ 140 mmol l^{-1} KCl containing pCa 6. The pCa was calculated according to Bers (1982). A rise of Ca^{2+} from 0 to pCa 6 gave an apparent extra Na^+ activity of about 1 mmol l^{-1}. Since in the experiments described below contractures never developed, the contribution of intracellular Ca^{2+} to the a^i_{Na} measurements must have been negligibly small which justified the omission of $k_{Na,Ca}$ and a^i_{Ca} in calculating a^i_{Na} in Equation 1.

Conventional and Na^+-sensitive microelectrodes were connected to a dual channel high impedance electrometer (model F-223 A, WP Instruments, New Haven, Conn., U.S.A.). The signals were displayed separately and electronically subtracted on digital panel meters. The panel meter readings were used for calculating a^i_{Na}. Potential and force measurements were also displayed on a Digital Oscilloscope (Nicolet, Madison WI, U.S.A.) and a chart recorder for data recording. Electronic differentiation was used to obtain the first time derivative of the action potential upstroke, $\dot{V}_{max}$. In some experiments the K^+ concentration of the bath solution was raised to 24 mmol l^{-1} in order to inactivate the Na^+ channels. Under this condition, a 2–3 times higher intensity of stimulation was necessary to evoke slow action potentials.

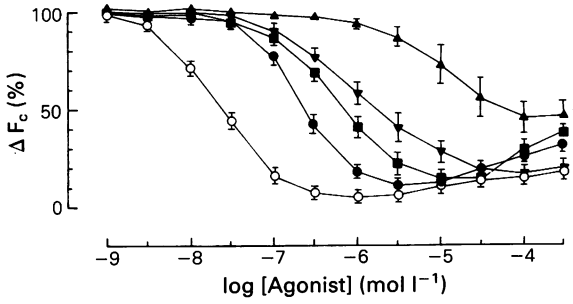

Figure 1 The negative inotropic effect of oxotremorine and of choline esters in the presence of 3-isobutyl-l-methyl xanthine (IBMX). Cumulative concentration-effect curves in the presence of 100 μmol l^{-1} IBMX for oxotremorine (○), carbachol (●), methacholine (■), acetylcholine + 10 μmol l^{-1} physostigmine (▼) and for bethanechol (▲). Means, with s.e.mean indicated by vertical lines if it exceeds the size of the symbol, are shown (5 muscles). Ordinate scale: decrease of the positive inotropic effect of 100 μmol l^{-1} IBMX (ΔFc 100% = 13.1 ± 1.8 mN (○), 15.3 ± 2.5 mN (●), 16.7 ± 3.2 mN (■), 15.9 ± 4.3 mN (▼), 14.0 ± 1.4 mN (▲). Pre-drug control of F_c before the application of IBMX was 2.8 ± 0.5 mN (○), 4.8 ± 1.2 mN (●), 2.3 ± 0.9 mN (■), 4.2 ± 1.5 mN (▼), 2.7 ± 0.6 mN (▲). Reserpine-pretreated guinea-pigs were used for all experiments. Contraction frequency 0.2 Hz. $[Ca^{2+}]_o$ = 3.2 mmol l^{-1}.

Materials

The drugs used were: carbamylcholine chloride (carbachol), acetyl-β-methylcholine chloride (methacholine), acetylcholine chloride, carbamyl-β-methylcholine chloride (bethanechol), oxotremorine sesquifumarate and 3-isobutyl-l-methyl xanthine (Sigma München, FRG); reserpine (dissolved in 5% ascorbic acid) was obtained from Serva (Heidelberg, FRG). The ion exchanger resin ETH 227 was a gift from Prof. Simon, Zürich.

Statistics and calculations

The data are presented as arithmetic means ± s.e.mean. Significance tests were performed by Student's *t* test. Significance was assumed when $P < 0.05$. Competitive antagonism was calculated according to the proposal of Waud (1975). Calculations were performed on a PDP-11 (Digital Equipment Corporation).

Results

The negative inotropic effect of choline esters and of oxotremorine

Figure 1 shows that oxotremorine and choline esters antagonized the positive inotropic effect of the phosphodiesterase inhibitor 3-isobutyl-l-methyl xanthine (IBMX). The concentrations that half-maximally inhibited the inotropic effect of 100 μmol l^{-1} IBMX (log EC_{50}) were −7.72 ± 0.05 for oxotremorine, −6.66 ± 0.06 for carbachol, −6.35 ± 0.1 for methacholine, −5.96 ± 0.15 for acetylcholine (in the presence of 10 μmol l^{-1} physostigmine) and −5.01 ± 0.12 for bethanechol. Oxotremorine and carbachol were about equally efficacious, they inhibited the IBMX-induced effect by 95 and 89%, respectively. Methacholine, acetylcholine (in the presence of 10 μmol l^{-1} physostigmine) and bethanechol caused an inhibition by 85, 81 and 54%, respectively. As can be seen from Figure 1, application of high concentrations of oxotremorine or choline esters

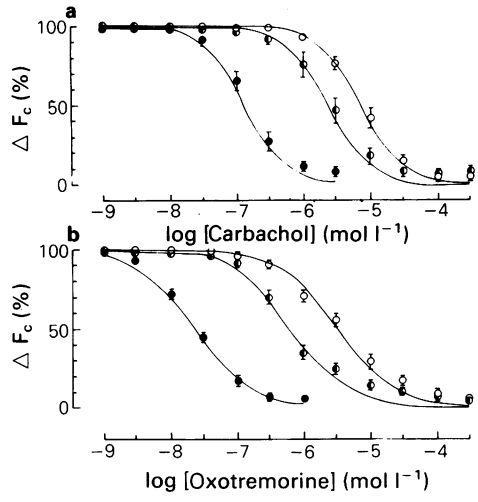

Figure 2 Competitive antagonism by atropine of the negative inotropic effect of (a) carbachol and (b) oxotremorine. Three cumulative concentration-effect curves were obtained for either oxotremorine or carbachol from each muscle, first in the absence (●), and then in the presence of 3 (◐) and 10 nmol l^{-1} atropine (○), respectively. Means, with s.e.mean indicated by vertical lines if it exceeds the size of the symbol, are shown (5 muscles). The data were fitted by theoretical curves describing competitive antagonism. Ordinate scales: decrease of the positive inotropic effect of 100 μmol l^{-1} 3-isobutyl-l-methyl xanthine (IBMX) (ΔF_c 100% before application of oxotremorine = 12.8 ± 2.3 mN in the absence, 14.9 ± 2.8 mN and 15.2 ± 2.2 mN in the presence of 3 and 10 nmol l^{-1} atropine, respectively; ΔF_c 100% before application of carbachol = 17.3 ± 2.5 mN in the absence, 21.5 ± 3.1 mN and 22.0 ± 3.0 mN in the presence of 3 and 10 nmol l^{-1} atropine, respectively). Predrug control of F_c before application of IBMX was 2.9 ± 0.7 mN, 3.0 ± 0.8 mN and 2.9 ± 0.8 mN in the oxotremorine series and 4.3 ± 1.2 mN, 4.8 ± 1.2 mN and 4.5 ± 1.0 mN in the carbachol series. Reserpine-pretreated guinea-pigs were used for all experiments. Contraction frequency 0.2 Hz. $[Ca^{2+}]_o$ = 3.2 mmol l^{-1}.

resulted in a decrease of the negative inotropic effect. At an agonist concentration of 300 μmol l^{-1}, this decrease was significant for oxotremorine, carbachol and methacholine. Inhibition of the negative inotropic effects of oxotremorine and of choline esters by atropine is evidence for the involvement of muscarinic receptors. Figure 2 shows that atropine (3 and 10 nmol l^{-1}) in the presence of 100 μmol l^{-1} IBMX shifted the concentration-effect curves of oxotremorine and of carbachol parallel to higher concentrations. The experimentally obtained data could be well fitted by a competitive antagonism. The curves shown in in Figure 2 were calculated according to the method proposed by Waud (1975). The dissociation constants for the atropine-receptor complex, K_B, were 0.18 ± 0.01 nmol l^{-1} in the presence of carbachol and 0.17 ± 0.01 nmol l^{-1} in the presence of oxotremorine. The stoichiometric coefficient for the binding of atropine to the receptor was close to 1 in the presence of carbachol (0.96 ± 0.02). In the presence of oxotremorine, however, a coefficient of 1.41 ± 0.02 was found indicating that the antagonism increased with increasing concentrations of atropine.

In order to determine whether the negative inotropic effects of oxotremorine and of choline esters in the presence of IBMX were due to a decrease in slow Ca^{2+} inward current, the effects of both muscarinic receptor agonists were studied on slow Ca^{2+}-dependent action potentials in potassium-depolarized preparations. As shown in Figure 3, oxotremorine and carbachol at concentrations that produced a 50% decrease of the positive inotropic effect of IBMX, attenuated $\dot{V}_{max}$, overshoot and duration of slow Ca^{2+}-dependent action potentials which had been enhanced in the presence of 100 μmol l^{-1} IBMX. As summarized in Table 1, results similar to those of Figure 3 were also obtained in 3 other catecholamine-depleted papillary muscles.

The positive inotropic effect of choline esters and of oxotremorine

In order to test whether the small increase in force of contraction induced by high concentrations of oxotremorine and choline esters in the presence of IBMX was due to the receptor-inactivation, concentration-effect relationships were obtained for muscarinic receptor agonists in the absence of cyclic AMP-mediated effects. As shown in Figure 4, acetylcholine and its congeners carbachol, methacholine and bethanechol produced a concentration-dependent positive inotropic effect in the noradrenaline-depleted papillary muscle of the guinea-pig. Half-maximal effective concentrations (log EC_{50}) were − 4.55 ± 0.1 for carbachol, − 4.46 ± 0.03 for methacholine, − 4.35 ± 0.04 for acetylcholine and − 3.96 ± 0.23 for bethanechol (n = 6 for each choline ester). As can be seen from Figure 4, carbachol and methacholine had the same inotropic efficacy, force of contraction increased by 6.87 ± 1.35 and 6.86 ± 1.4 mN, respectively. In contrast, the inotropic efficacy of acetylcholine and bethanechol was only 64 and 58% that of a maximally effective concentration of carbachol. Concentration-effect curves which were obtained for acetylcholine after the muscles had been pre-incubated with 10 μmol l^{-1} physostigmine for 40 min showed a slightly higher mean log EC_{50} (− 4.38 ± 0.05) than those obtained in the absence of the esterase inhibitor. The inotropic efficacy of acetylcholine was not in-

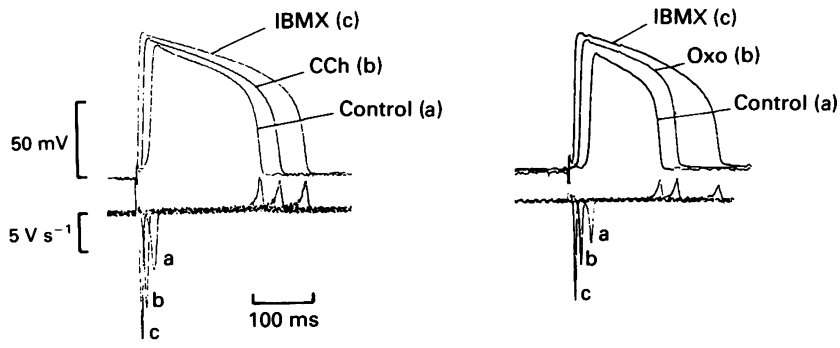

Figure 3 The effect of 100 μmol l^{-1} 3-isobutyl-l-methyl xanthine (IBMX) on the slow action potential and maximum rate of depolarization (V s^{-1}) and the attenuation of these effects by 0.3 μmol l^{-1} carbachol (CCh) or 30 nmol l^{-1} oxotremorine (Oxo). The concentrations correspond to EC_{50} values obtained from Figure 1. Different noradrenaline-depleted papillary muscles were used in the carbachol and oxotremorine experiment. Records are from one continuous microelectrode impalement. IBMX was allowed to act for 30 min before the record was taken. The effects of oxotremorine and of carbachol were recorded 5 min after drug-application. $[K^+]_o$ = 24 mmol l^{-1}. $[Ca^{2+}]_o$ = 3.2 mmol l^{-1}. Contraction frequency 0.2 Hz.

creased in the presence of physostigmine. The positive inotropic effect of choline esters started within 10 s after application, was maximal within 5 to 6 min and remained constant thereafter. The time parameters of the isometric contraction curve (time to peak force and relaxation time) were not changed by any of the choline esters up to a concentration of 1 mmol l^{-1}. The concentration-effect curves of choline esters were shifted to higher concentrations by 10 nmol l^{-1} atropine and were almost completely suppressed in the presence of 100 nmol l^{-1} atropine. This effect of atropine (100 nmol l^{-1}) is shown in Figure 4 for one representative choline ester. The muscarinic receptor agonist oxotremorine also produced a concentration-dependent positive inotropic effect (0.1–300 μmol l^{-1}) in the noradrenaline-depleted papillary muscle. As can be seen from Figure 5a, the maximum positive inotropic effect induced by 300 μmol l^{-1} oxotremorine

Table 1 Effects of carbachol and oxotremorine on slow Ca^{2+}-dependent action potentials in the presence of 100 μmol l^{-1} 3-isobutyl-l-methyl xanthine (IBMX)

Condition‡	*Resting potential* (mV)	*Overshoot* (mV)	*Max. rate of rise* (Vs^{-1})	*Duration of action potential at 90% repolarization* (ms)
Control				
24 mmol l^{-1} K^+	−49.2 ± 0.5	37.4 ± 1.5	8.9 ± 1.7	175.3 ± 30.7
+ 100 μmol l^{-1} IBMX (4)	−48.1 ± 0.3	46.5 ± 2.0	23.9 ± 6.1	257.2 ± 22.7
+ 30 nmol l^{-1} oxotremorine‡§	−49.2 ± 0.5	39.9 ± 1.5*	12.4 ± 4.1*	200.1 ± 12.9*
Control				
24 mmol l^{-1} K^+	−49.4 ± 0.9	38.7 ± 0.5	7.6 ± 0.4	181.0 ± 6.1
+ 100 μmol l^{-1} IBMX (4)	−49.4 ± 1.0	47.4 ± 0.5	21.3 ± 0.3	259.5 ± 6.0
+ 0.3 μmol l^{-1} carbachol‡§	−49.4 ± 1.0	41.6 ± 0.6*	14.2 ± 0.4*	212.6 ± 8.8*

Preparations obtained from reserpine-pretreated guinea-pigs (5 mg kg^{-1} body weight reserpine injected intraperitoneally 24 h before the experiment); stimulation frequency 0.2 Hz. Mean values ± s.e.mean are presented. Numbers in parentheses give the number of experiments.

‡ Electrode impalements were maintained during drug interventions.

§ Concentration that inhibits the positive inotropic effect by 50%.

* Significant ($P < 0.001$) vs the values in the presence of IBMX alone.

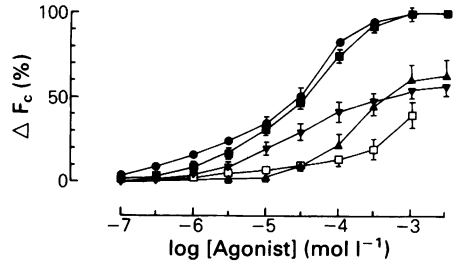

Figure 4 The positive inotropic effect of choline esters. Two cumulative concentration-effect curves were obtained from each muscle, one for carbachol (●) the other either for methacholine (■), acetylcholine (▼) or bethanechol (▲). A concentration-effect curve for methacholine in the presence of a 100 nmol l^{-1} atropine (□) is also shown. Means (with s.e.mean indicated by vertical lines if it exceeds the size of the symbol) for carbachol (18 muscles) and the other choline esters (6 muscles) are shown. Ordinate scale: positive inotropic effect, ΔF_c, expressed as % of the value observed with a maximally effective concentration of carbachol (100% = 6.9 ± 1.4 mN). Pre-drug control of F_c was 5.9 ± 0.9 mN (●), 6.6 ± 0.8 mN (▼), 9.1 ± 1.3 mN (■) 6.1 ± 0.6 mN (▲), 5.4 ± 1.6 mN (□). Reserpine-pretreated guinea-pigs were used for all experiments. Contraction frequency 0.2 Hz. $[Ca^{2+}]_o$ = 6.2 mmol l^{-1}.

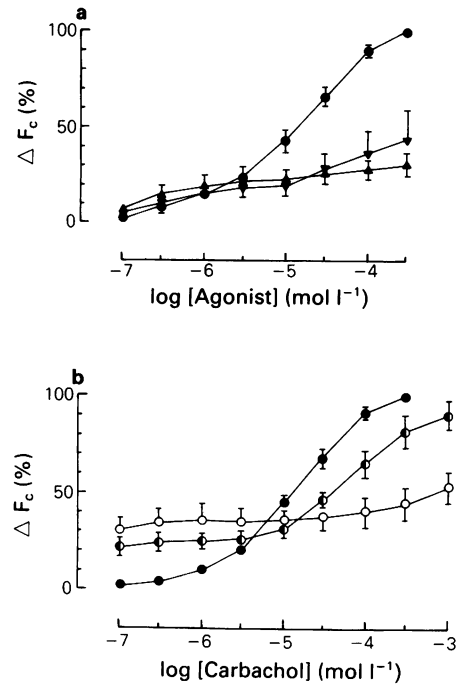

Figure 5 (a) Failure of atropine to inhibit the positive inotropic effect of oxotremorine. Three cumulative concentration-effect curves were obtained from each muscle, one for carbachol (●) the others for oxotremorine in the absence (▲) and in the presence of 100 nmol l^{-1} atropine (▼). Means, with s.e.mean indicated by vertical lines if it exceeds the size of the symbol (6 muscles) are shown. Ordinate scale: positive inotropic effect, ΔF_c, expressed as % of the value obtained with 300 μmol l^{-1} carbachol (100% = 6.5 ± 1.0 mN). Pre-drug control of F_c was 6.0 ± 0.4 mN (●), 5.3 ± 0.5 mN (▲), 6.2 ± 1.3 mN (▼). (b) Inhibition of the positive inotropic effect of carbachol by oxotremorine. Three carbachol concentration-effect curves were obtained from each muscle, one without oxotremorine (●), the two others in the presence of 1 μmol l^{-1} (◐) and 100 μmol l^{-1} oxotremorine (○), respectively. Ordinate scale: positive inotropic effect, ΔF_c, expressed as % of the value obtained with 300 μmol l^{-1} carbachol (100% = 6.4 ± 0.9 mN). Pre-drug control of F_c was 7.3 ± 2.9 mN (●), 6.2 ± 2.3 mN (◐), 6.8 ± 2.2 mN (○). Reserpine-pretreated guinea-pigs were used in all experiments depicted in (a) and (b). $[Ca^{2+}]_o$ = 6.2 mmol l^{-1}. Contraction frequency of 0.2 Hz.

was only 30% that of a maximal effective concentration of carbachol, when determined on the same preparation. The positive inotropic effect of oxotremorine developed with a time course comparable to that of choline esters. Figure 6 shows that the shape of the isometric contraction curve was unaffected by oxotremorine up to a concentration of 300 μmol l^{-1}. Figure 6 also shows (substantiated in 5 other muscle preparations) that 300 μmol l^{-1} oxotremorine did not alter resting membrane potential (V_m), overshoot (OS) and maximum rate of depolarization ($\dot{V}_{max}$), but prolonged significantly action potential duration at 0 (APD_o) and at 90% repolarization (APD_{90}). The values (n = 6) before and 10 min after the addition of 300 μmol l^{-1} oxotremorine were −82.3 ± 1.8 and −82.0 ± 1.6 mV for V_m, 46.2 ± 1.2 and 46.0 ± 0.8 mV for OS, 209.0 ± 16.0 and 208.0 ± 15.6 V s^{-1} for $\dot{V}_{max}$, 157.5 ± 4.3 and 168.6 ± 3.8 ms for APD_o ($P < 0.05$) and 211.8 ± 5.3 and 229.8 ± 4.3 ms for APD_{90} ($P < 0.05$), respectively. In contrast to choline esters, the positive inotropic effect of oxotremorine was not inhibited after prior incubation of the muscles with 10 or 100 nmol l^{-1} atropine (see Figure 5a). Furthermore, blockade of nicotinic receptors, histamine H_2-receptors, α- and β-adrenoceptors with 300 μmol l^{-1} hexamethonium bromide, 100 μmol l^{-1} cimetidine, 3 μmol l^{-1} phentolamine and 3 μmol l^{-1} (±)-propranolol, respectively, failed to antagonize the positive inotropic effect of oxotremorine. Tetrodotoxin (10 μmol l^{-1}) likewise did not attenuate the oxotremorine-induced inotropic effects. To determine whether oxotremorine interacted with mus-

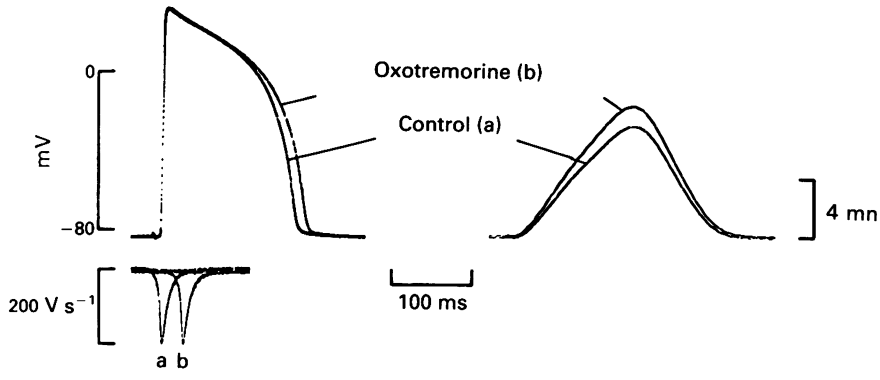

Figure 6 Effect of oxotremorine on the transmembrane action potential. Original records of the transmembrane action potential, maximum rate of depolarization ($V s^{-1}$) and isometric contraction of a noradrenaline-depleted papillary muscle before (control) and 10 min after the addition of 300 μmol l^{-1} oxotremorine. Records from the same microelectrode impalement. Contraction frequency 0.2 Hz. $[Ca^{2+}]_o = 6.2$ mmol l^{-1}.

carinic receptors responsible for the positive inotropic effect of choline esters, carbachol concentration-effect curves were obtained in the presence of 2 different oxotremorine concentrations. Figure 5b shows that oxotremorine inhibited the positive inotropic effect of carbachol and shifted the concentration-effect curves to higher carbachol concentrations. Due to the positive inotropic effect of 1 and 100 μmol l^{-1} oxotremorine, concentration-effect curves for carbachol intersected with the control curve at 2 different levels. As can be further seen from Figure 5b, the antagonism, at least in the presence of 1 μmol l^{-1} oxotremorine, was surmountable.

Intracellular Na^+ activity

Figure 7 shows the effects of 300 μmol l^{-1} acetylcholine (a) and of 300 μmol l^{-1} oxotremorine (b) on a^i_{Na} and resting membrane potential in a quiescent papillary muscle before and after the addition of 100 nmol l^{-1} atropine. Application of acetylcholine to the superfusing solution containing 6.2 mmol l^{-1} Ca^{2+} caused a^i_{Na} to increase from 5.8 mmol l^{-1} to 7.2 mmol l^{-1} within 6 min. After a^i_{Na} was stable for 15 min, 100 nmol l^{-1} atropine was added to the acetylcholine-containing solution, and a^i_{Na} returned within 15 min to the control level. Membrane resting potential remained at −82.8 mV throughout the experiment. In contrast to acetylcholine, oxotremorine did not increase a^i_{Na} significantly (Figure 7b). The values for a^i_{Na} before and 15 min after the addition of 300 μmol l^{-1} oxotremorine were 5.7 mmol l^{-1}, respectively. Additional application of 100 nmol l^{-1} atropine was also without effect on a^i_{Na}. Membrane resting potential remained stable at −83.1 mV throughout the experiment. Table 2 summarizes the effects of acetylcholine and oxotremorine on resting potential and a^i_{Na} obtained from 3 catecholamine-depleted papillary muscles bathed in 6.2 mmol l^{-1} Ca^{2+}.

Discussion

The present study demonstrates that muscarinic receptor agonists produced two distinct inotropic effects in guinea-pig ventricular myocardium which were opposite in direction and differed markedly in their concentration-dependence. The half-maximal effective concentrations needed to decrease force of contraction were about 100 fold lower than those necessary to elicit positive inotropic effect.

It is well known that the negative inotropic effect of muscarinic receptor agonists depends on the presence of drugs which increase force of contraction by augmenting cellular cyclic AMP (for review see Löffelholz & Pappano, 1985). In order to increase the cellular cyclic AMP level, the phosphodiesterase inhibitor 3-isobutyl-1-methyl xanthine (IBMX) was used in the present study. This compound has been shown to increase the cyclic AMP content in guinea-pig papillary muscles and, in contrast to other phosphodiesterase inhibitors,to mimic the mechanical and membrane electrical effects of catecholamines (Korth, 1978; Korth & Engels, 1981; Brückner *et al.*, 1985). Because of the stability of its positive inotropic effect, IBMX was preferred to catecholamines. With respect to its antagonistic effect on force of contraction and on slow Ca^{2+}-dependent action potential, oxotremorine was more potent than choline esters, probably due to

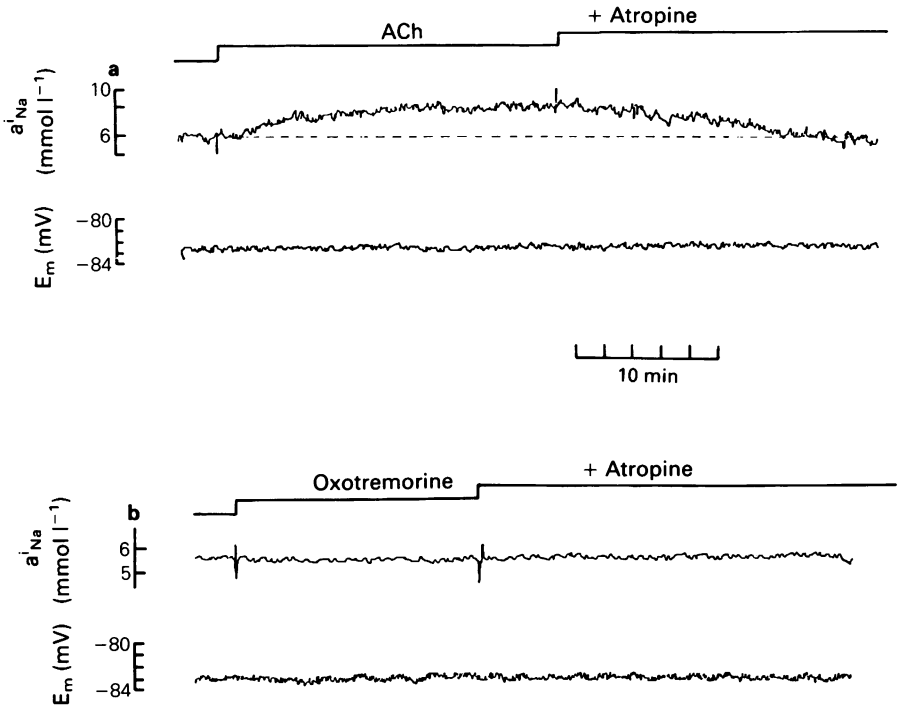

Figure 7 (a) Acetylcholine (ACh, 300 μmol l^{-1})-induced increase in intracellular Na^+ activity. Increase in a^i_{Na} during superfusion of a resting noradrenaline-depleted papillary muscle with acetylcholine and its reversal by 100 nmol l^{-1} atropine. The resting membrane potential (E_m) remained unchanged. (b) Failure of oxotremorine (300 μmol l^{-1}) to induce an increase in intracellular Na^+ activity. No change of resting membrane potential was observed. $[Ca^{2+}]_o$ = 6.2 mmol l^{-1} in (a) and (b).

Table 2 Effects of acetylcholine and oxotremorine on a^i_{Na} in quiescent guinea-pig papillary muscles exposed to a 6.2 mmol l^{-1} Ca^{2+}-containing solution

Condition	*Resting potential* (mV)	a^i_{Na} (mmol l^{-1})	Δa^i_{Na}‡ (mmol l^{-1})
Control	−83.4 ± 0.8	5.5 ± 0.6	
300 μmol l^{-1} acetylcholine (3)	−83.6 ± 0.8	7.6 ± 0.8*	1.6 ± 0.4
+ 100 nmol l^{-1} atropine	−83.5 ± 0.7	5.5 ± 0.6	
Control	−84.1 ± 1.2	5.3 ± 0.5	
300 μmol l^{-1} oxotremorine (3)	−84.2 ± 1.2	5.3 ± 0.5	—
+ 100 nmol l^{-1} atropine	−84.3 ± 1.2	5.3 ± 0.5	

Results are expressed as mean ± s.e.means. Preparations obtained from reserpine-pretreated animals (5 mg kg^{-1} body weight reserpine injected intraperitoneally 24 h before the experiment).
Numbers in parentheses give the number of experiments.
* $P < 0.01$ vs control.
‡ Increase in a^i_{Na} above control.

its higher potency at inhibiting cyclic AMP formation (Delhaye *et al.*, 1984; Brown & Brown, 1984). Atropine was a competitive inhibitor of the negative inotropic action of oxotremorine and of choline esters in the presence of IBMX. From the identical dissociation constants of atropine, it can be concluded that oxotremorine and choline esters interacted with the same population of muscarinic receptors. When applied at high concentrations, either in the absence or in the presence of IBMX, choline esters produced, via muscarinic receptors, a concentration-dependent positive inotropic effect. It has previously been proposed that the positive inotropic effect of high concentrations of carbachol is due to an increase in intracellular Na^+ activity, a^i_{Na}, which enhances force of contraction by stimulating Na^+-Ca^{2+} exchange (Korth & Kühlkamp, 1985). This hypothesis is strengthened by the present study; acetylcholine which was only half as effective as carbachol on force of contraction, induced an increase in a^i_{Na} which was 50% of that of a maximally effective concentration of carbachol (compare Korth & Kühlkamp, 1985). Since the carbachol-induced increase in force of contraction is strenghthened by raising the extracellular Ca^{2+} concentration (Korth & Kühlkamp, 1985), the positive inotropic effect and a^i_{Na} were determined in this study in the presence of 6.2 mmol l^{-1} Ca^{2+}. It should be noted, however, that raising or lowering Ca^{2+} in the bath solution had no influence on the EC_{50} values of the positive or the negative inotropic effect of the muscarinic receptor agonists. Among the choline esters, carbachol exhibited the highest positive inotropic potency and efficacy, followed by methacholine, acetylcholine and bethanechol. Oxotremorine produced a comparatively small increase in force of contraction, which, in contrast to the positive inotropic effect of choline esters, was not due to an increase in a^i_{Na} and was not antagonized by atropine. On the other hand, oxotremorine inhibited in a concentration-dependent manner the positive inotropic effect of carbachol and thus oxotremorine must have occupied the same muscarinic receptor as carbachol but, like an antagonist, failed to exhibit intrinsic activity. Failure of various antagonists to inhibit the oxotremorine-induced increase in force of contraction, excludes the involvement of specific membrane receptors which are known to mediate positive inotropic effects in heart muscle. On the other hand, oxotremorine prolonged the duration of the transmembrane action potential at 0 and 90% repolarization, and it may be speculated that a prolonged Ca^{2+} influx during depolarization was, at least in part, responsible for the small increase in force. The atropine-resistant positive inotropic effect of oxotremorine offers a straightforward explanation for the increase in antagonist potency of increasing concentrations of atropine in the presence of oxotremorine and IBMX. Since atropine did not prevent oxotremorine from counteracting its negative inotropic effect, the concentration-effect curves were shifted farther to the right and a stoichiometric coefficient of atropine binding greater than 1 was obtained.

In a recent study, Brown & Brown (1984) have found that carbachol elicited two biochemical effects in embryonic chick heart cells: inhibition of catecholamine-stimulated cyclic AMP formation and stimulation of phosphoinositide hydrolysis. The half-maximal effective concentrations (EC_{50}) for these biochemical effects were 0.2 and 20 μmol l^{-1}, respectively, and thus remarkably close to the EC_{50} values of 0.25 and 32 μmol l^{-1}, determined for the negative and for the positive inotropic effect of carbachol in guinea-pig heart. In accordance with the present results, Brown & Brown (1984) have also observed that oxotremorine, which was more effective than carbachol at inhibiting cyclic AMP formation, failed to stimulate phosphoinositide turnover. In the mammalian heart, muscarinic receptors are known to exhibit high and low affinity states for agonists, and it has been proposed that the high affinity state may represent receptors that can couple to and inhibit adenylate cyclase, while the low affinity state may be associated with effects on phosphoinositide turnover (Quist, 1982; Brown & Brown, 1983; 1984; McMahon & Hosey, 1985). As an alternative explanation, Brown & Brown (1984) suggested that inhibition of adenylate cyclase and activation of phosphoinositide metabolism by muscarinic receptor agonists could result from actions on the same receptor state, which is efficiently coupled to the former and inefficiently coupled to the latter effect.

While all available evidence indicates that inhibition of adenylate cyclase is the cause for the negative inotropic effect of choline esters and of oxotremorine, phosphoinositide metabolism and the positive inotropic effect of high choline ester concentrations may be merely epiphenomena. It should be noted, however, that inositol 1,4,5-trisphosphate which is one of the products of phosphoinositide hydrolysis, is known to mobilize intracellular Ca^{2+} from non-mitochondrial Ca^{2+} stores in various cell types, including the sarcoplasmic reticulum of canine heart (Hirata *et al.*, 1984; Streb *et al.*, 1983; Volpe *et al.*, 1985; Hashimoto *et al.*, 1986). Although it seems to be very unlikely that release of Ca^{2+} from cardiac sarcoplasmic reticulum was responsible for the long-lasting positive inotropic effect of choline esters, an increased Ca^{2+}-influx across the cardiac plasma membrane in response to an accumulation of inositol 1,4,5-triphosphate should be taken into consideration (see also Berridge, 1984). Whether the increase in intracellular Ca^{2+} occurs only secondary to an increase in intracellular Na^+ via Na^+-Ca^{2+} exchange (this study; Korth & Kühlkamp, 1985)

or is also due to an increased permeability of the sarcolemma for Ca^{2+} remains to be determined. Alternatively, the second product of phosphoinositide hydrolysis, 1,2-diacylglycerol, which has been shown to activate a membrane-bound pump that exchanges H^+ for Na^+ (for review, see Macara, 1985), could have increased intracellular pH and a^i_{Na} which in turn was then exchanged for extracellular Ca^{2+}.

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A comparison between the pattern of dopamine and noradrenaline release from sympathetic neurones of the dog mesenteric artery

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1 The release of dopamine and noradrenaline (NA), from the main trunk of the mesenteric artery and its proximal branches elicited by electrical nerve stimulation and K^+, has been measured by using high pressure liquid chromatography with electrochemical detection.

2 Both stimuli released dopamine and NA. With the main trunk of the mesenteric artery, dopamine represented 8% of the NA tissue content; the dopamine/NA ratio in the catecholamine overflow caused by nerve stimulation or K^+-induced depolarization also averaged 8%. For the proximal branches the tissue dopamine/NA ratio was significantly greater than that observed to occur in the overflow caused by nerve stimulation and K^+.

3 When the perifusion with a K^+-enriched medium was extended to 120 min the amount of NA released from both the main trunk and the proximal branches progressively declined. The same pattern of release was observed for dopamine in the main trunk, whereas for the proximal branches dopamine overflow did not decline throughout the perifusion period. The addition of α-methyl-*p*-tyrosine did not change the pattern of amine overflow.

4 Our interpretation of these results is that both dopamine and NA are derived from the same sympathetic neurone. In the proximal branches of the mesenteric artery dopamine and NA appear to be in two different storage structures, whereas in the main trunk both dopamine and NA are located in only one storage structure.

Introduction

In previous studies we have identified a noradrenaline-independent dopamine pool, as defined by selective 6-hydroxydopamine depletion of noradrenaline (NA), in the proximal branches of the mesenteric artery but not in the main trunk from the same blood vessel (Soares-da-Silva & Davidson, 1985; Caramona & Soares-da-Silva, 1985). Further investigations on this subject, in order to define the nature and cellular localization of this NA-independent dopamine pool, have provided evidence against the presence of independent dopaminergic neurones in this vascular area and indicated that this NA-independent dopamine pool is probably a non-precursor store of dopamine in noradrenergic neurones supplying the proximal branches of the mesenteric artery (Soares-da-Silva, 1986a, b). Taken together these results led us to suggest that in the proximal branches of the mesenteric artery the non-precursor dopamine pool and NA, though present in the very same neurone, are stored in two different storage structures; whereas in the main trunk of the mesenteric artery both dopamine and NA are probably stored in one common storage structure, since no evidence has been found in favour of a non-precursor dopamine pool (Soares-da-Silva, 1986b).

Although other evidence is needed in order to confirm this theory, the resolution of this problem clearly has important implications, since, until recently, dopamine in peripheral tissues was assumed to be a precursor for the synthesis of ß-hydroxylated catecholamines in adrenergic neurones and the adrenal medulla or a transmitter in its own right in independent dopaminergic neurones distributed to some well-defined areas (Bell, 1982). Thus, if there is a different storage structure for the non-precursor dopamine pool in noradrenergic neurones supplying the proximal branches of the mesenteric artery, but in the main trunk from the same blood vessel both dopamine and NA are located in one common storage structure, the proportional release of dopamine and NA in these two vascular tissues should vary according to the amine storage structure mobilized.

We have investigated this problem by studying the amount of both amines released from the main trunk of the mesenteric artery and its proximal branches during electrical stimulation or K^+-induced depolarization.

Methods

Mongrel dogs of either sex weighing 14–23 kg were anaesthetized with sodium pentobarbitone (30 mg kg^{-1} i.v., injected in the forelimb) and the main trunk of the anterior mesenteric artery and its proximal branches removed, stripped of their mesentery, rinsed free from blood and cut longitudinally. Each segment, weighing about 40 mg in the case of proximal branches of the mesenteric artery or up to 100 mg for samples of the main trunk and 4 cm long, was incubated for 30 min in 5 ml of Krebs solution, at 37°C, gassed with 95% O_2 and 5% CO_2, in the presence of 55 μM hydrocortisone and 0.1 mM pargyline (in order to block extraneuronal uptake and monoamine oxidase, respectively). The Krebs solution had the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.4, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2 and glucose 11. EDTA 0.04 mM was added to the Krebs solution in order to prevent oxidative destruction of catecholamines.

After the incubation period, segments of proximal branches and main trunk of the mesenteric artery were continuously perifused for 90 min in a 1 ml organ bath; aerated (95% O_2 and 5% CO_2) and warmed (37°C) Krebs solution (containing hydrocortisone as above) was pumped through the bath by means of a Harvard Peristaltic Pump (model 1210) at a constant rate of 0.3 ml min^{-1}, and the overflow was collected. In all experiments cocaine (10 μM) was added to the perifusion fluid from 0 min onwards. In some experiments phentolamine (0.2 μM) was also added at this time. Electrical stimulation (from t = 60 to t = 90 min) was applied by means of thin platinum electrodes attached to both ends of the preparation. The stimuli were rectangular pulses of alternating polarity, 2 ms duration, 100 V intensity at 1 Hz frequency (Hugo Sachs Stimulator, model 215/II). The fluid was collected in 10 ml cooled vials containing 0.8 ml 1.0 mM perchloric acid. At the end of the collection period 50 mg alumina was added and the pH of the sample immediately adjusted to pH 8.6. Mechanical shaking for 10 min was followed by centrifugation and the supernatant discarded. The adsorbed catecholamines were then eluted from the alumina with 150 μl 0.1 mM perchloric acid on Millipore microfilters (MF 1); 50 μl of the eluate was injected into a high pressure liquid chromatograph with electrochemical detection (BAS model 304 LC 4B) and the dopamine and NA measured. A 5 μM ODS column of 25 cm length was used. The mobile phase was degassed solution of monochloroacetic acid (0.15 mM), sodium octylsulphate (0.3 mM) and EDTA (2 mM), pH 3.0, pumped at a rate of 1.8 ml min^{-1}. A carbon paste electrode was used and the detector potential was + 0.65 V. Dihydroxybenzylamine was used as an internal standard. Peak height increased linearly with the concentration of NA and dopamine. The interassay coefficient of variation was less than 5%. Under our conditions, the lower limits of detection for NA and dopamine were 10 and 30 pg per sample, respectively.

The spontaneous loss from t = 30 to t = 60 min (in the absence of nerve stimulation) was also measured. This value was subtracted from the nerve-stimulation overflow, allowing the overflow as a result of nerve stimulation to be calculated.

After the stimulation period tissues were removed from the organ bath, blotted with filter paper, weighed, minced with fine scissors and homogenized with a Duall-Kontes homogenizer in 2.0 ml 0.1 mM perchloric acid. The homogenates were centrifuged (10000 r.p.m., 15 min, 0°C) and the supernatant decanted. Aliquots of 1.5 ml supernatant were placed in 5 ml conical glass vials with 50 mg alumina and the pH adjusted to 8.6. Mechanical shaking for 10 min was followed by centrifugation, the supernatant discarded and subsequently treated like samples of bathing fluid.

In another set of experiments tissues were perifused with a K^+-enriched Krebs solution; 40% of the NaCl was replaced by KCl in the KCl-enriched medium giving final concentrations of NaCl and KCl of 71 and 52 mM, respectively. Throughout these experiments, the main trunk of the mesenteric artery and its proximal branches were handled as described above, except that from t = 60 to t = 90 min or to t = 180 min K^+-enriched Krebs solution was pumped through the perifusion organ bath; from t = 0 to t = 60 min tissues were perifused with normal Krebs solution. Similar to the nerve stimulation experiments the spontaneous loss from t = 30 to t = 60 min was also measured and this value subtracted from the K^+-induced release to give the extra overflow as a result of catecholamine release during the depolarization period.

Differences between two means were estimated by Student's *t* test for unpaired data; a probability of less than 0.05 was assumed to denote a significant difference.

Drugs

Drugs used were: cocaine hydrochloride (Uquipa, Lisboa, Portugal), dopamine hydrochloride (Sigma, St. Louis, MO, U.S.A.), ethylenediaminetetracetic acid disodium salt (EDTA, Sigma), hydrocortisone

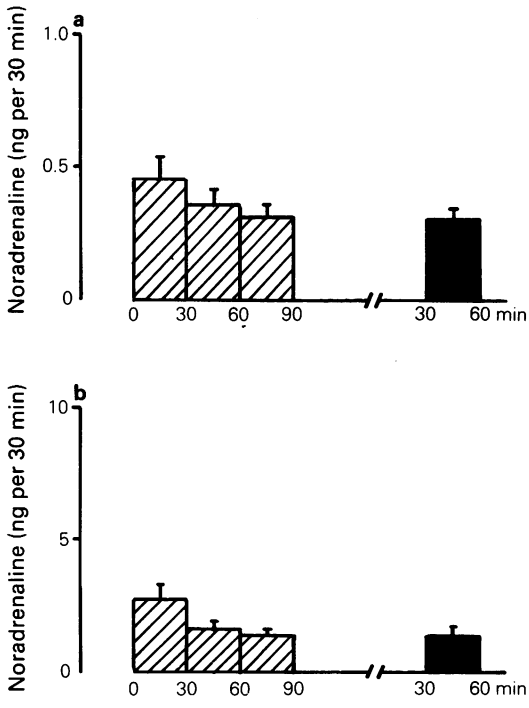

Figure 1 Progressive decline with time of the spontaneous loss of noradrenaline in (a) the main trunk of the mesenteric artery and (b) its proximal branches. The hatched columns represent the means of three experiments and the solid columns represent the mean noradrenaline spontaneous loss in ten similar experiments at least 30 min after setting up the preparations.

phosphate (Sigma), 1-α-methyl-*p*-tyrosine (Sigma), (−)-noradrenaline bitartrate (Sigma), pargyline hydrochloride (Sigma) and phentolamine hydrochloride (Regitin, Ciba, Switzerland).

Results

Spontaneous loss and nerve stimulated release

Both dopamine and NA were detectable in samples of the perifusion fluid obtained during nerve stimulation, but not in spontaneous overflow, where only NA was found. The amounts were greatest immediately after setting up the preparations and declined to a lower stable value from t = 30 to t = 60 min. In preliminary experiments the collection period was extended to t = 90 min. Although the spontaneous loss of NA appeared to be slightly decreased from t = 60 to t = 90 min, the difference was not found to be significant (Figure 1).

Electrical stimulation increased, by ten times, the NA overflow and dopamine was also released from both preparations under study, the main trunk of the mesenteric artery and its proximal branches. As shown in Table 1, the amount of NA released during nerve stimulation of the main trunk of the mesenteric artery was about three times less than that released from the proximal branches. However, when NA overflow values are expressed as fractional release no obvious difference was found between the two tissues studied (data not shown). The proportion of dopamine to NA released, which averaged 8.2% for the main trunk of the mesenteric artery, was about half that value for the proximal branches. Even when values of the released dopamine were expressed as a fraction of the tissue amine content a significant difference was found between the main trunk of the mesenteric artery and its proximal branches. Quite unexpectedly, whereas for the main trunk of the mesenteric artery both dopamine/NA ratios in tissue and nerve stimulation overflow averaged 8%, in the proximal branches the tissue dopamine/NA ratio was greater than that for nerve stimulation overflow ($P < 0.05$).

In another five experiments phentolamine was added to the perifusion fluid. Phentolamine had an increasing effect on the overflow of both amines during nerve stimulation of both tissues. However, in the main trunk phentolamine-induced increase of NA and dopamine release was of about 50 and 60%, respectively. In the proximal branches the effect of phentolamine was considerably more pronounced. The proportion of dopamine to NA in the overflow remained unchanged in the presence of phentolamine compared to control conditions (Table 1).

Spontaneous loss and K^+-induced release

The experiments where the main trunk of the mesenteric artery and its proximal branches were exposed to a K^+-enriched Krebs solution from t = 60 to t = 90 min showed that both dopamine and NA were released. As in experiments with nerve stimulation, dopamine was not found to occur in detectable amounts in spontaneous overflow (Table 2). Dopamine and NA overflow levels in experiments with K^+-enriched medium were similar to those for nerve stimulation; dopamine/NA ratios were about the same. With the main trunk of the mesenteric artery the proportion of dopamine to NA released by K^+ was the same as that found in the tissue. However, for the proximal branches the proportion of dopamine to NA released was lower than that found in the tissue (Table 2).

In five further experiments, the effect of phentolamine was examined. Phentolamine increased the

Table 1 Absolute and relative contents of dopamine (DA) and noradrenaline (NA) (ng g^{-1}) and spontaneous loss and nerve stimulated release of DA and NA (ng per 30 min) from (a) the main trunk of the mesenteric artery and (b) its proximal branches

	Spontaneous loss	*Nerve stimulation release*	*Tissue levels*
a *Main trunk of the mesenteric artery*			
NA	0.3 ± 0.04	4.1 ± 0.3	1214.8 ± 40.2
DA	-	0.3 ± 0.03	96.6 ± 6.1
DA/NA × 100	-	8.2 ± 1.3	8.0 ± 0.7
Phentolamine (0.2 μM) present throughout			
NA	0.4 ± 0.04	6.3 ± 0.7	1229.4 ± 115.8
DA	-	0.5 ± 0.06	83.4 ± 6.5
DA/NA × 100	-	8.0 ± 0.8	7.5 ± 0.7
b *Proximal branches of the mesenteric artery*			
NA	1.2 ± 0.11	13.4 ± 0.4	3403.7 ± 70.9
DA	-	0.4 ± 0.04	178.4 ± 17.0
DA/NA × 100	-	3.0 ± 0.35*	5.5 ± 0.4
Phentolamine (0.2 μM) present throughout			
NA	1.5 ± 0.18	28.6 ± 1.5**	3457.0 ± 105.3
DA	-	1.1 ± 0.18**	194.9 ± 18.3
DA/NA × 100	-	4.1 ± 0.3*	5.8 ± 0.5

Values are means ± s.e.mean of five experiments.
**Significantly different from control values ($P < 0.01$).
*Significantly different from values for tissue DA/NA ratios ($P < 0.05$).

overflow of both amines by about two times in the proximal branches, but the overflow dopamine/NA ratio remained almost the same as under control conditions. Interestingly, in the main trunk of the mesenteric artery, just as with nerve stimulation, phentolamine increased both dopamine and NA release by 50 and 60%, respectively. The overflow dopamine/NA ratio remained constant at 8%.

Table 2 Absolute and relative contents of dopamine (DA) and noradrenaline (NA) (ng g^{-1}) and spontaneous loss and K^+-evoked release of DA and NA (ng per 30 min) from (a) the main trunk of mesenteric artery and (b) its proximal branches

	Spontaneous loss	*K^+-evoked release*	*Tissue levels*
a *Main trunk of the mesenteric artery*			
NA	0.3 ± 0.03	5.2 ± 0.4	1272.0 ± 164.0
DA	-	0.4 ± 0.04	107.4 ± 11.9
DA/NA × 100	-	7.5 ± 0.7	8.2 ± 0.7
Phentolamine (0.2 μM) present throughout			
NA	0.3 ± 0.03	7.4 ± 0.6	1367.2 ± 105.3
DA	-	0.6 ± 0.05	105.4 ± 10.6
DA/NA × 100	-	8.0 ± 0.8	7.8 ± 0.6
b *Proximal branches of the mesenteric artery*			
NA	1.0 ± 0.09	13.8 ± 1.4	3315.4 ± 393.3
DA	-	0.5 ± 0.04	184.5 ± 18.4
DA/NA × 100	-	3.4 ± 0.30*	5.5 ± 0.5
Phentolamine (0.2 μM) present throughout			
NA	1.1 ± 0.10	26.4 ± 2.5**	3226.6 ± 282.9
DA	-	1.0 ± 0.09**	173.2 ± 18.8
DA/NA × 100	-	3.5 ± 0.3*	5.4 ± 0.5

Values are means ± s.e.mean of five experiments.
**Significantly different from control values ($P < 0.01$).
*Significantly different from values for tissue DA/NA ratio ($P < 0.05$).

Table 3 Absolute and relative contents of dopamine (DA) and noradrenaline (NA) ($ng\,g^{-1}$) and the effect of prolonged K^+-induced depolarization on the DA and NA overflow (ng per 30 min) from (a) the main trunk of the mesenteric artery and (b) its proximal branches

	Spontaneous loss	*DA and NA overflow in each period of depolarization*				*Tissue levels*
		First	*Second*	*Third*	*Fourth*	
a *Main trunk of the mesenteric artery*						
NA	0.4 ± 0.04	6.8 ± 0.7	4.6 ± 0.5*	3.8 ± 0.4**	3.0 ± 0.3**	1089.0 ± 90.3
DA	-	0.5 ± 0.06	0.3 ± 0.03*	0.2 ± 0.02**	0.2 ± 0.02**	94.3 ± 9.8
DA/NA × 100	-	8.1 ± 0.8	7.5 ± 0.8	6.1 ± 0.6	6.5 ± 0.6	8.5 ± 0.9
b *Proximal branches of the mesenteric artery*						
NA	0.9 ± 0.1	27.3 ± 1.9	12.6 ± 1.3**	10.2 ± 1.1**	8.3 ± 0.9**	2698.0 ± 70.9
DA	-	0.8 ± 0.07	0.9 ± 0.08	1.1 ± 0.1*	1.1 ± 0.09*	228.0 ± 29.7
DA/NA × 100	-	3.2 ± 0.3	7.3 ± 0.7	10.5 ± 1.2**	13.3 ± 0.4**	8.4 ± 0.9

Values are means ± s.e.mean of five experiments.
Significantly different from values for the first period of depolarization, $*P<0.05$; $**P<0.01$.

The effect of prolonged K^+-induced depolarization on dopamine and noradrenaline overflow

We extended the perifusion period with the K^+-enriched Krebs solution from t = 60 to t = 180 min and the overflow was collected in four equal fractions. In these experiments phentolamine (0.2 μM) was added to the perifusion fluid from t = 0 min onwards. As shown in Table 3, the amount of NA released, both from the main trunk of the mesenteric artery and its proximal branches, progressively declined in each period of depolarization so that in the fourth the overflow was only 40% of the first. With the main trunk the amount of dopamine released also progressively declined from the first period of depolarization to the fourth, as did NA. However, for the proximal branches, dopamine overlfow values progressively increased from the first to the last sample obtained during depolarization.

In the main trunk of the mesenteric artery the overflow dopamine/NA ratios were similar at all collection periods, though a slight decrease of the overflow dopamine/NA ratio was observed during the course of the experiment. On the other hand, for the proximal branches the overflow dopamine/NA ratios progressively increased from the first to the fourth period of depolarization.

The effect of α-methyl-p-tyrosine on the K^+-evoked release of dopamine and noradrenaline

The results in the preceding paragraph, namely those concerning dopamine overflow from the proximal branches of the mesenteric artery, led us to hypoth-

Table 4 Absolute and relative contents of dopamine (DA) and noradrenaline (NA) ($ng\,g^{-1}$) and the effect of α-methyl-*p*-tyrosine on the K^+-induced release of DA and NA (ng per 30 min) from (a) the main trunk of the mesenteric artery and (b) its proximal branches

	Spontaneous loss	*DA and NA overflow in each period of depolarization*				*Tissue levels*
		First	*Second*	*Third*	*Fourth*	
a *Main trunk of the mesenteric artery*						
NA	0.4 ± 0.04	6.1 ± 0.6	3.5 ± 0.3*	2.5 ± 0.3*	1.9 ± 0.2*	1033.9 ± 97.4
DA	-	0.4 ± 0.04	0.2 ± 0.02*	0.15 ± 0.02*	0.2 ± 0.02*	39.4 ± 3.8
DA/NA × 100	-	6.0 ± 0.6	5.8 ± 0.6	6.2 ± 0.6	5.3 ± 0.5	3.8 ± 0.4
b *Proximal branches of the mesenteric artery*						
NA	0.8 ± 0.09	15.9 ± 0.5	8.7 ± 0.4*	6.6 ± 0.3*	5.2 ± 0.3*	2344.2 ± 210.4
DA	-	0.4 ± 0.03	0.4 ± 0.04	0.4 ± 0.06	0.4 ± 0.04	44.8 ± 6.7
DA/NA × 100	-	2.5 ± 0.2	4.5 ± 0.4*	4.5 ± 0.3*	7.9 ± 1.0*	1.9 ± 0.2

Values are means ± s.e.mean of five experiments.
Significantly different from values for the first depolarization period, $*P<0.01$.

esize that an increase in catecholamine synthesis was occurring during the prolonged depolarization to which tissues were submitted. For this reason experiments with α-methyl-*p*-tyrosine (which inhibits dopamine and NA synthesis) were performed. α-Methyl-*p*-tyrosine (2 μM) was added to the perifusion fluid from t = 0 min onwards, and the experimental arrangement was similar to that described in the preceding paragraph.

For the proximal branches of the mesenteric artery, α-methyl-*p*-tyrosine produced a significant decrease of both dopamine and NA overflow values at all four periods of depolarization. However, for the main trunk both dopamine and NA overflow levels only became significantly reduced after t = 90 min, as shown in Table 4. Contrary to the progressive decline of NA overflow from the first to the fourth period of depolarization, in the proximal branches of the mesenteric artery dopamine overflow remained unchanged throughout the perifusion period despite the inhibition of catecholamine synthesis.

Surprisingly, only the dopamine content in both blood vessels was found to be significantly decreased after α-methyl-*p*-tyrosine, whereas NA content was unaffected.

Discussion

Nerve stimulation and K^+-induced depolarization caused a simultaneous release of both dopamine and NA. However, for the proximal branches of the mesenteric artery the proportion of dopamine released was signficantly lower than that found in the tissue, whereas for the main trunk the proportion of dopamine to NA released was similar to that found in the tissue. Therefore, it is possible that in the proximal branches of the mesenteric artery dopamine and NA do not constitute a homogeneous mixture.

The most important result needed to elucidate whether dopamine is present in neurones or in extraneuronal structures is that phentolamine, a drug known to act on adrenergic nerves, produced a similar increase of dopamine and NA release, so that the proportion of dopamine to NA in the overflow remained unchanged. This also suggests, though it does not prove, that dopamine and NA are released from the same neurone. As is well known, evidence favours the view that the increase in NA overflow by phentolamine is due to blockade of α-adrenoceptors present on sympathetic nerve terminals (Langer, 1977; Starke, 1977). In a dopaminergic nerve terminal there would be no NA release to activate the α-adrenoceptor mediated feedback system. Therefore, it is unlikely that dopamine comes from dopaminergic nerves but almost certain that both dopamine and NA are released from the same sympathetic neurone. This also agrees with previous results, using a different experimental approach, where evidence against the presence of independent dopaminergic neurones in this vascular area was presented (Soares-da-Silva, 1986a, b).

Assuming that the proportion of the two amines in the overflow from the proximal branches does reflect their release, then the higher proportion of NA might suggest that dopamine and NA in tissue stores are equally mobilized by nerve stimulation and K^+-induced depolarization. Expressed as a fraction of total tissue amine there is in fact a preferential release of NA. In addition, it has been shown that part of the total NA in tissue stores is present in a pool which is available to be depleted rapidly by nerve impulses and another pool which is less available for depletion (Trifaró & Cubeddu, 1979; Fried, 1980). Thus, if NA is mainly released from the former pool there would be a preferential release of NA and for the same reason it would be this pool which would be depleted by continuous K^+-induced depolarization, which was our finding. In contrast to the results with NA, dopamine overflow in the same blood vessel progressively increased or remained about the same when tyrosine hydroxylase was inhibited throughout the perifusion period, thus suggesting that a pool less available for depletion is probably the dominating storage form for dopamine. Thus, the possibility that there are two different types of storage structures for dopamine and NA inside sympathetic nerves supplying the proximal branches of the mesenteric has to be faced.

For the main trunk of the mesenteric artery, where all of the dopamine is a precursor for NA (Soares-da-Silva, 1986a, b), similar dopamine/NA ratios in the overflow and tissue stores might suggest that dopamine and NA in the tissue are equally available for the nerve impulse. These results agree with those found by Bell *et al.* (1984) for the rat and guinea-pig vas deferens, where innervation is purely noradrenergic (Bell & Gillespie, 1981) and both dopamine and NA are released from a common store during noradrenergic transmission.

The experiments with α-methyl-*p*-tyrosine also agree with the view that in the proximal branches of the mesenteric artery dopamine and NA in tissue stores are not equally mobilized by depolarization and dopamine is probably stored in a pool which is only slowly depleted, though more sensitive to tyrosine hydroxylase inhibition than the NA pool. These experiments further suggest that catecholamine synthesis occurs during K^+-induced depolarization of the dog mesenteric artery and also provide evidence in favour of a preferential release of newly synthesized amine, as desribed by Kopin *et al.* (1968) and Klein & Harden (1975). On the other hand, data from experiments with α-methyl-*p*-tyrosine also indicate that

the dopamine pool (slowly depleted), just as the NA pool (rapidly depleted), reside in cellular compartments equally involved in transmitter turnover. In addition, they agree with the occurrence of active catecholamine synthesis during *in vitro* nerve activation, as extensively reviewed by Weiner (1970).

Although it is not clear what role the dopaminergic mechanisms play in cardiovascular homeostatis and pathophysiology of cardiovascular disorders, there is evidence to suggest an involvement of the catecholamine dopamine in conditions such as hypertension, heart failure and cardiac ischaemia. In fact, the activity of the renin-angiotensin-aldosterone system may be regulated by the endogenous dopaminergic tone, since dopamine receptor antagonists modulate the aldosterone response to angiotensin II (A II) (Gordon *et al.*, 1983). On the other hand, at the advanced hypertensive stage, the effects of dopamine ß-hydroxylase and phenylethanolamine N-methyltranferase in spontaneously hypertensive rats (SHR) remained low, whereas that of tyrosine hydroxylase increased, indicating a non-uniform change in the synthetic enzyme activities during the course of hypertension (Grobecker *et al.*, 1982). A similar situation has been described in the failing hamster heart; increased tyrosine hydroxylase and decreased dopamine ß-hydroxylase activity with increased dopamine tissue content (Sole *et al.*, 1982). Other authors have observed increased tissue dopamine levels in the heart, kidney, adrenal medualla (Racz *et al.*, 1986) and sympathetic ganglia (Gianutsos & Moore, 1978). Also, increased release of dopamine from sympathetic nerves was observed in perinephritic hypertensive dogs (Branco & Osswald, 1986) and ischaemic myocardium of the rat (Schömig *et al.*, 1984). Moreover, it has been suggested that the down regulation of dopamine receptors in SHR (Beck & Sowers, 1984) could be due to an increased release of dopamine from sympathetic nerves (Racz *et al.*, 1986).

In conclusion, our interpretation of the results presented is that both dopamine and NA are derived from the same sympathetic neurones. In the proximal branches of the mesenteric artery dopamine and NA appear to be in two different storage structures with a different pattern of release which suggest that dopamine might play a role in mesenteric vascular homeostasis. In fact, preliminary reults have suggested that dopamine in this vascular area may have a functional role in controlling transmitter release since dopamine receptor antagonists increased NA outflow (Soares-da-Silva, 1986c). Thus it would be interesting to perform further investigations under conditions of altered cardiovascular homeostasis such as those occurring in hypertension and heart failure.

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[^{3}H]-verapamil binding to rat cardiac sarcolemmal membrane fragments; an effect of ischaemia

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1 The [^{3}H]-verapamil binding activity of rat cardiac sarcolemmal fragments was studied, using membranes harvested from non-perfused, aerobically-perfused and ischaemic hearts.

2 Glass-fibre filters were found to contain specific, high affinity–(K_D 38 ± 3.1 nM) [^{3}H]-verapamil binding sites–making them unsuitable for use in [^{3}H]-verapamil binding studies.

3 Incubation of membranes from non-perfused hearts in a medium containing 150 mM NaCl, 1 mM $CaCl_2$ and 50 mM Tris revealed two populations of [^{3}H]-verapamil binding sites. When centrifugation instead of filtration was used to separate bound and free [^{3}H]-verapamil, high affinity sites with a K_D of 0.57 ± 0.19 μM and a B_{max} of 38 ± 5.2 pmol mg^{-1} protein, and low affinity sites with a K_D of 78 ± 27.5 μM and a B_{max} of 2.9 ± 1.3 nmol mg^{-1} protein were detected. However, only low affinity binding sites could be detected in membranes which had been incubated in a cation-free medium containing 50 mM Tris.

4 [^{3}H]-verapamil binding to the low and high affinity sites was saturable, reversible, stereospecific and displaceable by D600 > diltiazem > Ca^{2+} but not by nifedipine, nitrendipine, nisoldipine or prazosin.

5 The two populations of binding sites survived aerobic perfusion and 60 min ischaemia at 37°C. Ischaemia reduced the B_{max} and K_D but selectivity was maintained.

Introduction

The Ca^{2+} antagonists (Ca^{2+} channel blockers) modulate slow channel function by interacting with specific, membrane-located sites (Bayer *et al.*, 1982; Lee & Tsien, 1983: Janis & Scriabine, 1983; Triggle & Janis, 1984; Schwartz & Triggle, 1984; Glossmann *et al.*, 1985). The binding sites for the dihydropyridine-based antagonists have been extensively investigated (Bellemann *et al.*, 1981; 1982; 1983; Janis *et al.*, 1982; Gould *et al.*, 1982; Williams & Tremble, 1982; Holck *et al.*, 1982; 1983; Murphy & Snyder, 1982; Ehlert *et al.*, 1982; DePover *et al.*, 1982; Bolger *et al.*, 1983; Fossett *et al.*, 1983; Sarmiento *et al.*, 1983; Janis & Triggle, 1984; Triggle & Janis, 1984; Striessnig *et al.*, 1985; Glossmann *et al.*, 1985), and two populations of binding sites have been identified: high affinity sites, with a dissociation constant (K_D) of 0.1–0.3 nM and a density (B_{max}) of 50–200 fmol mg^{-1} protein, and low affinity sites, with a K_D of 60 nM and a B_{max} of 35 pmol mg^{-1} protein. These binding sites are saturable and stereospecific, and the bound ligand–usually [^{3}H]-nitrendipine–is displaceable (Glossmann *et al.*, 1985).

As far as phenylalkylamine-based Ca^{2+} antagonists are concerned, their binding sites have been less thoroughly investigated. Hulthen *et al.* (1982) detected a small number of high affinity [^{3}H]-verapamil binding sites in frog hearts. By contrast, microsomal fractions harvested from skeletal muscle T-tubules (Reynolds *et al.*, 1983; Garcia *et al.*, 1984; Galizzi *et al.*, 1984a,b; Glossmann *et al.*, 1984) and cerebral cortex (Reynolds *et al.*, 1983) seem to contain a relatively large number of low affinity [^{3}H]-verapamil binding sites.

The following studies were undertaken to characterize further the specific [^{3}H]-verapamil binding sites in cardiac sarcolemmal fragments and to establish whether the binding activity of these sites is modified by sixty minutes of normothermic ischaemia.

Methods

Sarcolemmal membranes harvested from adult male Sprague-Dawley rats (250–300 g) were used. The rats

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were maintained on a standard diet, and tail cuff measurements showed them to be normotensive.

Perfusion sequence

The rats were lightly anaesthetized with a diethylether-O_2 mixture and treated with heparin. The hearts were then excised, immersed in ice-cold perfusion buffer and either immediately subfractioned or perfused in the Langendorff mode, as previously described (Nayler *et al.*, 1984). The perfusion buffer was a modified Krebs-Henseleit solution containing, in mM: NaCl 119.0, $NaHCO_3$ 25.0, KCl 4.6, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 1.3 and glucose 11.0. It was gassed with 95% O_2 + 5% CO_2 (pH 7.4), maintained at 37°C, and unless otherwise stated, delivered to provide a mean coronary flow of 10–12 ml min^{-1}. Spontaneously beating preparations was used to avoid any possibility of electrical stimulation releasing endogenous noradrenaline.

After 15 min aerobic perfusion the hearts were randomly divided into two groups. One group was perfused for another 60 min as described above. The other hearts were made globally ischaemic for 60 min, by totally occluding coronary flow whilst maintaining left ventricular wall temperature at 37°C, by means of temperature-controlled water-filled heating jackets.

Isolation of sarcolemmal membrane fragments

After the required period of aerobic perfusion or global ischaemia the hearts were immersed in ice-cold homogenizing medium containing 250 mM sucrose and 10 mM Tris HCl (pH 8.1). Freshly excised but unperfused hearts were treated in the same way. The ventricles were trimmed of atria, weighed and sarcolemmal membrane fragments isolated by differential centrifugation and sucrose gradient purification as described elsewhere (Daly *et al.*, 1984).

Characterization of membranes

The isolated membranes were enzymatically characterized in terms of their Na^+/K^+-ATPase (Daly *et al.*, 1984), 5′ nucleotidase (Edwards & Maguire, 1970) and succinate dehydrogenase (Cooperstein *et al.*, 1950) activity and sialic acid content (Warren, 1959).

Radioligand binding assay

Equilibrium binding studies

[^{3}H]-verapamil binding [^{3}H]-verapamil binding was monitored for 30 min at 25°C in triplicate, using a reaction mixture containing 150 mM NaCl, 1.0 mM $CaCl_2$, 50 mM Tris (pH 7.4) and a protein concentration of 0.05–0.1 mg ml^{-1} (Hulthen *et al.*, 1982). Bound and free [^{3}H]-verapamil were separated by filtration or centrifugation as indicated.

When separation was by filtration, 2–60 nM [^{3}H]-verapamil was used in the absence or presence of 20 μM unlabelled verapamil for total and non-specific binding respectively. The reaction was terminated by adding 3.5 ml of ice-cold 50 mM Tris buffer (pH 7.4), followed immediately by vacuum filtration across Whatman GF/B filters. The filters were washed twice with 3.5 ml of 50 mM Tris buffer, (pH 7.4) and the filter radioactivity counted (40–45% efficiency) in Filter Count Scintillant, using a Packard Tricarb Spectrometer.

When centrifugation was used, 15 nM [^{3}H]-verapamil, together with unlabelled verapamil over the concentration range 5 nM–1 mM was added for saturation, and 2 mM methoxyverapamil (D600) for non-specific binding. The whole reaction was carried out in siliconized Eppendorf tubes. After 30 min incubation at 25°C, the tubes were cooled on ice for 5 min before separating bound and free [^{3}H]-verapamil by centrifugation at 11,800 *g* for 15 min at 4°C in a Hicracus Christ microfuge (model 918). The tubes were then placed on ice. Five min later the supernatant was carefully but quickly removed and the pellet immediately washed with 0.5 ml ice-cold 50 mM Tris (pH 7.4). The washing solution was immediately removed by vacuum suction and the radioactivity of the pellet established by scintillation counting as described above.

[^{3}H]-nitrendipine binding To ensure that the sarcolemmal fragments used in these studies bound [^{3}H]-nitrendipine as described by other investigators, [^{3}H]-nitrendipine binding was characterized as outlined by Bellemann *et al.* (1981). Cardiac sarcolemmal membrane fragments were exposed to 0.05–12 nM [^{3}H]-nitrendipine for 90 min in an incubation medium containing 150 mM NaCl, 1 mM $CaCl_2$, 50 mM Tris (pH 7.4). Unlabelled nitrendipine, 20 μM, was used to establish non-specific binding and the bound and free [^{3}H]-nitrendipine were separated by vacuum filtration across Whatman GF/B filters. The filters were counted for radioactivity as described above.

Displacement studies The specificity of [^{3}H]-verapamil binding was determined by standard displacement techniques. Thus 0.1 μM–10 mM $CaCl_2$, 1 nM–1 mM D600, 0.1 μM–1 mM diltiazem or 0.1 nM–100 μM nifedipine, nisoldipine, nitrendipine, or prazosin was added to membranes which had been pre-incubated with 15 nM [^{3}H]-verapamil, together with either 45 nM or 60 μM unlabelled verapamil.

To establish whether the [^{3}H]-verapamil binding was stereo-selective, (+)-verapamil (1 nM–1 mM) and (−)-verapamil (1 nM–1 mM) were used as displacers of bound [^{3}H]-verapamil.

Protein determination

Protein content was estimated by the method of Lowry *et al.* (1951), using bovine serum albumin as standard.

Analysis of data

Initial estimates of equilibrium binding parameters (K_D and B_{max}) were obtained from Scatchard, Hill and Hofstee analysis, using the 'EBDA' programme (McPherson, 1983). A file was then produced and the data analysed with the aid of a weighed non-linear square computer curve fitting programme, 'LIGAND' (Munson & Rodbard, 1980). This was used to obtain final parameter estimates suitable for establishing whether the binding data best fitted a one or two site model – as assessed by the built in *F*-ratio test.

K_D is the equilibrium constant of the ligand for its receptor and is equal to the ratio of the forward rate constant versus the reverse rate constant. B_{max} is the maximum number of binding sites. K_i is the equilibrium dissociation constant for the interaction of the competing agent (I) with the receptor for the ligand (L). Thus

$$K_i = \frac{IC_{50}}{(1+{}^{[L]}/K_D)}$$

where the IC_{50} is the concentration of the competing ligand which displaces 50% of the bound ligand [L]. K_D refers to the dissociation constant of ligand (L).

The results are presented as mean ± s.e.mean of *n* determinations, the estimations for which were made in triplicate. Tests of significance were calculated by Student's *t* test taking $P<0.05$ as the limit of significance.

Chemicals and reagents

[^{3}H]-verapamil and [^{3}H]-nitrendipine were obtained from New England Nuclear (Boston, Mass) as N-methyl-[^{3}H]-verapamil hydrochloride and 5-methyl-[^{3}H]-nitrendipine with a specific activity of 75.0 and 70.0 Ci mmol^{-1} respectively.

Verapamil and methoxyverapamil (D600) were used either as the racemate hydrochlorides or as the (+)-and (−)-isomers, and were obtained as a gift from Knoll AG, Ludwigshafen, Germany. Nifedipine, nisoldipine and nitrendipine were obtained as gifts from Bayer AG, Germany, and diltiazem from Tanabe Laboratories, Japan. Prazosin-HCl was obtained as a gift from Pfizer Ltd, Sydney, Australia. Bovine serum albumin was obtained from the Commonwealth Serum Laboratories, Melbourne, Australia. All other reagents were from Sigma Chemical Company, St. Louis, USA.

Table 1 [^{3}H]-verapamil binding to cardiac sarcolemmal membranes isolated from non-perfused, aerobically-perfused and ischaemic hearts, using filtration to separate bound and free [^{3}H]-verapamil

Preparation	*Incubation medium*	K_D (nM)	B_{max} (pmol mg^{-1} protein)	*Hill coefficient*
Non perfused hearts				
	50 mM Tris 150 mM NaCl	24.0 ± 7.8	28.5 ± 7.5	1.006 ± 0.012
	1 mM $CaCl_2$ 50 mM Tris	39.0 ± 8.3	13.1 ± 6.6	1.012 ± 0.022
In the absence of sarcolemmal membranes (filter blank)				
	150 mM NaCl 1 mM $CaCl_2$ 50 mM Tris	38.0 ± 3.1	12.2 ± 1.9*	0.989 ± 0.05
75 min aerobic perfusion				
	150mM NaCl 1 mM $CaCl_2$ 50 mM Tris	43.0 ± 7.6	15.3 ± 1.0	0.997 ± 0.07
15 min aerobic perfusion + 60 min ischaemia				
	150 mM NaCl 1 mM $CaCl_2$ 50 mM Tris	34.9 ± 6.4	14.8 ± 3.7	0.991 ± 0.05
		(NS)	(NS)	(NS)

*Denotes [^{3}H]-verapamil binding in pmol per filter. Tests of significance relate to the significance of the difference in K_D, B_{max} and Hill coefficient caused by 60 min ischaemia, relative to the results obtained after aerobic perfusion. Each result is mean ± s.e.mean of 6 experiments. NS, not significant at $P<0.05$.

Table 2 % of [^{3}H]-verapamil and [^{3}H]-nitrendipine bound to filters and centrifugation tubes

Ligand bound to	*[^{3}H]-verapamil*		*[^{3}H]-nitrendipine*	
	A	*B*	*A*	*B*
Filters	12.74	1.74	5.4	5.04
Non-siliconized tubes	1.80	0.41	3.16	1.89
Siliconized tubes	0.46	0.10	1.07	1.70

Total [^{3}H]-verapamil used: 32730 d.p.m., with specific activity 75.0 Ci mmol^{-1}. Total [^{3}H]-nitrendipine used: 28765 d.p.m., with specific activity 70.9 Ci mmol^{-1}. The reaction mixture contained 150 mM NaCl, 1 mM $CaCl_2$ and 50 mM Tris, pH 7.4. Incubation was for 30 min at 25°C in the absence (A) and presence (B) of 20 μM cold verapamil or nitrendipine. Each result is the mean of three separate experiments. Bound and free ligand were separated by filtration.

Results

[^{3}H]-nitrendipine binding

In the sarcolemmal membrane fragments used here two populations of [^{3}H]-nitrendipine binding sites were detected. The high affinity sites had a K_D of 0.23 ± 0.04 nM and a B_{max} of 258 ± 87 fmol mg^{-1} protein. The low affinity sites had a K_D of 31.7 ± 5.5 nM and a B_{max} of 11.9 ± 1.2 pmol mg^{-1} protein. The binding was saturable, reversible and displaceable, with bound [^{3}H]-nitrendipine being selectively displaced by nifedipine > D600.

[^{3}H]-verapamil binding to cardiac sarcolemmal membrane fragments

Equilibrium binding studies When cardiac membrane fragments prepared from freshly excised hearts were incubated in 50 mM Tris (pH 7.4), either in the absence or presence of 150 mM NaCl and 1 mM $CaCl_2$, and the bound and free [^{3}H]-verapamil separated by filtration, a single population of high affinity [^{3}H]-verapamil binding sites was detected. Scatchard analysis of [^{3}H]-verapamil binding in membranes which had been incubated in 50 mM Tris without any added Na^+ or Ca^{2+}, revealed a single population of binding sites, with a K_D of 24 ± 7.8 nM and B_{max} of 28.5 ± 7.5 pmol mg^{-1} protein (Table 1). In the presence of Na^+ and Ca^{2+}, there was a significant ($P < 0.05$) reduction in the B_{max} (13.1 ± 6.6 pmol mg^{-1} protein) of the binding sites but the affinity (K_D 39 ± 8.3 nM) remained unchanged (Table 1).

When similar incubations were made but without any membrane fragments, and the incubation medium was 'filtered' exactly as described above, Scatchard analysis indicated that the [^{3}H]-verapamil had bound to the glass filters with a K_D of 38 ± 3.1 nM and a B_{max} of 12.2 ± 1.9 pmol per filter (Table 1). These values are close to those (Table 1) obtained for [^{3}H]-verapamil-

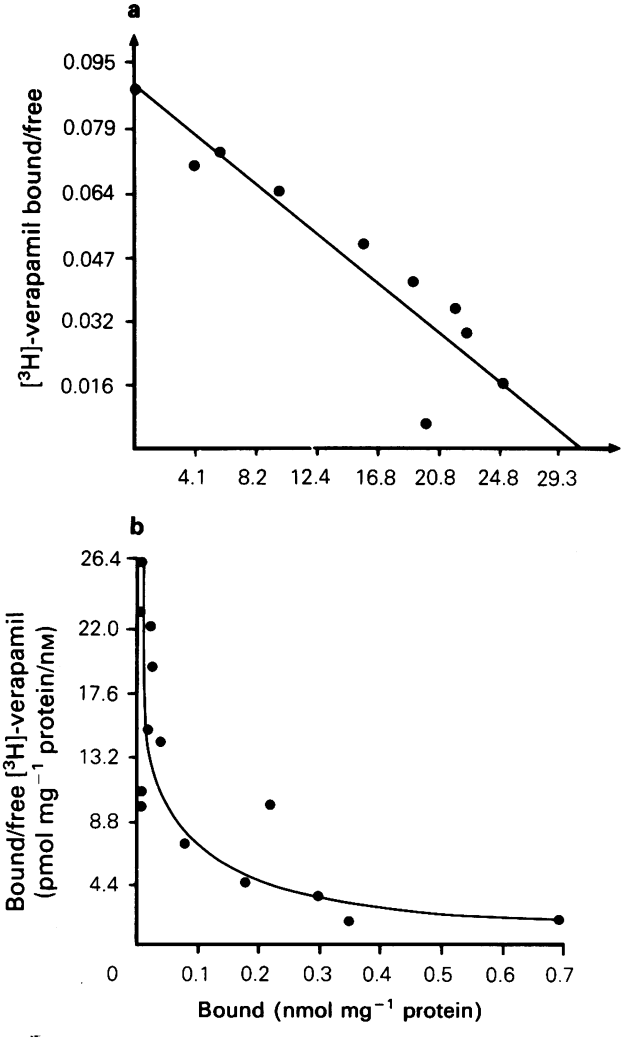

Figure 1 Scatchard plot of [^{3}H]-verapamil binding to rat isolated cardiac sarcolemmal membrane fragments incubated in (a) 50 mM Tris and (b) 150 mM NaCl, 1 mM $CaCl_2$, 50 mM Tris, pH 7.4 at 25°C for 30 min. Membrane bound and free [^{3}H]-verapamil were separated by centrifugation. The binding parameters are given in Table 3. Ordinate scales: bound/free [^{3}H]-verapamil; abscissa scales: bound [^{3}H]-verapamil (nmol mg^{-1} protein). Similar estimates were obtained from six separate experiments, using triplicate estimates for each point.

membrane binding. Table 2 shows that in contrast to [^{3}H]-nitrendipine, the [^{3}H]-verapamil which binds to GF/B filters is displaceable by unlabelled verapamil. Under these conditions, therefore, data obtained from binding studies in which bound and free [^{3}H]-verapamil have been separated by filtration across GF/B filters does not necessarily provide reliable data relating to the binding of [^{3}H]-verapamil to sarcolemmal membrane fragments. Because of this, and since [^{3}H]-verapamil binds with relatively low affinity (Reynolds *et al.*, 1983; Glossmann *et al.*, 1984; Garcia *et al.*, 1984; Galizzi *et al.*, 1984a or b) centrifugation (Bennett, 1978) rather than filtration seemed to provide a more appropriate method for separating bound and free [^{3}H]-verapamil. Table 2 shows that the background binding of [^{3}H]-verapamil to Eppendorf tubes is relatively small when compared to that bound by the filters and that siliconization of the tubes further reduces this binding.

When sarcolemmal membranes were incubated in 50 mM Tris (pH 7.4) and the bound and free [^{3}H]-verapamil separated by centrifugation, only low affinity [^{3}H]-verapamil binding sites, with a K_D of $68 \pm 5.5\,\mu$M and a B_{max} of 29.1 ± 5.5 nmol mg^{-1} nmol mg^{-1} protein, were detected (Figure 1a and Table 3). However, adding 150 mM NaCl and 1 mM $CaCl_2$ to the reaction mixture, and separating bound and free ligand by centrifugation, provided evidence of two populations of binding sites (Figure 1b and Table 3)–a population of relatively high affinity sites, with a K_D of $0.57 \pm 0.19\,\mu$M and a B_{max} of 38 ± 5.2 pmol mg^{-1} protein, and a population of lower affinity sites, with a K_D of $78 \pm 27.5\,\mu$M and a B_{max} of 2.9 ± 1.37nmol-mg^{-1} protein (Table 3).

Displacement studies Studies in which 15 nM [^{3}H]-verapamil and 45 nM unlabelled verapamil were added to membranes incubated in 150 mM NaCl, 1 mM $CaCl_2$ and 50 mM Tris and the bound and free [^{3}H]-verapamil separated by filtration, showed that D600 displaced bound verapamil with a K_i of $2.4 \pm 0.6\,\mu$M, and that neither nitrendipine, nisoldipine, nifedipine, Ca^{2+}, prazosin nor diltiazem were effective.

In contrast, under the same conditions of incubation but using centrifugation to separate bound and free [^{3}H]-verapamil, Ca^{2+}, diltiazem and D600 displaced bound [^{3}H]-verapamil with K_i values of 465 ± 15, 14.6 ± 3.82 and $4.1 \pm 1.61\,\mu$M, respectively

Table 3 [^{3}H]-verapamil binding to cardiac sarcolemmal membranes isolated from non-perfused, aerobically perfused and ischaemic hearts, using centrifugation to separate bound and free [^{3}H]-verapamil

	High affinity			*Low affinity*		
Reaction mixture	K_D (μM)	B_{max} (pmol mg^{-1} protein)	*Hill coefficient*	K_D (μM)	B_{max} (nmol mg^{-1} protein)	*Hill coefficient*
Membranes prepared from non-perfused hearts						
507mM Tris 150 mM NaCl 1 mM $CaCl_2$ 50 mM Tris	–	–	–	68.0 ± 5.5	29.1 ± 5.5	0.996 ± 0.004
	0.57 ± 0.19	38.00 ± 5.20	0.997 ± 0.008	78.00 ± 27.50	2.90 ± 1.30	0.993 ± 0.007
Membranes prepared after 75 min aerobic perfusion 150 mM NaCl 1 mM $CaCl_2$ 50 mM Tris	0.37 ± 0.10	22.36 ± 7.22	0.993 ± 0.002	74.86 ± 17.90	2.84 ± 0.77	0.995 ± 0.004
Membranes prepared after 15 min aerobic perfusion and 60 min ischaemia 150 mM NaCl 1 mM $CaCl_2$ 50 mM Tris	0.17 ± 0.04	3.88 ± 0.01	0.997 ± 0.01	34.45 ± 10.23	0.81 ± 0.39	0.970 ± 0.023
	($P < 0.05$)	($P < 0.02$)	(NS)	($P < 0.03$)	($P < 0.02$)	(NS)

Tests of significance relate to the significance of the changes in K_D, B_{max} and Hill coefficient caused by 60 min ischaemia, relative to the values obtained after aerobic perfusion. Each result is mean ± s.e.mean of 6 experiments. NS, not significant at $P < 0.05$.

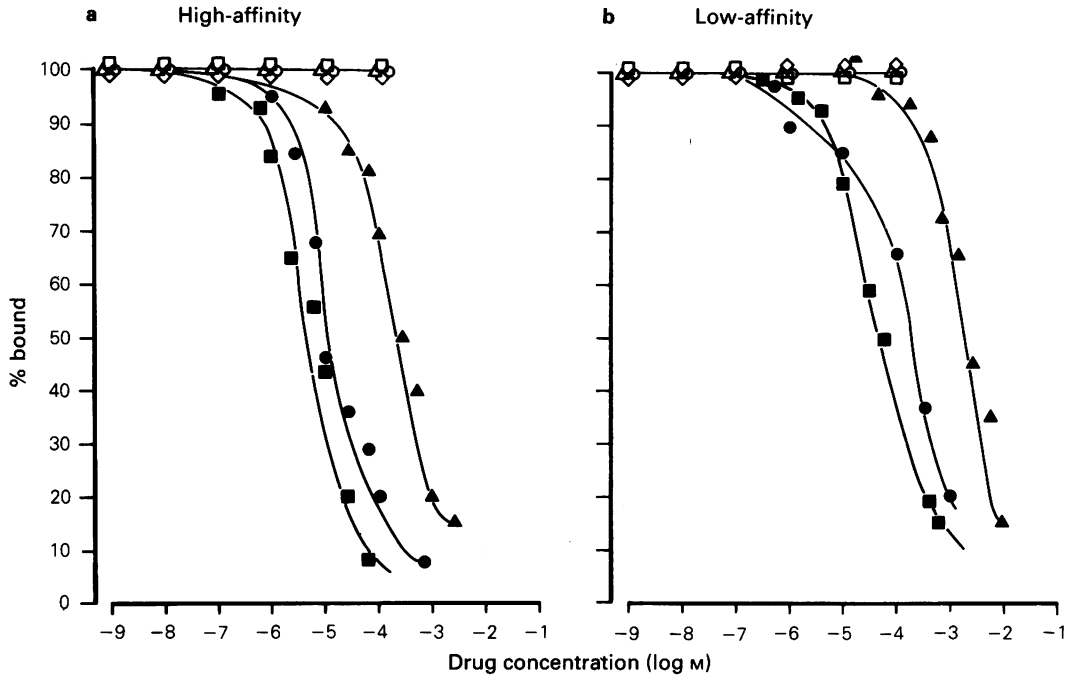

Figure 2 Displacement by Ca^{2+} (▲), diltiazem (●), D600 (■), nifedipine (◇), nitrendipine (□), nisoldipine (△) and prazosin (○) of [^{3}H]-verapamil bound to sarcolemmal membranes isolated from non-perfused hearts. Membranes were incubated in 150 mM NaCl, 1 mM $CaCl_2$ and 50 mM Tris (pH 7.4) at 25°C for 30 min with 15 nM [^{3}H]-verapamil and either (a) 45 nM cold verapamil (conditions suitable for high affinity binding), or (b) 60 μm cold verapamil conditions suitable for low affinity binding. Bound and free [^{3}H]-verapamil were separated by centrifugation. The displacement parameters are given in the text. Ordinate scales: % bound; abscissa scales: drug concentration (log M). Similar estimates were obtained from 3–4 separate experiments, using triplicate estimates for each point.

(Figure 2). Again, neither the dihydropyridines nor prazosin displaced the bound [^{3}H]-verapamil (Figure 2). Using 15 nM [^{3}H]-verapamil and 60 μM cold verapamil, (conditions that are suitable for low affinity binding) bound [^{3}H]-verapamil was displaceable by Ca^{2+} (K_i, 3940 ± 420 μM), diltiazem (K_i, 104 ± 6.3 μM), and D600 (K_i, 63.9 ± 1.5 μM) but not by nifedipine, nisoldipine, nitrendipine nor prazosin (see Figure 2).

Experiments in which the separate (+)- and (−)-isomers of verapamil were used to displace bound [^{3}H]-verapamil revealed stereospecificity, with a K_i of 10.2 ± 3.9 μM for the (+)-isomer and 4.1 ± 1.2 μM for the (−)-isomer, for the high 36.4 ± 15.2 mM and 6.4 ± 1.2 μM for the low affinity sites.

[^{3}H]-verapamil binding to sarcolemmal membranes harvested from aerobically perfused hearts

Experiments in which [^{3}H]-verapamil binding was monitored by using centrifugation to separate bound and free [^{3}H]-verapamil, revealed two populations of [^{3}H]-verapamil binding sites in sarcolemmal fragments harvested from aerobically perfused hearts (Figure 3). These were high affinity sites – with a K_D of 0.37 ± 0.10 μM and a B_{max} of 22.36 ± 7.22 pmol mg^{-1} protein, and low affinity sites – with a K_D of 74.86 μM and a B_{max} of 2.84 ± 0.77 nmol mg^{-1} protein (Table 3). Bound [^{3}H]-verapamil was displaceable by diltiazem (with a K_i of 2.58 ± 0.91 μM for the high and 69.5 ± 8.5 μM for the low affinity sites) and by D600 (3.55 ± 1.95 μM and 60.3 ± 7.5 μM for the high and low affinity sites, respectively). Nitrendipine, however, did not displace bound [^{3}H]-verapamil.

Effect of ischaemia on [^{3}H]-verapamil binding

Separation by centrifugation Sarcolemmal membranes prepared from hearts which had been ischaemic for 60 min at 37°C retained two populations of [^{3}H]-verapamil binding sites (Figure 3). The K_D of the high affinity sites was significantly reduced (by 54% ($P < 0.05$)) relative to the values obtained after aerobic perfusion, and there was an 82.7% ($P < 0.02$)

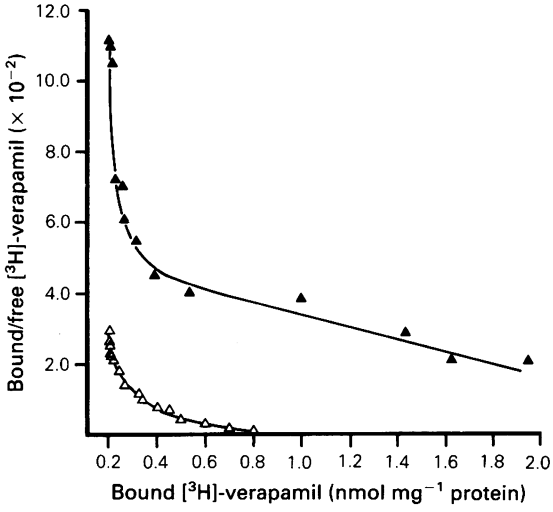

Figure 3 Scatchard plot for [^{3}H]-verapamil binding to cardiac sarcolemmal membranes fragments isolated from aerobically perfused (▲, 75 min aerobic perfusion) and globally ischaemic (△, 15 min aerobic perfusion + 60 min ischaemia) hearts. Membranes were incubated in 150 mM NaCl, 1 mM $CaCl_2$ and 50 mM Tris (pH 7.4) at 25°C for 30 min. Membrane bound and free [^{3}H]-verapamil were separated by centrifugation. The curves were fitted by non-linear regression analysis. The binding parameters are given in Table 3. Ordinate scale: bound/free [^{3}H]-verapamil ($\times 10^{-2}$); abscissa scale: bound [^{3}H]-verapamil (nmol mg^{-1} protein). Similar estimates were obtained from six separate experiments, using triplicate estimates for each point.

reduction in the B_{max} (Table 3), relative to the values obtained after aerobic perfusion. The K_D and the B_{max} of the low affinity sites were also altered, the K_D being reduced by 54% ($P<0.03$) and the B_{max} by 71.5% ($P<0.02$) (Table 3). However, the binding sites retained their selectivity; bound [^{3}H]-verapamil being displaced by diltiazem (high affinity sites: K_i 0.37 ± 0.03 μM; low affinity sites: K_i 32 ± 0.06 μM) and D600 (K_i 1.36 ± 0.79 μM and 32.2 ± 3.8 μM for high and low affinity sites respectively) but not by nitrendipine. In summary, therefore, 60 min ischaemia significantly alters the [^{3}H]-verapamil binding activity of the sarcolemma but the specificity of binding sites remains unchanged.

To establish whether this ischaemia-induced change in the activity of the verapamil binding sites is due to an effect of ischaemia (or some consequence of it) and not simply an artifact due to an altered protein content, or the isolation of a different subcellular fraction, the sarcolemmal fragments obtained from the ischaemic and aerobically perfused hearts were characterized. The results (Table 4) show that the yield

Table 4 Sarcolemmal profile

Perfusion	*Aerobic*	*Ischaemic*
Yield (mg protein g^{-1} heart wt)	0.60 ± 0.02	0.58 ± 0.04 (NS)
Sialic acid content (nmol mg^{-1} protein)	126.3 ± 4.7	147.7 ± 1.7 ($P<0.01$)
Na^+K^+-ATPase (ouabain sensitive) (μmol P_i mg^{-1} protein h^{-1})	19.0 ± 1.1	11.3 ± 0.5 ($P<0.01$)
5' nucleotidase (μmol P_i mg^{-1})	14.0 ± 0.6	11.5 ± 0.7 ($P<0.05$)
Succinate dehydrogenase (μmol cytochrome C oxidized mg^{-1} protein h^{-1}	2.94 ± 0.2	3.04 ± 0.3 (NS)

Each result is mean ± s.e.mean of six separate experiments. Tests of significance relate to the significance of the change caused by ischaemia ($P<0.05$) with respect to controls (aerobically perfused series). NS, not significant.

of sarcolemma and the presence of subcellular contamination, as gauged by succinate dehydrogenase activity, was not significantly altered. At the same time, however, there was a significant reduction in the activity of ouabain sensitive ATPase and 5' nucleotidase enzymes, and a significant increase in sialic acid content (Table 4).

Separation by filtration In the ischaemia [^{3}H]-verapamil binding experiments described so far, bound and free [^{3}H]-verapamil were separated by centrifugation. In another series of experiments in which only high affinity [^{3}H]-verapamil binding sites were being monitored and where the bound and free [^{3}H]-verapamil could therefore be separated by filtration, 60 min normothermic ischaemia did not appear to have any significant effect on [^{3}H]-verapamil binding sites (Table 1). Table 1 shows that under these conditions only a single population of high affinity [^{3}H]-verapamil binding sites, with a K_D of 43.0 ± 7.6 nM and B_{max} of 15.3 ± 1.0 pmol mg^{-1} protein, could be detected in membrane fragments isolated from aerobically perfused hearts. After 60 min ischaemia at 37°C, comparable sarcolemmal membrane fragments still contained a single population of high affinity [^{3}H]-verapamil binding sites, with a K_D of 34 ± 6.5 nM and a B_{max} of 14.8 ± 3.7 pmol mg^{-1} protein (Table 1). Hence, when filtration was used to separate bound and free [^{3}H]-verapamil 60 min ischaemia did not seem to have any significant effect on the high affinity [^{3}H]-verapamil binding sites. This

contrasts with the data obtained when centrifugation was used to separate the bound and free ligand (Table 3).

Discussion

These results show that enzymatically characterized sarcolemmal membranes which contain high and low affinity [^{3}H]-nitrendipine binding sites, also contain two populations of [^{3}H]-verapamil binding sites. These are high affinity sites – with a K_D of 0.57 μM and a B_{max} of 38 pmol mg^{-1} protein, and low affinity sites – with a K_D of 78 μM and a B_{max} of 2.9 nmol mg^{-1} protein, respectively. The high affinity sites could only be detected in membranes which had been incubated in the presence of cations. Both the low and high affinity [^{3}H]-verapamil binding sites were saturable and stereospecific. Moreover the bound [^{3}H]-verapamil was displaceable by D600 > diltiazem > Ca^{2+} but not by nifedipine, nitrendipine or nisoldipine nor by the α_1-adrenoceptor blocker, prazosin.

In the present study when glass fibre filters were used to separate bound and free [^{3}H]-verapamil, only a single population of relatively high affinity [^{3}H]-verapamil binding sites could be detected. Hulthen *et al.* (1982) Reynolds *et al.* (1983), Galizzi *et al.* (1984a,b), Glossmann *et al.* (1984) and Garcia *et al.* (1984) were also only able to detect a single population of high affinity sites (K_D 4–50 nM; B_{max} 50 fmol–13 pmol mg^{-1} protein) when they separated bound and free [^{3}H]-verapamil across glass fibre filters. However, the present results indicate that it is difficult to interpret the binding data obtained under these conditions because [^{3}H]-verapamil binds to glass fibre filters with an affinity and density comparable with that exhibited by membranes. Another factor which must be taken into consideration when selecting the most appropriate method for separating bound and free [^{3}H]-verapamil revolves around the relatively low affinity of [^{3}H]-verapamil binding. For radioligand binding studies in which the affinity of the ligand (K_D) exceed 10^{-8} M, the recommended method for separating bound and free ^{3}H-ligand is by centrifugation (Bennett, 1978). It was for this reason that we used this method to characterize low affinity [^{3}H]-verapamil binding sites. If instead of centrifugation, separation is by filtration then because the ligand dissociates from its binding sites at a relatively rapid rate when the filters are washed, it is difficult to obtain a true estimate of K_D and B_{max}. These methodological problems could explain why some investigators have only been able to detect a single population of [^{3}H]-verapamil binding sites in isolated membranes.

The present results show that even when centrifugation was used to separate bound and free [^{3}H]-verapamil only a single population of low affinity [^{3}H]-verapamil binding sites was detected when the membranes were incubated in 50 mM Tris. However, the inclusion of 150 mM NaCl and 1 mM $CaCl_2$ uncovered the second population of higher affinity binding sites. Galizzi *et al.* (1984a) have also described this second population of [^{3}H]-verapamil binding sites. Additional experiments are needed to explore this cation requirement of the high affinity sites. Presumably the low affinity [^{3}H]-verapamil binding cannot be attributed to a random accumulation of the ligand into the lipid bilayer, because [^{3}H]-verapamil binding at the low, as at the high, affinity sites was saturable, stereospecific and displaceable. It is reasonable to assume therefore that the binding is specific and that we are dealing with two populations of binding sites, with the B_{max} of the low affinity sites greatly exceeding that of the high affinity sites. However, each group (high and low affinity) contains a single population of sites, with a Hill coefficient approaching unity (Table 3).

The bound [^{3}H]-verapamil was not displaceable by the dihydropyridines or by prazosin. This inability of the dihydropyridines to displace [^{3}H]-verapamil cannot be attributed to an inability of the membranes to bind these compounds because both low and high affinity [^{3}H]-nitrendipine binding sites were present. It must be assumed, therefore, that the [^{3}H]-verapamil binding sites are distinct and separate from those occupied by the dihydropyridines, a finding which supports the conclusion reached by others (DePover *et al.*, 1982; Ferry & Glossmann, 1982; Millard *et al.*, 1983). The failure of prazosin to displace bound [^{3}H]-verapamil is surprising, because verapamil interacts with α_1-adrenoceptors (Glossmann *et al.*, 1980; Nayler *et al.*, 1982; Karliner *et al.*, 1982). One possible explanation for this could be that only a small proportion of the [^{3}H]-verapamil binding sites is associated with α_1-adrenoceptors. If this is indeed true, then any displacement of [^{3}H]-verapamil bound to these sites may be masked by a large population of [^{3}H]-verapamil binding sites not associated with these receptors.

Although our results show that cardiac sarcolemmal fragments contain both low and high affinity [^{3}H]-verapamil binding sites, and that these sites are distinct from those occupied by the dihydropyridines, the question which remains to be resolved relates to the physiological significance of the two populations of binding sites. The K_D of the high affinity sites falls within the concentration range which attenuates slow channel function (Kohlhardt *et al.*, 1975). By contrast, the K_D of the low affinity sites falls within the range at which verapamil acts as a local anaesthetic (Fairhurst *et al.*, 1980), blocks the fast inward Na^+ and slow outward K^+ currents (Kass & Tsien, 1975) and interacts with α-adrenoceptors (Glassmann *et al.*, 1980; Nayler *et al.*, 1982; Karliner *et al.*, 1982),

muscarinic and opiate receptors (Cavey *et al.*, 1977).

Our results show that sarcolemmal membranes which had been prepared from ischaemic hearts retained two populations of [^{3}H]-verapamil binding sites. Ischaemia did, however, cause a significant decrease in the density (B_{max}) and dissociation constant (K_D) of both the high and low affinity binding sites, but their selectivity was unchanged. This effect of ischaemia is not peculiar to the phenylalkylamine binding sites. The dihydropyridine binding sites behave in the same way (Nayler *et al.*, 1985). Reports have appeared in the literature describing a lack of or opposite effects of ischaemia on other receptor systems, e.g. unaltered muscarinic and increased β-adrenoceptor numbers after 60 min ischaemia (Mukherjee *et al.*, 1979), and increased α_1-adrenoceptor numbers after 30 min ischaemia (Corr *et al.*, 1981). Our inability to detect any effect of ischaemia on the profile of the high affinity binding sites, when the bound and free ligand was separated by filtration, probably reflects the difficulties that we encountered in obtaining true saturation kinetics under these conditions, because of the presence of high affinity [^{3}H]-verapamil binding sites in the filters.

The present results show that although 75 min of aerobic perfusion failed to cause a significant change in either the K_D or B_{max} of the [^{3}H]-verapamil binding sites being studied here, there was a trend towards a decrease in both the K_D and B_{max}. Despite this trend the overall specificity of the binding sites was retained, with nitrendipine failing to displace bound [^{3}H]-verapamil in both instances. It is worth noting, however, that the altered ability of diltiazem (as indicated by the changed K_i), but not of D600, to displace bound [^{3}H]-verapamil from membranes obtained from aerobically perfused relative to non-perfused hearts is indicative of the fact that aerobic perfusion does have an effect on the diltiazem recognition sites. Whether prolonged periods of aerobic perfusion, in excess of the 75 min used here, alters the ability of other phenylalkylamines to displace bound [^{3}H]-verapamil, requires further investigation.

An apparent hypersensitivity to verapamil has been reported for the ischaemic myocardium (Smith *et al.*, 1976; Smith & Briscoe., 1982). This hypersensitivity cannot be explained in terms of an ischaemia-induced increase in the number of verapamil binding sites, because the present results show that ischaemia reduces their number. However, we did find an increase in affinity associated with this decline in the number of binding sites. Possibly the affinity of the ligand for its binding site is functionally more important than the actual number of binding sites. Alternatively the hypersensitivity of the ischaemic myocardium to verapamil may involve mechanisms other than an increase in receptor number or affinity.

In conclusion, this study shows that the binding of [^{3}H]-verapamil to cardiac sarcolemmal fragments is complex. Two populations of binding sites are involved, and the binding is cation sensitive, saturable, reversible, stereoselective and displaceable by D600 > diltiazem > Ca^{2+} but not by the dihydropyridines nor by prazosin. These binding sites survive a prolonged period of normothermic ischaemia, but with a reduced B_{max} and K_D. Their response to ischaemia therefore, resembles that of the dihydropyridine binding sites (Nayler *et al.*, 1985).

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A pharmacological study of the rabbit saphenous artery *in vitro*: a vessel with a large purinergic contractile response to sympathetic nerve stimulation

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1 Mechanical responses to transmural electrical stimulation were recorded in isolated transverse ring preparations of rabbit saphenous artery. Electrical stimulation for a period of 1 s produced a rapid monophasic contraction and, for a period of 1 min, a biphasic contraction consisting of a rapid constriction followed by a slower sustained constriction. All contractions were abolished in the presence of tetrodotoxin (1 $\mu g\,ml^{-1}$) or guanethidine (4 μM).

2 After desensitization of the P_2-purinoceptor with α,β-methylene ATP, contractions to electrical stimulation for 1 s were reduced significantly at all frequencies tested: responses evoked by stimulation at 4 Hz were usually almost completely inhibited, whereas those evoked by stimulation at 64 Hz were only partially inhibited. On the other hand, in the presence of the α-adrenoceptor antagonist, prazosin, neurogenic contractions were only partially reduced: at 4 Hz there was no significant reduction in the neurogenic contractions while at 32 and 64 Hz, contractions were reduced by an average of 20 and 28% respectively. Usually all contractions were abolished by a combination of the two drugs.

3 Prazosin antagonized contractions of the vessel to exogenously applied noradrenaline but not to ATP, whereas desensitization of the P_2-purinoceptor with α,β-methylene ATP blocked responses to ATP but not those to noradrenaline. The concentration-response curve to histamine was not affected by treatment of the vessel with prazosin, or after desensitization of the P_2-purinoceptor with α,β-methylene ATP.

4 These results suggest that noradrenaline and ATP are co-released from sympathetic nerves supplying the rabbit saphenous artery, both substances being involved in the mechanical contractions of this tissue. Further, the ratio of ATP to noradrenaline involved in these mechanical contractions is dependent upon the frequency of stimulation, but at all frequencies tested the purinergic component is greater than the adrenergic component.

Introduction

Non-adrenergic, non-cholinergic nerves have been shown to have a widespread occurrence in the autonomic nervous system of vertebrates (Burnstock, 1969). Burnstock (1972) proposed that adenosine 5'-triphosphate (ATP), or a related nucleotide, is the neurotransmitter released from some of these nerves and that they be termed 'purinergic'. Since then evidence has accumulated that ATP is a cotransmitter, with noradrenaline, in sympathetic nerves supplying a number of smooth muscle preparations (see Burnstock, 1976; 1982; 1985a,b). Studies on the vas deferens and the cat nictitating membrane with the P_2-purinoceptor antagonist arylazidoaminoproprionyl ATP ($ANAPP_3$) or involving desensitization of the P_2-purinoceptor with α,β-methylene ATP, have demonstrated that ATP acts as a contractile cotransmitter with noradrenaline during sympathetic neurotransmission (Fedan *et al.*, 1981; Sneddon *et al.*, 1982; Meldrum & Burnstock, 1983; Westfall *et al.*, 1983; Sneddon & Burnstock, 1984a; Duval *et al.*, 1985). A number of studies on different blood vessels have demonstrated, both mechanically and electrically, neurogenic responses that are not antagonized by α-adrenoceptor antagonists (see Burnstock & Kennedy, 1986). It has been suggested that purines might play a role as cotransmitters with noradrenaline in some vessels (Su, 1975; Head *et al.*, 1977; Katsuragi & Su, 1981; Muramatsu *et al.*, 1981; Sedaa *et al.*, 1986). Recently, with the use of α,β-methylene ATP, there has been direct evidence that ATP acts as a cotransmitter

with noradrenaline in the contractile responses of the rat tail artery (Sneddon & Burnstock, 1984b; Vidal *et al.*, 1986), the mesenteric artery (Ishikawa, 1985; Kügelgen & Starke, 1985; Muramatsu, 1986), and the rabbit ear artery (Kennedy *et al.*, 1986).

The rabbit saphenous artery is a muscular vessel well innervated with noradrenergic nerve fibres, and produces large, rapid contractions to neurogenic stimulation (Gillespie & Rae, 1972). However, neurogenic contractions of this vessel are resistant to α-adrenoceptor antagonists (Holman & Suprenant, 1980). In the present study, the possibility that noradrenaline and ATP act as cotransmitters in the rabbit saphenous artery has been investigated over a range of stimulation frequencies and for short (1 s) and long (1 min) periods of time (see Kennedy *et al.*, 1986). The possibility that the non-adrenergic excitatory neurotransmitter is ATP has been investigated by using α,β-methylene ATP to desensitize the postjunctional P_2-purinoceptors.

Methods

Male New Zealand white rabbits (2.8–3.3 kg) were killed by a blow to the head and exsanguination. Two ring segments, 4 mm in length after excision, were removed from the proximal end of each saphenous artery. They were cleaned of excess connective tissue and mounted horizontally, under isometric tension in 15 ml organ baths, by inserting two tungsten wires into the lumen according to the method of Bevan & Osher (1972). The tissues were bathed in Krebs solution of the following composition (mM): NaCl 133, KCl 4.7, $NaHPO_4$ 1.35, $NaHCO_3$ 16.3, $MgSO_4$ 0.61, glucose 7.8 and $CaCl_2$ 2.52 (Bülbring, 1953). The solutions were aerated with 95% O_2 and 5% CO_2 and maintained at 37°C. Throughout the experiment the Krebs solution was changed every 5–10 min by an overflow method. Preparations were allowed to equilibrate for 1–2 h under a resting tension of 0.75 g. Contractions of the circular smooth muscle were recorded by use of a Grass FTO3C transducer and displayed on a Grass ink-writing oscillograph (model 79).

Electrical stimulation of intramural nerves was delivered to the tissue across two platinum wire electrodes placed parallel to, and on either side of, the vessel using a Grass S11 stimulator. The voltage (40–50 V) and the pulse duration (0.08–0.1 ms) at which the neurogenic responses of the tissue were just maximal were established for each tissue at the start of each experiment and then maintained constant throughout the experiment. With these parameters, the responses were blocked by tetrodotoxin (1 μg ml^{-1}) and were therefore due to nerve stimulation and not to direct stimulation of the muscle. The rabbit saphenous artery was stimulated electrically over a range of frequencies (4–64 Hz) for a period of 1 s or 1 min, with a 4 or 15 min interval between each stimulation, respectively. Stimulations were repeated to ensure consistency of the response. On each preparation, the same range of stimulation frequencies and the periods of stimulation were again repeated either in the presence of the α-adrenoceptor antagonist, prazosin, or after desensitization of the P_2-purinoceptor with α,β-methylene ATP, first at a concentration of 1 μM and then 10 μM and finally in the presence of both drugs, each at 10 μM. If any response remained after treatment with both prazosin and α,β-methylene ATP, stimulations were repeated after a 10 min incubation with tetrodotoxin (1 μg ml^{-1}) to determine whether the residual response was neural or myogenic. The second component of the biphasic contraction induced by electrical stimulation for a period of 1 min was often variable and difficult to assess. Therefore, in order to standardize the method of calculation, the sustained contraction was taken as that response produced after 1 min of continuous stimulation. Desensitization of the P_2-purinoceptor was achieved by three or four exposures of the vessel to α,β-methylene ATP for 5 min at 10 min intervals, until no further contractile response was elicited and tone declined to its resting level. Tissues were incubated for 20 min with prazosin before any prazosin-resistant responses were measured. Noradrenaline and histamine did not cause desensitization of the tissue and therefore could be added cumulatively to the bath. ATP, like α,β-methylene ATP, caused desensitization of the tissue and therefore, it was added at 40 min intervals with repeated washing in between each addition. The responses to cumulative additions of noradrenaline (0.1–30 μM) and non-cumulative additions of ATP (0.1–3 mM) were tested before and after exposure to prazosin (10 μM) and before and after desensitization to α,β-methylene ATP (10 μM). Histamine concentration-response curves (0.1–100 μM) at the start and finish of the experiment served as control responses to ensure that prazosin, α,β-methylene ATP, and nerve stimulations were acting in a specific manner and did not cause a general desensitization of the tissue.

Drugs

Adenosine 5′-triphosphate (sodium salt) (ATP), α,β-methylene adenosine 5′-triphosphate (lithium salt) (α,β-methylene ATP), (−)-noradrenaline bitartrate, tetrodotoxin, and histamine dihydrochloride were all obtained from Sigma Chemical Company. Prazosin hydrochloride was a gift from Pfizer Ltd. Guanethidine sulphate (Ismelin) was obtained from Ciba. All drugs were dissolved in distilled water. Ascorbic acid (100 μM) was added to the noradrenaline solution. All drugs except tetrodotoxin and guanethidine were prepared freshly each day.

Statistical analysis of results

Responses to nerve stimulation were expressed as a percentage of the maximal contraction obtained to histamine. The responses at each stimulus frequency have been expressed as a mean ± standard error of mean (s.e.mean). The pD_2 value for a drug, under a particular set of conditions, was calculated from the mean log (concentration of the drug) ± standard error (s.e.mean) which produced 50% of its maximal response. The slopes of concentration-response curves were calculated from the regression of the percentage responses obtained at each log concentration of the drug. Mean slopes ± s.e.mean, were used for testing parallelism between concentration-response curves in the presence and absence of prazosin and before and after desensitization to α,β-methylene ATP. Results have been analysed using Students *t* test (paired and unpaired as appropriate) and a probability of less than 0.05 was considered significant.

Results

Neurogenic contractions of the vessel

Transmural electrical stimulation of the rabbit isolated saphenous artery for a period of 1 s (4–64 Hz) induced a rapid, frequency-dependent contraction. Near maximal contraction was attained at 64 Hz (Figure 1a,b) and this response was approximately 50–60% of the maximal contraction to exogenous histamine. Stimulation for a period of 1 min (4, 8, 16 Hz) produced a response which varied from preparation to preparation (Figure 1c,d). In all preparations there was an initial rapid, transient contraction that reached a maximum within 1 or 2 s. In some preparations this was followed by a sustained contraction that was greater than or equivalent to the initial contraction. This sustained contraction developed in less than 1 min from the start of electrical stimulation and lasted for the duration of the stimulation. In some preparations the initial transient contraction was followed by a much smaller contraction which, in some cases, was sustained for the 1 min of stimulation or in others very gradually decreased towards baseline (Figure 1c,d). In the latter case, this second phase of the contraction was difficult to assess, and, therefore, to standardize the method of calculation of responses to periods of stimulation of 1 min, the sustained contraction was defined as that contractile response produced after 1 min of continuous stimulation. All contractions fell back rapidly to base line at the end of the stimulation period and responses could be repeated every 3–4 min for the 1 s trains, or 10–15 min for the 1 min trains of stimulation without fatigue or desensitization being evident. All responses were abolished by tetrodotoxin (1 μg ml^{-1}) ($n = 5$) and also by guanethidine (4 μM) ($n = 4$).

Effect of prazosin on neurogenic contractions

Neurogenic contractions were compared before (control) and after preincubation of the vessel for 20 min with prazosin (1 μM). In the presence of prazosin, an α_1-adrenoceptor antagonist, there was a small reduction of the neurogenic contractions. This antagonism was directly related to the frequency of stimulation. For the 1 s train of stimulation, at 32 and 64 Hz there was a significant reduction in the contraction by an average of 20 and 28% respectively, whereas at lower frequencies of stimulation any reductions in contractions were not significant (Figures 1a, 2a, 4). Neither

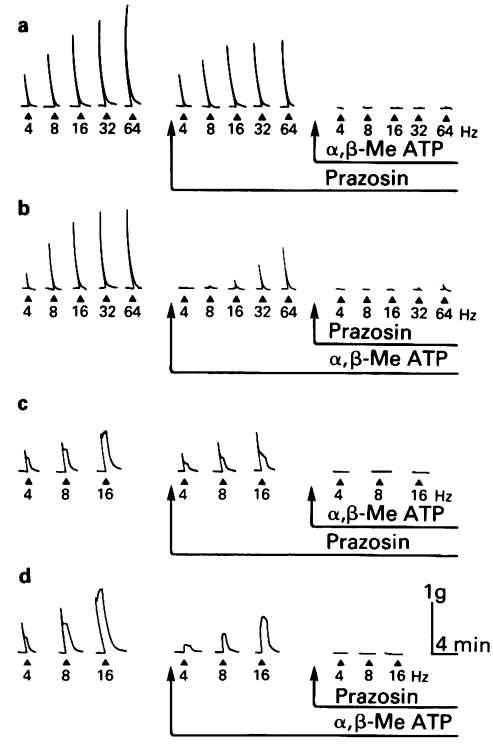

Figure 1 Contractions produced in the isolated saphenous artery of the rabbit on neurogenic transmural stimulation (0.08–0.1 ms; supramaximal voltage) for 1 s (a,b) and 1 min (c,d) at the frequencies (Hz) indicated (▲). Nerve stimulations were repeated in the presence of 10 μM prazosin added before (a,c) or after (b,d) desensitization of the P_2-purinoceptor with 10 μM α,β-methylene ATP (α,β-Me ATP) as indicated on the figure by the arrowed lines. The horizontal bar signifies 4 min and the vertical bar 1 g.

the initial rapid component nor the sustained component of the neurogenic contractions produced by 1 min trains of stimulation at 4, 8 and 16 Hz was significantly altered in the presence of prazosin (Figures 1c, 3a). When, in the absence of prazosin, the sustained contraction was greater than the initial rapid contraction, then this sustained contraction was often reduced in the presence of prazosin. After preincubation for 20 min with a higher concentration of prazosin (10 μM), there was no further change in the neurogenic contractions to 1 s or 1 min trains of stimulations at all frequencies tested (Figures 2a, 3a).

Effect of desensitization of the P_2-purinoceptor on the prazosin-resistant neurogenic contractions

Prazosin-resistant neurogenic contractions were compared before and after desensitization of the P_2-purinoceptor with α,β-methylene ATP (10 μM). Prazosin-resistant contractions to 1 s and 1 min trains of stimulation were usually abolished after desensitization of the P_2-purinoceptor (Figures 1, 2a, 3a). Any residual response was blocked by tetrodotoxin (1 μg ml^{-1}).

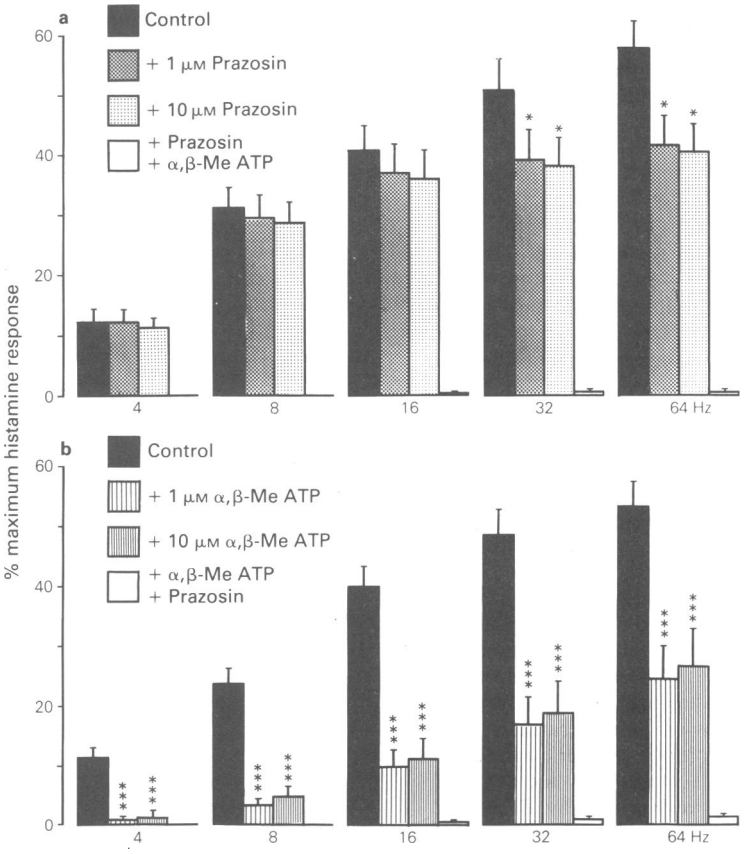

Figure 2 Contractions of the isolated saphenous artery of the rabbit to 1 s periods of perivascular nerve stimulation (0.08–0.1 ms, supramaximal voltage, 4–64 Hz) expressed as a percentage of the maximal contraction to histamine. (a) Contractions produced in the absence of any drug (control) are compared with those obtained in the presence of prazosin, 1 and 10 μM. Note that the prazosin-resistant contractions were abolished (4, 8 Hz) or almost totally inhibited (16, 32, 64 Hz) after desensitization by 10 μM α,β-methylene ATP (α,β-Me ATP) ($P < 0.001$) ($n = 14$). (b) Contractions produced in the absence of drug (control) are compared with those obtained after the P_2-purinoceptors have been desensitized with 1 and then with 10 μM α,β-methylene ATP (α,β-Me ATP). Note that the α,β-methylene ATP-resistant contractions were abolished (4, 8 Hz) or almost totally inhibited (16, 32, 64 Hz) in the presence of 10 μM prazosin ($P < 0.001$) ($n = 15$). Vertical bars denote s.e.mean. Significant differences ($^*P < 0.05$; $^{***}P < 0.001$) between control and experimental contractions were calculated by paired t tests.

Effect of desensitization of the P_2-purinoceptor on the neurogenic contractions

Neurogenic contractions were compared before (control) and after desensitization of the P_2-purinoceptor with α,β-methylene ATP (1 μM). After desensitization of the P_2-purinoceptor there was a significant reduction in the contraction to neurogenic stimulations for periods of 1 s and 1 min at each frequency tested (Figures 1, 2b, 3b). In the case of stimulation for a period of 1 min, there was no longer a biphasic response, only a sustained contraction remained. For

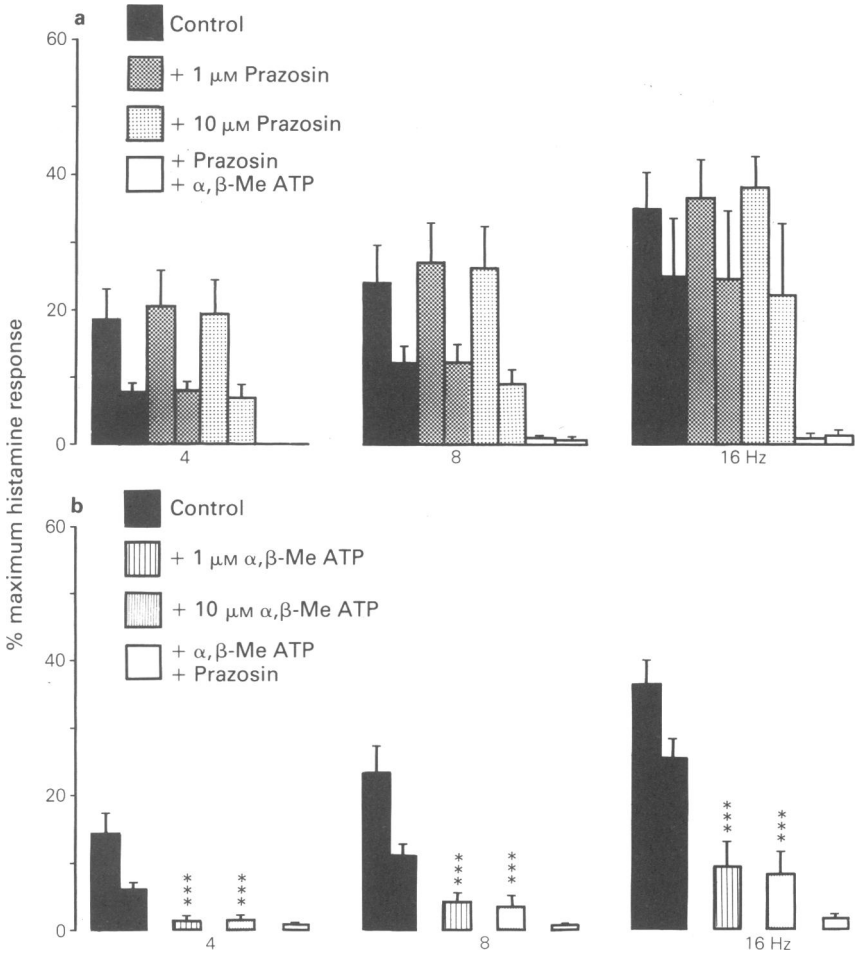

Figure 3 Biphasic contractions of the isolated saphenous artery of the rabbit to 1 min perivascular nerve stimulation (0.08–0.1 ms; supramaximal voltage; 4, 8 and 16 Hz) each expressed as a percentage of the maximal contraction to histamine. The left hand column of each pair of responses under a particular set of conditions represents the initial contraction, and the right the sustained contraction. (a) Contractions produced in the absence of any drug (control) are compared with contractions produced in the presence of prazosin, 1 and 10 μM. Note that prazosin-resistant contractions to perivascular nerve stimulation were abolished (4 Hz) or almost totally inhibited (8, 16 Hz) after desensitization of the P_2-purinoceptor with α,β-methylene ATP, 10 μM ($P < 0.001$) ($n = 10$). (b) Contractions produced in the absence of drugs (control) are compared with those produced after the vessel has been desensitized to α,β-methylene ATP (α, β-Me ATP), 1 and 10 μM. Note that the α,β-methylene ATP-resistant contractions to perivascular nerve stimulation were almost totally inhibited in the presence of prazosin, 10 μM ($P < 0.001$) ($n = 10$). It was not possible to differentiate any component of the rapid contraction from the maintained contraction, hence only the latter was measured. Vertical bars denote s.e.mean. Significant differences (***$P < 0.001$) between control and experimental contractions were calculated by paired t tests.

1 s and 1 min trains of stimulation, the magnitude of the α,β-methylene ATP-resistant contractile component, expressed as a fraction of the whole contraction at a given frequency of stimulation, was directly related to the frequency of stimulation (Figure 4). A further desensitization of the P_2-purinoceptor with 10 μM α,β-methylene ATP caused no further reduction in the neurogenic contraction (Figures 2b, 3b). There was a large variation in the contribution of the α,β-methylene ATP-sensitive component of the neurogenic response and this became more apparent at the higher stimulus frequencies. In some preparations after desensitization of the P_2-purinoceptor with α,β-methylene ATP, the response to stimulation for 1 s was almost totally blocked at 4 Hz, but only partially blocked at 64 Hz. In other cases there was an almost total block at both 4 and 64 Hz. This large variation was also observed during long-term neurogenic stimulation. Generally, if the sustained component were as great or greater than the initial rapid response then this contraction was more resistant to α,β-methylene ATP desensitization than average, whereas if this component were small and not well sustained, it was largely due to an α,β-methylene ATP-sensitive (purinergic) component.

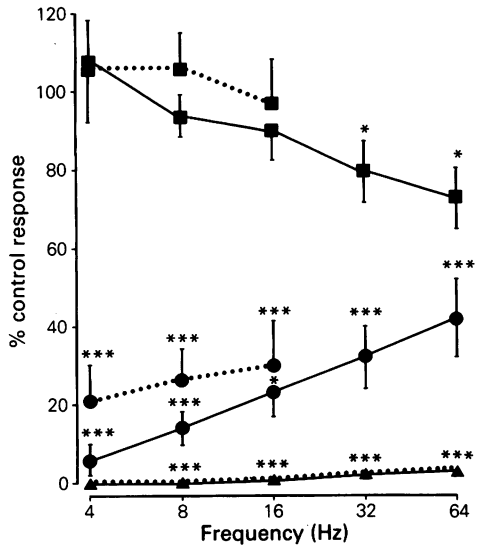

Figure 4 Contractions of isolated saphenous artery of rabbit produced by 1 s and 1 min perivascular nerve stimulation (0.08–0.1 ms, supramaximal voltage) after preincubation with prazosin, 1 μM (■) ($n = 10–14$) or after desensitization by α,β-methylene ATP, 1 μM (●) ($n = 10–15$) or after treatment with both drugs (▲) ($n = 20–29$). Results produced by 1 s and 1 min periods of stimulation (the sustained component only) are represented by points joined by solid and broken lines respectively. Results for each frequency are expressed as a percentage of the neurogenic contraction produced in the absence of drug. Vertical bars denote s.e.mean. Significant differences ($^*P < 0.05$; $^{***}P < 0.001$) between control and experimental contractions were calculated by paired t tests.

Effect of prazosin on neurogenic contractions of vessels in which the P_2-purinoceptors have previously been desensitized

α,β-Methylene ATP-resistant neurogenic contractions were compared before and after incubation of the vessel with prazosin (10 μM) for 20 min. α,β-Methylene ATP-resistant contractions produced by 1 s and 1 min trains of stimulation were usually abolished after incubation with prazosin (Figures 1, 2b, 3b). Any residual response was blocked by tetrodotoxin (1 μg ml^{-1}).

Responses to drugs

Histamine and noradrenaline each produced reproducible, concentration-dependent, sustained contractions of the rabbit saphenous artery. The maximal contractions evoked by the two drugs did not differ significantly; the maximal contraction to histamine was 3.47 ± 0.20 g ($n = 7$) and that to noradrenaline was 3.32 ± 0.23 g ($n = 9$). Although the pD_2 value for each was in a similar concentration range, that for histamine was significantly greater than that for noradrenaline (Table 1). Unlike noradrenaline or histamine, ATP and α,β-methylene ATP produced rapid, transient concentration-dependent contractions that were reproducible after a 30–40 min period of repeated washing. The pD_2 value for contraction of the vessel to α,β-methylene ATP was 5.92 ± 0.08 ($n = 6$). The vessel was far less sensitive to ATP and hence contraction to ATP at the concentrations tested (0.03–3 mM) did not reach maximal response and therefore a pD_2 could not be attained. However, comparison of the pD_2 value of α,β-methylene ATP with the ATP concentration required to produce an equivalent contraction suggests that α,β-methylene ATP is approximately 3500 times more effective than ATP at causing a contraction of the vessel. The concentration-response curve to noradrenaline was significantly shifted to the right after preincubation of the tissue with prazosin (1 μM) for 20 min. On increasing the concentration of prazosin in the bath to 10 μM, there was a further significant shift in the concentration-response curve to noradrenaline (Table 1). The concentration-response curve to noradrenaline was not significantly altered from control values after desensitization of the P_2-purinoceptor with 10 μM α,β-methylene ATP (Table 1). On the other hand, contrac-

tile responses produced by ATP were abolished after P_2-purinoceptor desensitization but were not significantly antagonized in the presence of prazosin (Table 1). After a combined treatment with prazosin and desensitization with α,β-methylene ATP, neither the maximal response nor the pD_2 value of the contraction to histamine was significantly altered from control values (Table 1). From this result it can be concluded that there was no general desensitization of the tissue due to prazosin or α,β-methylene ATP.

Discussion

These results demonstrate that electrical field stimulation of the perivascular nerves of the rabbit isolated saphenous artery produces contractions that are largely mediated by ATP, or a related purine, at P_2-purinoceptors and, to a lesser extent, by noradrenaline at α_1-adrenoceptors. The relative contribution of these two components to the over-all contraction of the vessel is dependent upon the parameters of stimulation applied. Since guanethidine abolishes all neurogenic contractions of this vessel, it is likely that ATP and noradrenaline function as cotransmitters from sympathetic perivascular nerves innervating the arterial smooth muscle, as has been proposed for other blood vessels (Sneddon & Burnstock, 1984b; Ishikawa, 1985; Kügelgen & Starke, 1985; Kennedy *et al.*, 1986; Muramatsu, 1986; Vidal *et al.*, 1986).

The rabbit saphenous artery contracted well to exogenously applied noradrenaline. Prazosin, a selective postjunctional α_1-adrenoceptor antagonist (Cavero & Roach, 1980), significantly antagonized this response. On the other hand, contractions to electrical stimulations were only partially antagonized

Table 1 Comparison of drug responses in rabbit isolated saphenous artery

(a) *Noradrenaline*

	pD_2	*Slope*	*Relative antagonism*
Control	5.05 ± 0.04(7)	60.30 ± 3.5(7)	
+ 1 μM Prazosin	3.87 ± 0.14(8)***‡	67.75 ± 11.9(8) NS‡	15
+ 10 μM Prazosin	3.52 ± 0.12(6)***‡§	60.60 ± 6.5(6) NS‡	34
+ α,β-Me ATP	4.99 ± 0.06(7) NS†	59.90 ± 3.4(7) NS†	1.1

(b) *Histamine*

	pD_2	*Slope*	*Relative shift*
Start	5.32 ± 0.08(6)	48.35 ± 5.5(6)	
Finish	5.36 ± 0.19(6) NS†	49.90 ± 6.3(6) NS†	0.91

(c) *ATP*

	-log EC_{30}	*Slope*	*Relative antagonism*
Control	2.56 ± 0.08(6)	21.38 ± 1.4(6)	
+ Prazosin	2.64 ± 0.07(6) NS†	27.56 ± 1.8(6)*	0.83
+ α,β-Me ATP	Not reached (6)***†	0	Infinite

All values are given as mean ± s.e.mean with number of observations (n) in parentheses.
(a) pD_2 values and mean slopes for the concentration-response curve to noradrenaline in the absence of any other drug (control), in the presence of 1 and 10 μM prazosin and after desensitization of the tissue to 10 μM α,β-methylene ATP (α,β-Me ATP).
(b) pD_2 values and mean slopes for the concentration-response curve to histamine in rabbit isolated saphenous artery at the start of the experiment and after the tissues have been treated with α,β-methylene ATP and prazosin.
(c) Contractions to ATP expressed as $-\log$ (30% maximal contraction to histamine) ($-\log EC_{30}$) in the absence of any other drug (control), in the presence of 10 μM prazosin, and after desensitization of the tissue to 10 μM α,β-methylene ATP. Note that, at the concentrations of ATP used (0.03–3 mM), pD_2 values were not attained.
Relative antagonism (or shift) in the concentration-response curve between control and experimental conditions (or start and finish of the experiment) were calculated by the antilog (pD_2 control − pD_2 experimental).
Significant differences (*$P<0.05$; ***$P<0.001$; NS = no significant difference) between control and experimental conditions were calculated by paired and unpaired t tests as indicated on the table.

Note:- †: paired statistical analysis; ‡: unpaired statistical analysis; §: there was a significant difference between pD_2 values of noradrenaline in the presence of 1 and 10 μM prazosin, $P<0.05$, unpaired statistical analysis.

by prazosin, and this was significant at the higher frequencies of stimulation. A higher concentration of prazosin caused a further significant rightward shift in the concentration-response curve to exogenous noradrenaline but was no more effective in blocking the neurogenic contraction of the rabbit saphenous artery. These prazosin-resistant mechanical contractions to neurogenic stimulation are probably mediated by the prazosin-resistant excitatory junction potentials described in the rabbit saphenous artery by Holman & Surprenant (1980). Desensitization of the P_2-purinoceptor with α,β-methylene ATP almost totally inhibited these large, prazosin-resistant neurogenic contractions. These results suggest that, although exogenous noradrenaline acts postjunctionally at α_1-adrenoceptors, contractions produced by nerve stimulation do not involve, to a great extent, these receptors and appear largely purinergic in nature. The proportion of the neurogenic response mediated via α_1-adrenoceptors is directly related to the frequency of stimulation. Any residual contraction that might remain after prazosin and α,β-methylene ATP treatment is most likely to be due to an incomplete action of these two drugs. However, one cannot rule out the possibility of a third neurotransmitter, as has been suggested in the guinea-pig vas deferens (Stjärne & Åstrand, 1985).

While it is generally recognised that the excitatory junction potentials recorded on smooth muscle cells of a number of sympathetically innervated vessels are prazosin-resistant (see Burnstock & Kennedy, 1986), it has been noted that a mechanical prazosin-resistant component of the response to sympathetic stimulation is not always evident (see for example the rabbit ear artery; Allcorn *et al.*, 1985). An explanation proposed by Kennedy *et al.* (1986), is that the prazosin-resistant (purinergic) component of the sympathetic nerve-mediated responses is favoured by short (1 s) periods of stimulation, while the noradrenaline component is favoured by longer stimulation periods. In addition it appears that the proportions of noradrenaline and ATP differ in different sympathetic nerves, so that sometimes the mechanical components to ATP and noradrenaline are clearly distinguishable as in the vas deferens (Meldrum & Burnstock, 1983) and the rabbit saphenous artery (present study), while in others such as the rabbit ear artery (Kennedy *et al.*, 1986) and rat tail artery (Vidal *et al.*, 1986) the prazosin-resistant (purinergic) component is less apparent except with short periods of stimulation (Kennedy *et al.*, 1986).

ATP and α,β-methylene ATP both produce concentration-dependent, rapid, transient vasoconstrictor responses in the rabbit saphenous artery. α,β-Methylene ATP is approximately 3500 times more effective than ATP at causing contraction of the vessel. On repeated administration of the drug, α,β-methylene ATP caused desensitization of its contractile response. After total desensitization to α,β-methylene ATP, contractions to ATP up to a concentration of 3 mM were totally abolished and in some cases a relaxation was observed, whereas there was no change in the sensitivity of the vessel to noradrenaline. It has been reported in other vessels and other tissues that α,β-methylene ATP is far more potent than ATP in causing a contraction and that it also selectively desensitizes the P_2-purinoceptor (see Burnstock & Kennedy, 1985). The results from the present study demonstrate that in the rabbit saphenous artery there are also postjunctional P_2-purinoceptors and that the desensitizing action of α,β-methylene ATP was selective for these receptors. Evidence suggests that α,β-methylene ATP acts only postjunctionally and any purine-mediated prejunctional inhibitory actions of perivascular nerve activity appear to be mediated by P_1-purinoceptors rather than P_2-purinoceptors (Burnstock & Brown, 1981). Also it has been shown that α,β-methylene ATP has little or no effect on stimulation-induced release of [^{3}H]-noradrenaline from sympathetic nerves of guinea-pig and mouse vas deferens (Stjärne & Åstrand, 1985; Westfall *et al.*, 1986), rabbit mesenteric artery (Kügelgen & Starke, 1985) or rat tail artery (Vidal *et al.*, 1986). In the present study, in support of this view, after pretreatment of the rabbit saphenous artery with prazosin, the neurogenic contraction was partially reduced, but only significantly at the higher frequencies, whereas exogenous noradrenaline was significantly antagonized at all concentrations. This suggests that only a small adrenergic component, but a large non-adrenergic component, was being stimulated to evoke the neurogenic contraction, and that any prejunctional inhibition of noradrenaline release would account for only a small reduction in the contractile response. The inhibitory effect of α,β-methylene ATP on neurogenic contractions, therefore, appears to be due to specific desensitization of postjunctional P_2-purinoceptors, although some prejunctional action has not been wholly discounted. Neurogenic contractions, in the absence of prazosin, were substantially reduced after desensitization of the P_2-purinoceptor with α,β-methylene ATP. The relative contribution of the α,β-methylene ATP-sensitive (purinergic) component to the overall contraction for a given frequency was inversely related to the frequency of stimulation. This was true both for the rapid contractions produced by a 1 s stimulation and for the sustained contraction produced by a 1 min stimulation. On stimulation for 1 s, $94 \pm 4\%$ of the response at 4 Hz, but only $58 \pm 10\%$ of the response at 64 Hz was purinergic. A similar frequency-dependent relationship has been demonstrated for ATP as a cotransmitter in the rabbit ear artery (Kennedy *et al.*, 1986) and the urinary bladder of various species (Moss & Burnstock, 1985). Desensitizing the P_2-purinoceptor of the rabbit saphenous artery with a higher

concentration of α,β-methylene ATP did not alter the contractions. However, this α,β-methylene ATP-resistant response was almost completely abolished after incubation of the tissue with prazosin. Also, the whole neurogenic contraction was abolished by guanethidine. Hence, on looking at neurogenic responses either in terms of prazosin-resistant contractions or in terms of α,β-methylene ATP-sensitive contractions, from each approach it can be concluded that a large component of the contraction of the rabbit saphenous artery in response to sympathetic nerve stimulation is purinergic in nature, but that noradrenaline also plays a role, especially at the higher frequencies of stimulation.

Electrical stimulation of the rabbit saphenous artery for 1 min produces a biphasic contraction consisting of an initial rapid contraction followed by a sustained contraction. Biphasic contractions are also seen in the guinea-pig vas deferens, and it has been proposed that in this tissue the first phase is mediated mainly by ATP and the second mainly by noradrenaline (Sneddon *et al.*, 1982; Burnstock & Sneddon, 1985). On the other hand Stjärne & Astrand (1985) have presented evidence that noradrenaline and ATP each contribute to both phases of the contractile response of the vas deferens, and that the ability to produce a biphasic response is a built-in property of the muscle. Likewise, in the rabbit ear artery, each of the components of the biphasic contraction are probably mediated by both noradrenaline and ATP (Kennedy *et al.*, 1986). In the rabbit saphenous artery, the rapid contraction produced by a 1 s train of stimulation was mostly mediated by ATP but also, to a lesser extent, by noradrenaline. Likewise, the sustained contraction of the biphasic response was mediated largely by ATP, but also by noradrenaline. Hence, considering both these results, it is concluded that in the rabbit saphenous artery ATP and noradrenaline each contribute to both components of the biphasic response, as is the case for the rabbit ear artery. The contribution of the two transmitters to each component of the biphasic contraction is dependent upon the frequency of stimulation, but unlike the rabbit ear artery, ATP plays the predominant role at each of the frequencies tested.

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Nerve pathways involved in adrenergic regulation of electrical and mechanical activities in the chicken rectum

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1 Peripheral nerve pathways responsible for adrenergic inhibition of mechanical and electrical activities in the chicken rectum and receptors mediating the adrenergic inhibition were investigated in isolated extrinsically-innervated rectum of the chicken.

2 Electrical stimulation of the anal end (Ra) or the ileal cut end (Ri) of Remak's nerve, or perivascular nerves (P) elicited relaxation of the rectum pretreated with atropine (0.5 μM) and hexamethonium (0.3 mM) to block the cholinergic and non-cholinergic, non-adrenergic excitatory innervations. Ri stimulation was much less effective than Ra and P stimulations. The relaxation was shown to be related to cessation of spontaneous spike discharge of the longitudinal muscle which was accompanied by membrane hyperpolarization.

3 The inhibitory effects elicited by Ra and P stimulations, which were prolonged beyond the period of the stimulation, were converted to transient ones by propranolol (3.4 μM). Phentolamine (2.6 μM) reduced effectively the residual effects. In contrast, the effects of Ri stimulation were little affected by these drugs.

4 The present results provide evidence for the existence of two nerve pathways responsible for direct adrenergic inhibitory innervation to the chicken rectum, one running orally in Remak's nerve trunk, leaving it and descending in the branches to the rectum, and the other running as the perivascular nerves along the arterial supplies of the rectum. The direct innervation is mediated predominantly by β-adrenoceptors.

Introduction

Morphological studies (Bennett & Malmfors, 1970; Akester, 1979) have suggested that the rectum of the chicken receives adrenergic innervation via the following three pathways: (1) a pathway which ascends the nerve of Remak and then leaves the nerve trunk as fine bundles extending to the organ; (2) another which descends this nerve trunk, leaves the trunk and reaches the organ; and (3) one which descends along the arteries as the perivascular nerves.

The aim of the present experiments was to demonstrate peripheral nerve pathways responsible for adrenergic inhibition of mechanical and electrical activities in the chicken rectum and to investigate receptors mediating the inhibition. It has been shown that the rectal region of Remak's nerve contains cholinergic and non-cholinergic nerve pathways mediating excitatory responses of the rectum (Bartlet & Hassan, 1971; Takewaki *et al.*, 1977; Komori & Ohashi, 1982), and that perivascular nerves of the caudal mesenteric artery are composed of cholinergic fibres in addition to adrenergic fibres (Everett, 1968). To avoid involvement of responses of the rectum mediated by these excitatory nerves when Remak's nerve and the perivascular nerves were stimulated, the present experiments were performed after blockade of these innervations with atropine and hexamethonium (Kanazawa *et al.*, 1980), unless otherwise stated.

Methods

Male chickens, aged more than 100 days, were obtained from commercial sources. The precise genetic background was not known, though they represented hybrids of White Leghorn and other strains. Birds were stunned and bled to death. The rectum was removed together with Remak's nerve and the vessels supplied. A portion of the anal end of Remak's nerve, 1–2 cm in length, was separated from the serosal

surface of the isolated rectum. A glass cannula of 1.0 mm i.d. and another glass cannula of 2.0 mm i.d. were inserted into the caudal mesenteric artery and vein, respectively, through which the preparation was perfused. The rectal artery, which arises from the caudal mesenteric artery, was ligated with about a 4 cm length measured from the origin, from which the rectal artery gives off 4 or 5 branches to the rectum.

Recording of mechanical responses

The isolated rectum preparation was mounted in a 150 ml organ bath filled with Krebs Henseleit solution containing atropine (0.5 μM) and hexamethonium (0.3 mM) and was also perfused at a constant rate of 1–3 ml min^{-1} by means of a roller pump via the arterial cannula with the same solution. The venous effluent was collected in a small compartment in the organ bath and sucked away. The bathing and perfusing solutions were aerated and maintained at 30°C to obtain constant responses to nerve stimulation over the period of an experiment (up to 3 h). Mechanical changes in the longitudinal direction of the rectum were recorded isotonically by a writing lever with 8 fold magnification on smoked paper or by a mechanoelectronic transducer (Nihon Kohden, TD-112S) on a pen recorder (Hitachi, 056). The resting tone of the preparations was adjusted by applying a load of 5 g to the levers.

Recording of membrane potential

The isolated rectum was sectioned lengthwise and pinned serosal side up on a rubber board and mounted in an organ bath with two compartments, as illustrated in Figure 1. In order to minimize variation in tone of the rectal muscle from one experiment to another, this procedure was done in the Krebs Henseleit solution containing isoprenaline (0.2 μM). The compartment in which the rectum was immersed was perfused at 5–8 ml min^{-1} with the solution containing atropine (0.5 μM). The other compartment was filled with the solution containing hexamethonium (0.3 mM). The tissue located just on the wall dividing two compartments and not immersed in either solution was kept wet by covering with a piece of wet filter paper. Hexamethonium (0.3 mM) was also applied to the ganglia on Remak's nerve by perfusing the solution containing this drug at 4 ml min^{-1} for 15 min by means of a roller pump via the arterial cannula inserted into the caudal mesenteric artery. This has been shown to be effective in blocking ganglionic transmission in the non-cholinergic excitatory nerve pathway to the rectum (Kanazawa *et al.*, 1980). In the centre of the rectal wall, a small circle (5 mm in diameter) was described by pinning with finer pins and usually membrane potential was recorded intracellularly from smooth muscle cells of the longitudinal layer inside the circle. Glass microelectrodes filled with 3 M KCl, with resis-

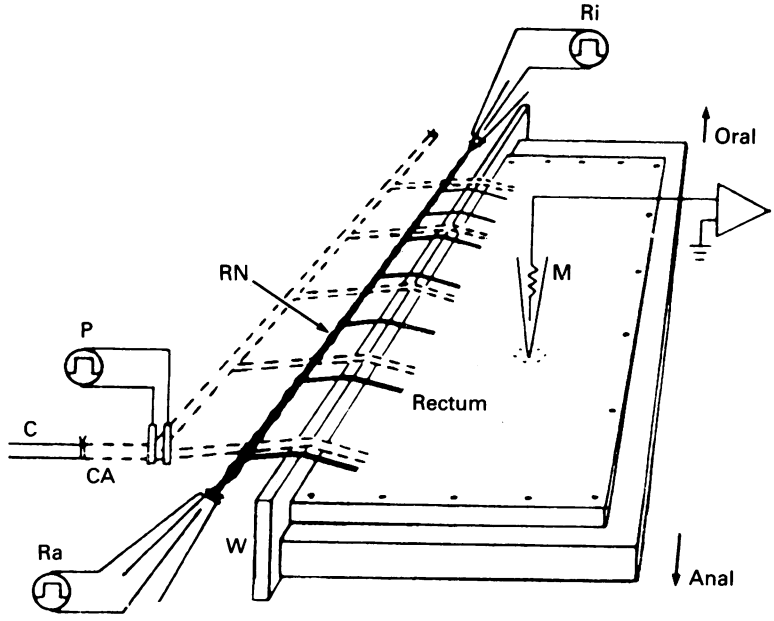

Figure 1 Diagrammatic sketch of the apparatus used for recording changes in membrane potential. RN, Remak's nerve trunk; CA, caudal mesenteric artery; C, arterial cannula; M, microelectrode; W, partition wall; Ra, stimulation site at the anal end of Remak's nerve trunk; Ri, stimulation site at the ileal cut end of Remak's nerve trunk; P, stimulaton site for the perivascular nerves of the caudal mesenteric artery; broken line, arterial supplies to the rectum.

tances of 40–80 Mohms were used. Changes in the membrane potential were displayed on an oscilloscope and photographed. All solutions used for this series of experiments were aerated and maintained at 37°C.

Nerve stimulation

Electrical stimulation was applied to the anal end of Remak's nerve (Ra), the ileal cut end of this nerve (Ri) and the perivascular nerves at the caudal mesenteric artery just before the branching off of the rectal artery (P). A bipolar suction electrode was used for Ra and Ri stimulations and a pair of ring electrodes of the type described by Burn & Rand (1960) for P stimulation. Rectangular pulses of 0.5 ms in duration and supramaximal intensity were delivered at varied frequencies using a stimulator (Nihon Kohden, SEM 3201). The stimulation period was 10 s for eliciting mechanical responses and it was 5 s for eliciting electrical responses.

Solution and drugs

Krebs Henseleit solution was of the following composition (mM): NaCl 118.9, KCl 4.6, $CaCl_2$ 1.8, $MgSO_4$ 2.4, KH_2PO_4 1.2, $NaHCO_3$ 25.0 and glucose 11.1, bubbled with a gas mixture of 95% O_2 and 5% CO_2. The following drugs were used: atropine sulphate (Tanabe), hexamethonium bromide (Yamanouchi), (−)-isoprenaline sulphate (Merck), propranolol hydrochloride (Sigma) and phentolamine mesylate (Ciba Geigy).

Statistical analysis

Mean data are presented with their standard errors and number of measurements. Student's t test was used for all comparisons between mean values. Statistical significance is determined as $P < 0.05$.

Results

Mechanical responses to nerve stimulation

Electrical stimulation of the anal end (Ra) or the ileal cut end (Ri) of Remak's nerve, or the periarterial nerves (P) elicited relaxation of the rectum. The lowest frequencies to produce the mechanical response were 1 or 2 Hz, regardless of the stimulating site, and the frequencies to elicit the maximal response were 30 or 40 Hz for both Ra stimulation and P stimulation and 20 or 30 Hz for Ri stimulation. The delay in response after the start of nerve stimulation was shortened with increasing stimulus frequency. For example, the delay of 5–8 s for responses to Ra stimulation at 2 Hz decreased to 1–2 s when the stimulus frequency was increased to 30 Hz. Figure 2 shows responses to nerve

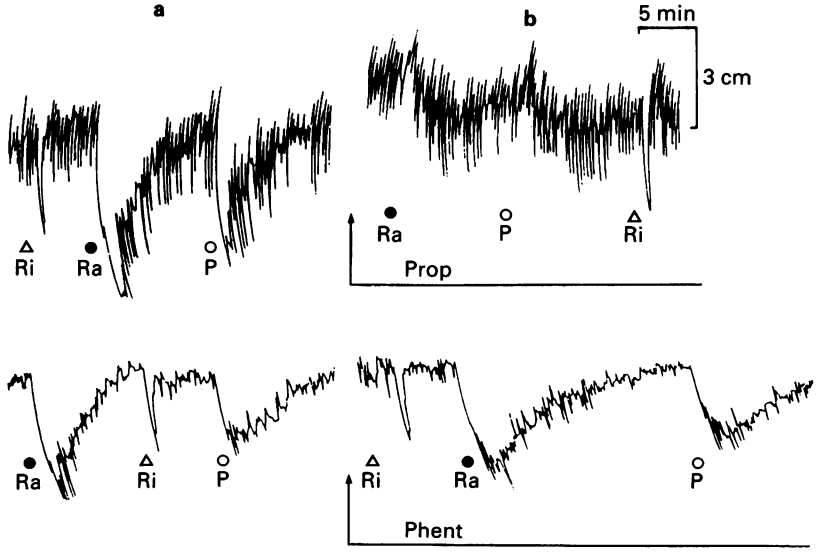

Figure 2 Mechanical responses of the isolated, perfused rectum of the chicken to stimulation (square pulses of 0.5 ms duration at 30 Hz for 10 s) of the anal end (Ra, ●) and ileal cut end (Ri, △) of Remak's nerve trunk, and of the perivascular nerves of the caudal mesenteric artery (P, o), and the effects of propranolol (3.4 μM) (Prop) and phentolamine (2.6 μM) (Phent) on these responses. (a) Control; (b) 30 min after propranolol or phentolamine. The bathing and perfusing solutions containing atropine (0.5 μM) and hexamethonium (0.3 mM) were used throughout the experiment. The responses were recorded isotonically with 8 fold-magnification on smoked paper.

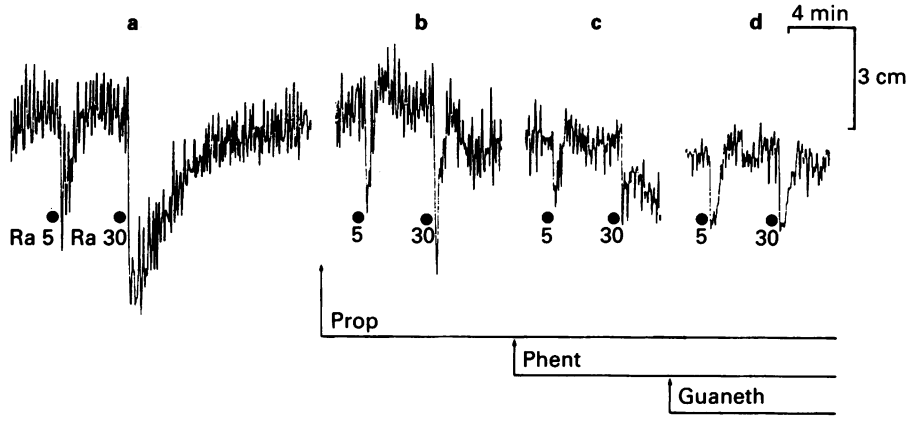

Figure 3 The effects of propranolol, phentolamine and guanethidine on the mechanical responses of the isolated, perfused rectum of the chicken to stimulation (square pulses of 0.5 ms duration at 5 Hz, Ra 5 ●, and 30 Hz, Ra 30 ●, for 10 s) of the anal end of Remak's nerve trunk. (a) Control; (b) 15 min after propranolol (3.4 μM) (Prop); (c) 20 min after additional application of phentolamine (2.6 μM) (Phent); (d) 30 min after additional application of guanethidine (17 μM) (Guaneth). The responses were recorded isotonically on the potentiometric recorder. Throughout the experiment the bathing and perfusing solutions contained atropine (0.5 μM) and hexamethonium (0.3 mM).

stimulation at 30 Hz at the three different sites. The response to Ra stimulation sustained and reached its peak relaxation within 60 s after cessation of the nerve stimulation. Then the tension returned gradually to the initial level. The time to decay completely from the peak relaxation was 6.3 ± 0.8 min ($n = 12$) at 30 Hz. The response to Ri stimulation was a relaxation of short duration. Usually the response reached its peak during the stimulation period of 10 s and subsided immediately after the stimulation was turned off; in some preparations the relaxation was followed by a transient contraction. Similar results to those produced by Ra stimulation were obtained with P stimulation, although P stimulation was slightly less effective than Ra stimulation. The relative values of Ri stimulation- and P stimulation-evoked responses to Ra stimulation-evoked response were measured in terms of the size and duration. The sizes were 60.5 ± 4.6% ($n = 5$) for the Ri stimulation and 70.0 ± 9.5% ($n = 6$) for the P stimulation, and the durations were 15.1 ± 4.7% for the Ri stimulation and 78.5 ± 6.6% for the P stimulation.

Effects of α- and β-adrenoceptor blocking agents on the mechanical responses

Phentolamine, an α-receptor blocker, and propranolol, a β-receptor blocker, were applied to the preparation by adding them to the bathing and perfusing solutions for 15 to 30 min. The responses to Ra and P stimulation were either reduced in duration to 15.8 ± 2.1% ($n = 6$) and 19.8 ± 3.1% ($n = 5$), respectively, or completely blocked after application of propranolol (3.4 μM), as shown in Figure 2. However, the degree of inhibitory effect on the size varied from one experiment to another, the percentage reduction ranging from 30 to 90%. Additional application of phentolamine (2.6 μM) invariably resulted in further reduction of the size to 20 to 50% of that before application of this drug (Figure 3). Phentolamine, 2.6 or 5.2 μM, alone had little effect on the responses, but in some experiments it slightly increased the duration by prolonging the time to reach its peak relaxation and the time to return to the initial tone (Figure 2). Propranolol was effective in inhibiting the responses in the presence of phentolamine, like those in normal solution. Relaxation, still produced by nerve stimulation in the simultaneous presence of phentolamine and propranolol, remained unchanged after application of guanethidine (up to 17 μM) (Figure 3). In contrast, the response to Ri stimulation remained almost unaltered after application of these drugs (Figure 2).

Electrical responses of the longitudinal muscle to nerve stimulation

The rectal muscle showed spontaneous electrical activity, as in other intestinal smooth muscles. The muscle discharged spike potentials either singly or in bursts 30 to 60 min after mounting in the organ bath and spontaneous discharge of spikes in bursts was usually superimposed on a small depolarization of the membrane for 5 or 10 s. Thirty or sixty minutes later, spike discharges occurred continuously without any

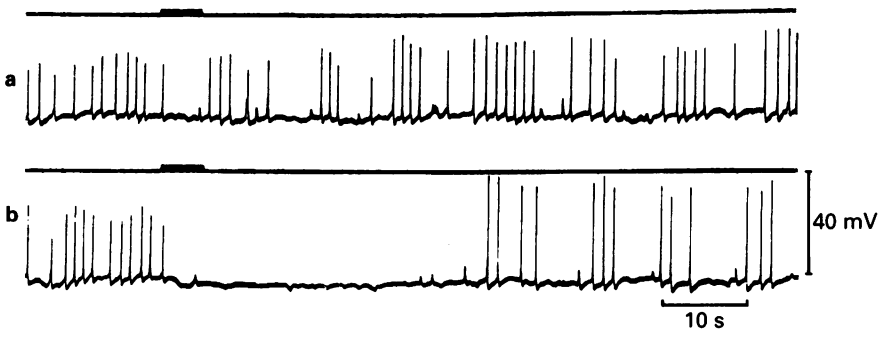

Figure 4 The inhibitory effects of stimulation (square pulses of 0.5 ms duration at 30 Hz for 5 s) of the ileal cut end (a) and anal end (b) of Remak's nerve trunk on the intracellularly recorded spontaneous spike discharge of the longitudinal smooth muscle of the chicken isolated rectum. (a) and (b), Records from the same cell with about 3 min interval. In each case, the upper horizontal line corresponds to the zero potential of the cell. The bathing solution for the rectum of the preparation contained atropine (0.5 μM) and that for Remak's nerve trunk contained hexamethonium (0.3 mM).

sustained depolarization and the discharge frequency varied from 0.4 s^{-1} to 1.2 s^{-1} in different cells, giving a mean frequency of 0.78 ± 0.02 s^{-1} (n = 35). The electrical activity was confirmed to be in the longitudinal muscle, since the circular muscle did not discharge spike potentials but slow waves of 20 to 40 mV in amplitude and 2 to 5 s in duration (Komori & Ohashi, unpublished observations). The amplitude of the spontaneous spikes varied in different cells, and was dependent on the resting membrane potential ranging from −35 to −55 mV measured as the maximal membrane potential between spike potentials. In general, the greater the resting membrane potential, the larger the spike amplitude. In most cells, spike potentials were preceded by a slow depolarization (pre-potential) as observed on other smooth muscles (Bülbring, 1955; Gillespie, 1962; Bennett *et al.*, 1966).

Nerve stimulation with single stimuli had no effect on the electrical activity of the smooth muscle. Nerve stimulation with repetitive stimuli at frequencies higher than 3 Hz, regardless of the stimulating site, caused cessation of spike discharges and the most effective stimulus frequency was 30 Hz as in the mechanical response of the rectum. Figure 4 shows typical responses to Ra stimulation. On Ra stimulation, spike discharge was blocked and the effect survived after termination of the nerve stimulation. In contrast to this, the effect of Ri stimulation started about 2 s after commencement of stimuli and usually disappeared within the period of the nerve stimulation. The response to Ri stimulation remained almost unchanged after prolonging the stimulus period for up to 10 s. Similar results to those with Ra stimulation were obtained with P stimulation. The time required to restore spike discharge after termination of nerve stimulation at 30 Hz for 5 s was 40.8 ± 2.4 s (n = 21) for Ra stimulation, being slightly longer than the 37.1 ± 2.5 s (n = 14) for P stimulation.

In 50% of the cells examined, before restoration of spike discharges prepotential-like depolarizations appeared, gradually increasing in amplitude and frequency with time, and finally reaching the threshold for generation of a spike potential. In the remaining cells, spike discharges occurred without any changes in the resting membrane potential. In general, spikes immediately after restoration were frequently larger in amplitude than the control (Figure 4), and more than 10 s was taken to return to the initial frequency of spike discharges.

In some preparations to which hexamethonium was not applied to block ganglionic transmission, effects of Ri stimulation and P stimulation on spike discharge were not different from those obtained in the presence of the ganglion blocking agent. There were some cells in which a few spikes were discharged within 10 s after termination of P stimulation (rebound spike discharges?). Ra stimulation elicited a contraction which was strong enough to dislodge the microelectrode. However, by inserting the microelectrode into another cell, it was confirmed that this stimulation caused cessation of spike discharges. The time taken for recovery from the inhibitory effect was 45.6 ± 4.6 s (n = 5), which was not significantly different from that in the presence of hexamethonium.

It is now clear that there are few adrenergic fibres which run aborally in Remak's nerve and extend to the rectal wall. Thus, investigation of adrenoceptors involved in the membrane potential changes was performed for Ra and P stimulations.

Hyperpolarization of longitudinal smooth muscle cells

Ra stimulation and P stimulation at frequencies higher than 5 Hz were both effective in producing an increase

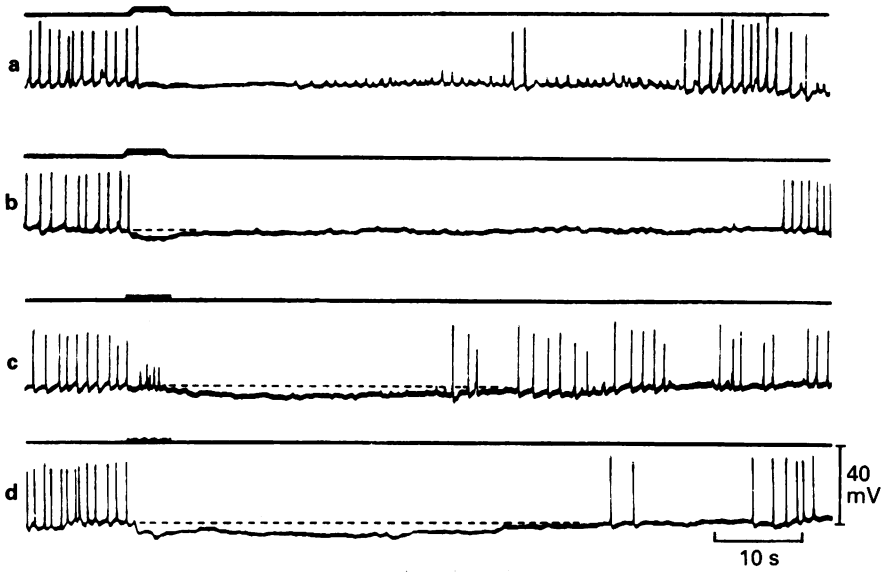

Figure 5 Comparison of the inhibitory effects of stimulation (square pulses of 0.5 ms duration at 30 Hz for 5 s) of the anal end of Remak's nerve trunk on the intracellularly recorded membrane potential of the longitudinal smooth muscle of the chicken isolated rectum. (a) No membrane hyperpolarization; (b) a transient membrane hyperpolarization; (c) a prolonged membrane hyperpolarization; (d) a mixed type of (b) and (c). (a and b) Records from the different cells in the same preparation; (c and d) records in other different preparations. In each case, the horizontal line corresponds to the zero potential of the cell and the broken line indicates the suspected level of the membrane potential without hyperpolarization. The bathing solution for the rectum preparation contained atropine (0.5 μM) and that for Remak's nerve trunk contained hexamethonium (0.3 mM).

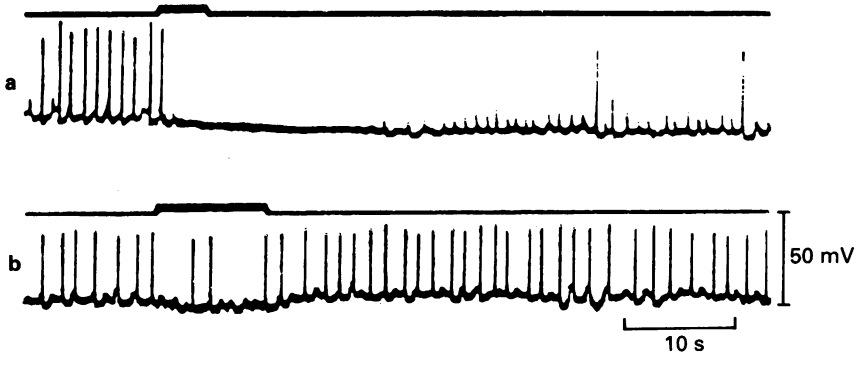

Figure 6 The effect of propranolol on inhibition of spontaneous spike discharge of the longitudinal smooth muscle of the chicken isolated rectum elicited by stimulation (square pulses of 0.5 ms duration at 30 Hz for 5 s in (a) and 10 s in (b)) of the anal end of Remak's nerve trunk. (a) Control; (b) 30 min after application of propranolol (3.4 μM) to the rectum. (a and b) Records from the same cell. In each case, the upper horizontal line corresponds to the zero potential of the cell. The bathing solution for the rectum of the preparation contained atropine (0.5 μM) and that for Remak's nerve trunk contained hexamethonium (0.3 mM).

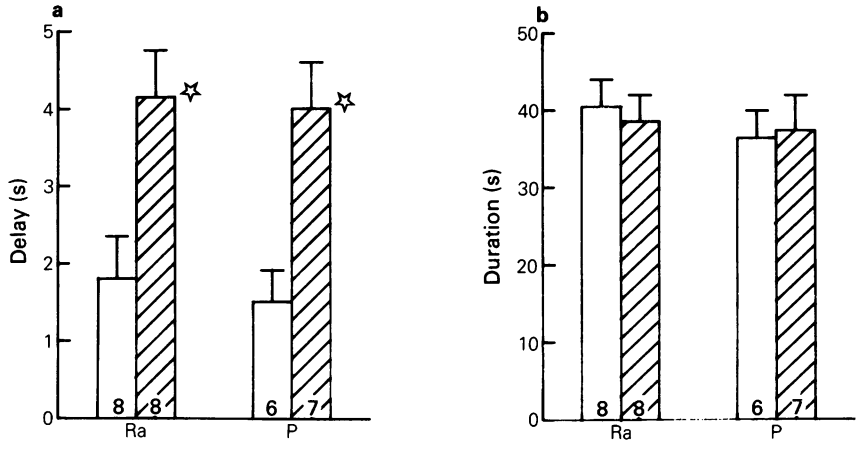

Figure 7 The effects of phentolamine on the delay and duration of inhibitory responses to stimulation (square pulses of 0.5 ms duration at 30 Hz for 5 s) of the anal end of Remak's nerve trunk (Ra) and of the perivascular nerves of the caudal mesenteric artery (P) in the chicken isolated rectum. (a) Delay measured as the time (s) taken from start of nerve stimulation to onset of the response; (b) duration measured as the time (s) taken from termination of nerve stimulation to restoration of spike discharge. Each column represents the mean of six to eight separate measurements (see figures at base of each column). Open columns, control; hatched columns, 30 min or more after application of phentolamine (2.6 μM). The vertical lines show s.e. *Statistically significant difference from the control value ($P < 0.01$).

in membrane potential (hyperpolarization). A maximal hyperpolarization was obtained when the stimulus frequency was 30 Hz for both Ra and P stimulations. However, no membrane hyperpolarization was observed on the records from 15% of the cells showing blockade of spike discharges. In 12% of the cells, membrane hyperpolarization began 1–2 s after the start of nerve stimulation and decayed within the stimulation period (a transient membrane hyperpolarization) and in 31% it developed gradually and was sustained beyond the period of nerve stimulation (a prolonged membrane hyperpolarization). In the remaining cells (42%), a mixed response of both transient and prolonged types of membrane hyperpolarization was elicited. The three types of hyperpolarizing responses to Ra stimulation are presented in Figure 5. The prolonged membrane hyperpolarization reached its peak (up to 9 mV) 9.5 ± 2.0 s ($n = 11$) after termination of nerve stimulation. Usually spike discharges were restored after recovery from hyperpolarization, but in some cells this occurred during the period of increased membrane potential.

Propranolol (3.4 μM) itself caused a small membrane depolarization (less than 10 mV) accompanied by slightly increased spike discharge frequency. The inhibitory effects of Ra stimulation and of P stimulation on electrical activity were markedly reduced, especially in duration, and converted to transient effects lasting only for the stimulation period or shorter (Figure 6). Phentolamine (2.6 μM) slightly prolonged the time course of the spike potential but had no effect on the membrane potential. The effects of this drug on the responses to nerve stimulation were to increase the delay of their onset (Figure 7) and to abolish the transient component of membrane hyperpolarization. Combined application of propranolol and phentolamine resulted in abolition of the inhibitory responses in most cells, and rarely a small and transient inhibitory effect still remained.

Discussion

The present results demonstrate two different adrenergic nerve pathways involved in inhibitory innervation of the chicken rectum. One of them runs orally in Remak's nerve trunk, leaves the nerve trunk, and extends in branches to the rectum and the other runs in the perivascular nerves along the arteries supplying the rectum. This is supported by histochemical observations (Bennett & Malmfors, 1970) that ascending fluorescent fibres are present in the rectal portion of Remak's nerve trunk and in the perivascular nerves of the caudal mesenteric artery. Bennett & Malmfors (1970) have also found fluorescent nerve cells in Remak's ganglia and suggested that the adrenergic fibres innervating the rectum originate predominantly from these nerve cells and with some coming from the cells of the paravertebral sympathetic chains. However, the present results are not in agreement with this suggestion, since the adrenergic response to Ra stimulation was observed in the presence of a ganglion

blocking agent, hexamethonium, and it was also confirmed that the adrenergic responses were very similar between before and after treatment with hexamethonium. Thus, the adrenergic fibres involved in the response appear to be postganglionic and pass Remak's ganglia. The fluorescent cells previously observed in Remak's ganglia (Bennett & Malmfors, 1970) may be involved in innervation of other tissues or might not be adrenergic neurones.

The gross anatomy of the sympathetic innervation of visceral organs of the chicken have revealed anastomoses of the rectal portion of Remak's nerve trunk with the paravertebral sympathetic chains, caudal mesenteric plexus and pelvic plexus (Watanabe, 1972; Akester, 1979). Combining these data with the present results, the origin of the adrenergic fibres innervating the rectum via Remak's nerve trunk seems to be in the cell bodies of the synsacral 9–14 sympathetic ganglia. The postganglionic fibres are comprised in the pelvic splanchnic (pudenal) nerves, take part in forming the pevlic plexus, leave the plexus to descend in the pelvic nerves, and finally enter Remak's nerve trunk just above the dorsal wall of the cloaca. The adrenergic fibres which run as perivascular nerves seem to originate predominantly from the cells of the thoracic 5–7 and synsacral 1–3 sympathetic ganglia, with some coming from the cells of the suprarenal ganglia or cranial mesenteric ganglia. Before reaching the rectum, the postganglionic fibres participate in forming extensive vascular plexuses present along the aorta, and extend to the vascular plexus of the caudal mesenteric artery.

Stimulation of nerve pathways which run aborally in Remak's nerve trunk caused a weak inhibition of electrical and mechanical activities in the rectum, but the inhibitory effect was unaffected by phentolamine and propranolol, suggesting involvement of non-adrenergic inhibitory nerves (Takewaki *et al.*, 1977). However, evidence for the existence of decending adrenergic fibres in Remak's nerve has been provided by earlier workers: there are descending fluorescent fibres and their terminals are in close contact to ganglion cells (Bennett & Malmfors, 1970). Preceding stimulation of the ileal cut end of Remak's nerve leads to inhibition of the responses to subsequent preganglionic stimulation of non-adrenergic and non-cholinergic (NANC) excitatory nerves, inhibition of the contraction of the rectum, excitatory junction potentials, and discharges in the post-ganglionic axons, and these inhibitory effects are eliminated after application of adrenergic neurone blocking drugs or α-adrenoceptor blocking drugs (Ishizuka *et al.*, 1982). It seems likely therefore that the adrenergic fibres descending in Remak's nerve may function exclusively as modulators of the synaptic transmission of NANC nerves in Remak's ganglia and may not reach the rectum.

Repetitive stimuli, but not single stimuli, were effective in producing relaxation, membrane hyperpolarization and inhibition of spontaneous spike discharge in the rectal longitudinal muscle. As the stimulus frequency was increased, the onset time of responses decreased and the responses increased in size and duration. Substantially similar results have been described for the rabbit distal colon (Gillespie, 1962) and guinea-pig taenia coli (Bennett *et al.*, 1966). These properties are taken as evidence for an innvervation like the model C classified by Burnstock (1970): most of the smooth muscle cells are not in close contact with nerve terminals and there are gaps of up to 300 nm to 1000 nm between nerve and smooth muscle membrane.

The present results show that the transmitter released from the adrenergic nerve terminals produces membrane hyperpolarization which is mediated mainly by β-receptors. Similar effects have been obtained with exogenous adrenaline or noradrenaline in longitudinal muscle strips of the chicken rectum (Komori *et al.*, 1980).

β-Receptor-mediated membrane hyperpolarization has been shown to be small or absent in intestinal smooth muscles (Bülbring *et al.*, 1980). In chicken rectum, β-receptors are of the β_2-type (Komori *et al.*, 1980) and it has been demonstrated that preferential release of adrenaline rather than noradrenaline is elicited by stimulation of Remak's nerve trunk (Komori *et al.*, 1979). Thus, this tissue is different from mammalian intestines in which β_1-receptors are present (Lands *et al.*, 1967) and noradrenaline functions as the neurotransmitter. There are certain smooth muscle preparations in which activation of β-receptors induces membrane hyperpolarization (rat uterus, Marshall, 1968; mouse uterus, Magaribuchi & Osa, 1971; rabbit pulmonary artery, Somlyo *et al.*, 1970; rat mesenteric artery, Mulvany *et al.*, 1982; cat trachea, Ito & Takeda, 1982). Since adrenoceptors for relaxation of uterus, trachea and blood vessels are classified as the β_2-type (Lands *et al.*, 1967), β_2-receptors, unlike β_1-receptors, may produce membrane hyperpolarization.

Interestingly, the membrane hyperpolarization recorded from smooth muscle cells of the longitudinal layer of the chicken rectum following nerve stimulation could be divided into three types in terms of duration and susceptibility to blockade by α- and β-adrenoceptor antagonists. In addition, no membrane hyperpolarization was observed in some cells. This variation would be due to different distribution of α- and β-receptors among cells, as described for substance P-, bradykinin- and ATP-receptors in the longitudinal layer of guinea-pig ileum (Bauer & Kuriyama, 1982). Cessation of spontaneous spike discharges without membrane hyperpolarization can be explained by assuming a pacemaker region for

spontaneous spike discharge (Tomita, 1970) in which smooth muscle cells have a high sensitivity to the transmitter associated with a dense distribution of adrenoceptors. If this is the case for these tissues with cable-like properties (Komori *et al.*, 1980), there may occur cells to which spike potentials can be conducted, but membrane hyperpolarization cannot spread.

In conclusion, the rectum of the chicken receives adrenergic inhibitory innervation in two ways. One of them causes membrane hyperpolarization that is effective in inhibiting spontaneous spike discharge in the smooth muscle and in turn produces relaxation. Nerves running orally in Remak's nerve trunk and extending to the rectum, and the periarterial nerves are responsible for this function. The other inhibits ganglionic transmission for the NANC excitatory nerves for which nerve fibres descending in Remak's nerve trunk appear to be responsible.

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Electrical changes produced by injury to the rat myocardium *in vitro* and the protective effects of certain antiarrhythmic drugs

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1 Glass microelectrodes were used to record intracellular electrical activity from rat isolated and superfused atrial myocardium during external electrical stimulation.

2 After 2 h in normal oxygenated physiological salt solution the muscle was exposed for 30 min to a superfusate simulating the composition of extracellular fluid during myocardial ischaemia (SI). This fluid contained lactate (20 mM), a raised potassium concentration (7 mM), no glucose and a pH lowered to 6.4, and was gassed with N_2 in place of O_2 (hypoxia).

3 During SI the diastolic threshold voltage for stimulation increased, the speed of action potential conduction between the right and left atria slowed, and both the effective and functional refractory periods of the right atrium shortened, as did the duration of the right atrial action potential.

4 The only component of SI which separately caused electrical changes similar to those of the full simulation was hypoxia.

5 Addition to the superfusate of verapamil ($0.5\,\mu g\,ml^{-1}$), sulphinpyrazone ($1-20\,\mu g\,ml^{-1}$) or indomethacin ($10-20\,\mu g\,ml^{-1}$) attenuated many of the SI-induced electrical changes, although indomethacin was much less effective than the other two drugs.

6 Lowering the calcium concentration of the superfusate from 2 mM to 0.5 mM protected against the SI-induced electrical changes that were inhibitable with sulphinpyrazone and verapamil.

Introduction

During myocardial ischaemia, in several mammalian species, the extracellular concentrations of hydrogen and potassium ions rise, the availability of glucose and oxygen declines, while lactate accumulates. Moreover, these extracellular chemical changes, particularly in combination, tend to shorten the duration of myocardial action potentials and their associated refractory periods (Downar *et al.*, 1977; Morena *et al.*, 1980; Cobb *et al.*, 1985; Ferrier *et al.*, 1985) and tend to slow the rate of conduction (ROC) of myocardial action potentials (Nakaya *et al.*, 1980; 1981; 1982; Kimura *et al.*, 1982; 1983; Gettes *et al.*, 1985). These electrical disturbances are arrhythmogenic, particularly when they occur heterogeneously throughout the myocardium (Northover, 1986). The present experiments were designed to explore the extent to which these electrical changes, produced by the chemical abnormalities that characterize the myocardium during ischaemia, were able to be created in the rat atrium *in vitro*, and the extent to which they were able to be modified by certain antiarrhythmic drugs.

Methods

Rats of the Sprague-Dawley strain weighing 340–480 g were killed by a blow to the head. The heart was removed quickly, the atria separated from the ventricles and attached with the endocardial surface upwards to the base of a superfusion trough maintained at 34°C. The muscle was exposed, unless specified otherwise, to a normal superfusate (NS) of the following composition (mM): NaCl 138, KCl 4, $CaCl_2$ 2, $MgCl_2$ 1, NaH_2PO_4 0.5, $NaHCO_3$ 10 and glucose 10, and gassed with a mixture of 95% O_2 plus 5% CO_2, giving a pH of 7.3.

Transmembrane potentials were recorded from subendocardial muscle fibres by means of a pair of glass microelectrodes filled with a 3 M solution of KCl and having resistances of $1-2 \times 10^7\,\Omega$. One microelectrode was lowered vertically by means of a micromanipulator towards the endocardial surface of the right atrium. Voltages detected by this microelectrode were passed by a single-ended high input-impedence cou-

pler with facilities for capacitance neutralisation (Type 8124, made by C.F. Palmer) to both an oscilloscope and a transient store microprocessor (Type 140, made by Bioscience). Stored signals were able to be replayed from the latter device at speeds of up to 2,000 fold slower than those at which they were recorded, permitting action potentials to be printed without distortion on paper, using a conventional pen recorder and allowing the maximum rate of depolarization (MRD) during the upstroke of the action potential to be found. The muscle was stimulated throughout an experiment at 4 Hz via a pair of platinum wire electrodes placed on the right atrial appendage. Square wave pulses of current, each 2 ms in duration and isolated from earth were used at a voltage of twice the prevailing diastolic threshold (DT). The effective refractory period (ERP) of right atrial muscle was determined using paired stimuli, and was taken as the interval between the most closely spaced pair of stimuli both of which yielded action potentials. The interval separating the upstrokes of the pair of action potentials so obtained was taken as the functional refractory period (FRP). The rate of conduction (ROC) of action potentials between right and left atria was measured with the aid of the second glass microelectrode, which was inserted in the wall of the left atrium. Voltage signals from both microelectrodes were displayed on a dual channel oscilloscope. Knowing the distance between the tips of the two microelectrodes and the time interval between the upstrokes of the action potentials recorded from them, it was possible to calculate the apparent ROC. However, since action potentials may not have conducted via the most direct route between the two microelectrodes, the ROC values may have been underestimated.

At the start of each experiment a tissue was allowed to equilibrate in NS for 2 h after which time control values of right atrial action potential duration measured at 60% repolarization (APD), DT, MRD, ERP and FRP were determined, together with the ROC between right and left atria. The superfusate was then changed for 30 min to one of abnormal composition, simulating the extracellular fluid composition of ischaemic muscle in one or more respects, as suggested by Ferrier *et al.* (1985). The relevant compositional abnormalities of this fluid were a high KCl concentration (7 mM), the presence of sodium lactate (20 mM), the absence of glucose, a pH lowered to 6.4 by lowering the $NaHCO_3$ concentration to 4 mM, and hypoxia produced by gassing with N_2 in place of O_2, giving a Po_2 in the superfusate of < 25 mmHg. When all these abnormalities were present in combination it was considered that conditions of simulated ischaemia (SI) prevailed.

Verapamil hydrochloride (supplied by Abbott Laboratories) was dissolved in distilled water. Indomethacin (supplied by Merck Sharp and Dohme, Ltd) and sulphinpyrazone (supplied by Geigy Pharmaceuticals, Ltd) were converted to their sodium salts with the aid of an equivalent weight of Na_2CO_3 and then prepared as aqueous solutions at pH 7.5. Aliquots of concentrated drug solutions were added to the superfusate just before use.

Results

Simulated ischaemia and its components

Exposure of the myocardium to SI caused the DT to rise, maximum values being reached after 15–20 min. Thereafter the DT values remained substantially unchanged for the remainder of the 30 min period of testing (Figure 1). Coincident with the rise in DT there was a progressive slowing of the ROC and a shortening of APD (Table 1), ERP and FRP (Figure 1). Shortening of refractory periods was most marked 12–15 min after the start of exposure to SI. Thereafter, refractory periods rose slowly (Figure 1), although APD values showed no tendency to increase, suggesting the development of some post-repolarization refractoriness. In all preparations total failure of conduction of action potentials from the right to the left atrium occurred within the 30 min period of SI. The mean time required to produce total right to left atrial conduction block was 18 min. Despite this, the right atrium continued to generate action potentials throughout the 30 min period of SI provided that the stimulus voltage continued to be adjusted to twice the prevailing DT. During exposure to NS the MRD values were acceptably consistent both between different preparations and between different places in the atrial wall of any given preparation. Unfortunately, during exposure to SI, although MRD values always tended to decrease, the decrease was extremely variable, not only between different preparations but also even at different places within a single preparation. For this reason MRD values have not been presented.

Experiments were performed to identify which components of SI were responsible for the observed electrical changes. In the first series of such experiments each component of SI was tested separately in an otherwise normal superfusate. Hypoxia was the only component of SI which shortened refractory periods, raised the DT and slowed the ROC when tested alone in an otherwise normal superfusate (Figure 2). These changes were not as great, however, as those produced by full SI (Figure 2). In the second series of experiments superfusates containing all but one of the components of SI in turn were tested. Omission of single components produced smaller rises in DT, less slowing of the ROC and less shortening of the refractory periods than was produced by exposure to the full SI (Figure 2). Exceptions to this general

Table 1 The ability of sulphinpyrazone, indomethacin and verapamil to inhibit simulated ischaemia (SI)-induced shortening of the right atrial action potential duration and SI-induced slowing of the conduction of action potentials between the atria

Superfusate	*Drug*	*Concentration* ($\mu g\ ml^{-1}$)	*APD* (ms) *10 min*	*15 min*	*20 min*	*ROC* ($cm\ s^{-1}$) *5 min*	*10 min*	*15 min*	*Inter-atrial block* (min)†
NS	–	–	18.1 ± 2.3	18.2 ± 2.2	19.2 ± 2.5	57 ± 8	52 ± 9	60 ± 11	> 30
SI	–	–	11.3 ± 2.1	9.1 ± 2.0	7.8 ± 1.8	47 ± 10	26 ± 12	18 ± 8	18
NS	Sulphinpyrazone	20	19.5 ± 2.9	19.2 ± 2.3	18.6 ± 2.4	50 ± 7	53 ± 5	57 ± 12	> 30
SI	Sulphinpyrazone	1	16.7 ± 2.7*	14.5 ± 2.3*	11.2 ± 2.0	49 ± 10	40 ± 13	32 ± 6*	> 30
SI	Sulphinpyrazone	10	19.4 ± 2.7*	18.7 ± 2.4*	14.3 ± 1.9*	53 ± 12	54 ± 8*	43 ± 6*	> 30
SI	Sulphinpyrazone	20	19.0 ± 2.6*	17.9 ± 2.5*	14.1 ± 2.3*	55 ± 6	61 ± 12*	42 ± 10*	> 30
NS	Indomethacin	20	18.5 ± 2.8	17.0 ± 2.7	19.4 ± 2.2	54 ± 11	58 ± 8	50 ± 11	> 30
SI	Indomethacin	1	11.6 ± 2.2	10.3 ± 2.1	8.0 ± 1.6	51 ± 10	30 ± 7	19 ± 6	21
SI	Indomethacin	10	14.2 ± 2.3*	13.4 ± 2.6*	11.9 ± 2.0*	52 ± 9	38 ± 12	26 ± 8	24
SI	Indomethacin	20	14.0 ± 2.6	12.6 ± 2.7	10.8 ± 2.0	45 ± 7	40 ± 10	29 ± 5*	25
NS	Verapamil	0.5	19.5 ± 2.0	19.3 ± 2.8	20.1 ± 1.9	52 ± 5	50 ± 6	49 ± 12	> 30
SI	Verapamil	0.5	17.7 ± 1.8	15.6 ± 2.6*	12.5 ± 1.2*	58 ± 8	49 ± 8*	51 ± 14*	> 30

There were between 10 and 30 observations in each treatment group. *Indicates a statistically significant difference ($P < 0.05$) from the corresponding value in the group exposed to SI in the absence of a drug. Measurements of right atrial action potential duration measured at 60% repolarization (APD) and rate of conduction (ROC) were made at 1 min intervals throughout the experiments, and the values quoted (mean ± s.e.) were obtained after the listed duration of exposure to the superfusate (NS = normal superfusate). †Time to inter-atrial block is the mean duration of exposure to the superfusate required to produce total failure of conduction of action potentials from the right to the left atrium.

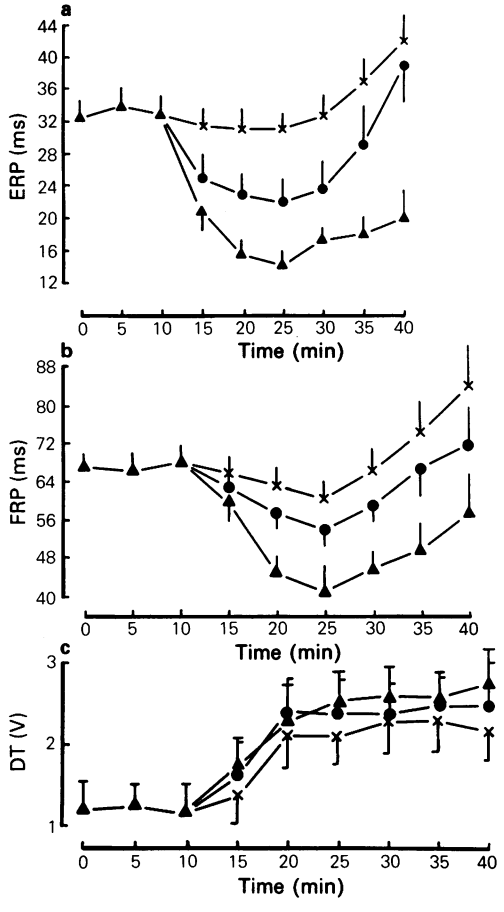

Figure 1 Effects of sulphinpyrazone and of indomethacin on SI-induced changes in the effective refractory period (ERP, a), functional refractory period (FRP, b) and diastolic threshold (DT, c). Tissues exposed to normal superfusate (NS) between 0 and 10 min, and to conditions of simulated ischaemia (SI) between 10 and 40 min. Each point represents the mean of between 8 and 30 observations. The superfusate contained no drug (▲), sulphinpyrazone 10 $\mu g\,ml^{-1}$ (×) or indomethacin 10 $\mu g\,ml^{-1}$ (●). Vertical lines represent s.e.

pattern were seen when the KCl concentration or the pH were kept at values chosen for the NS but the superfusate was made up to contain all the other components of SI. It is concluded, therefore, that the high concentration of KCl and the low pH of SI do not contribute greatly to the electrical disturbances produced by SI. Although restoring the glucose concentration during SI to the value prevailing during NS caused less electrical disturbance than was produced by the full simulation (Figure 2), this concentration of glucose was not fully protective. Nevertheless, at twice the concentration present in NS, glucose fully protected against the SI-induced shortening of ERP (Figure 2).

Omitting magnesium from the superfusate for 30 min during either SI or exposure to otherwise NS, produced slight and statistically insignificant changes in DT, APD, ROC and refractory periods. In contrast, lowering the calcium concentration from 2 mM to 0.5 mM, while having insignificant effects on the rise in DT caused by SI, greatly attenuated the associated shortening of APD and refractory periods (Figure 3), and completely prevented the block of action potential conduction between right and left atrium produced by exposure to SI. Reducing the calcium concentration of NS from 2 mM to 0.5 mM slightly but insignificantly reduced the DT but prolonged APD and refractory periods to an appreciable extent (Figure 3).

Effects of sulphinpyrazone, indomethacin and verapamil

At concentrations of between 1 and 20 $\mu g\,ml^{-1}$ sulphinpyrazone had only insignificant effects on the measured electrical parameters during superfusion with NS. In the same concentration range, however, the drug attenuated the slowing of the ROC and the shortening of both APD (Table 1) and refractory periods caused by exposure to SI (Figures 1 and 4), although the rise in DT was unaltered (Figure 1). The protective effects of sulphinpyrazone were concentration related. The lowest effective concentration tested was 1 $\mu g\,ml^{-1}$ with maximal protection at 10 $\mu g\,ml^{-1}$. The protection afforded by a concentration of 20 $\mu g\,ml^{-1}$ was no greater than that given by 10 $\mu g\,ml^{-1}$ (Figure 4 and Table 1).

Indomethacin was less protective than sulphinpyrazone. The lowest effective concentration of indomethacin tested was 10 $\mu g\,ml^{-1}$, with no greater protection afforded by 20 $\mu g\,ml^{-1}$ (Figure 4 and Table 1), based upon SI-induced shortening of ERP. Hypoxia-induced shortening of ERP was only marginally attenuated by indomethacin at 10 $\mu g\,ml^{-1}$ (Figure 5), whereas sulphinpyrazone at the same concentration gave significant protection (Figure 5).

Verapamil (0.5 $\mu g\,ml^{-1}$) protected against the shortening of both APD and refractory periods caused by exposure to SI and inhibited the associated slowing of the ROC (Table 1). In the presence of this concentration of verapamil exposure to SI for 30 min never produced failure of action potential conduction between the two atria (Table 1). Verapamil at this concentration in NS had no effect upon MRD, ERP, ROC or APD and only slightly and insignificantly prolonged the FRP. However, at a concentration of 5 $\mu g\,ml^{-1}$, verapamil in NS significantly slowed the ROC of action potentials and prolonged both the

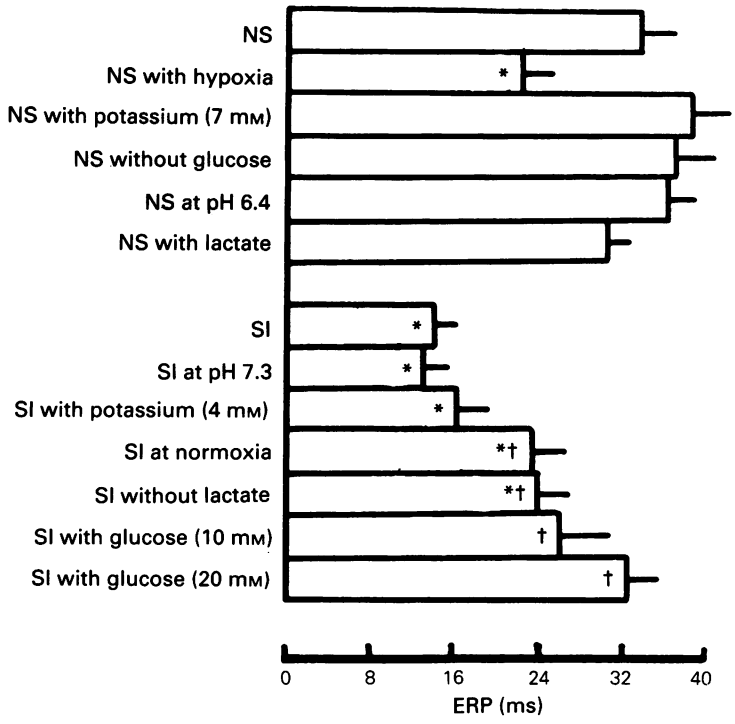

Figure 2 Effect on the effective refractory period (ERP) of exposure for 15 min to conditions of simulated ischaemia (SI) or to its various components. There were between 5 and 11 observations in each treatment group. The horizontal bars at the ends of the columns represent s.e. *Indicates a significant difference ($P < 0.05$) from the normal superfusate (NS) group. †Indicates a significant difference ($P < 0.05$) from the SI group.

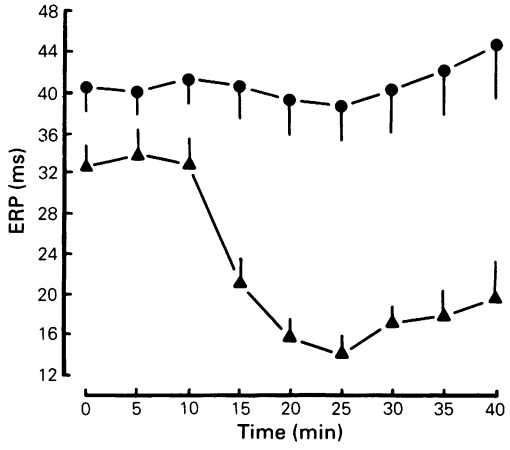

Figure 3 Effect of varying the extracellular calcium concentration on simulated ischaemia (SI)-induced changes in the effective refractory period (ERP). Tissues were exposed to normal superfusate (NS) between 0 and 10 min, and to SI between 10 and 40 min. Each point represents the mean of between 12 and 30 observations. The superfusates contained $CaCl_2$ 2 mM (▲) or 0.5 mM (●). Vertical lines represent s.e.

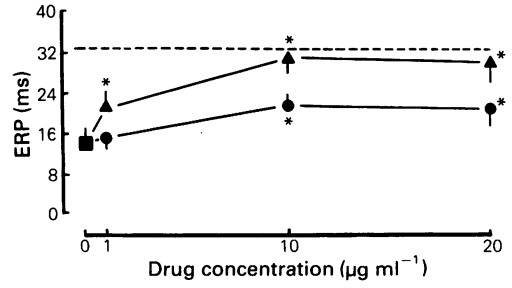

Figure 4 Effect of varying concentrations of sulphinpyrazone or indomethacin on simulated ischaemia (SI)-induced changes in the effective refractory period (ERP). Tissues were exposed to SI for 15 min. Superfusates contained no drug (■), sulphinpyrazone (▲), or indomethacin (●). *Value significantly different ($P < 0.05$) from that obtained in the absence of drugs. Vertical lines represent s.e. The horizontal broken line is the ERP value of tissues in normal superfusate (NS) without drugs.

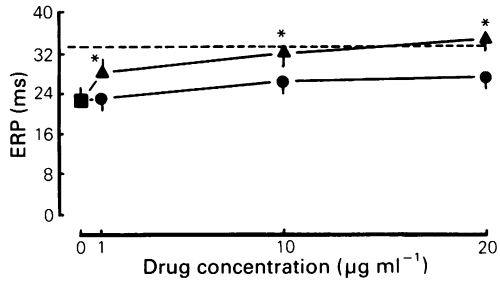

Figure 5 Effect of varying concentrations of sulphinpyrazone or indomethacin on hypoxia-induced changes in the effective refractory period (ERP). Tissues exposed to hypoxia in normal superfusate (NS) for 15 min. Superfusates contained no drug (■), sulphinpyrazone (▲) or indomethacin (●). *Value significantly different ($P < 0.05$) from that obtained in the absence of drugs. Vertical lines represent s.e. Each point represents the mean of between 5 and 12 observations. The horizontal broken line is the ERP value of tissues in NS without drugs.

APD and the refractory periods, the FRP more than the ERP.

Discussion

The present experiments have revealed that under conditions of SI *in vitro*, produced by a combination of abnormally raised concentrations of hydrogen, potassium and lactate ions and a deficiency of oxygen and glucose, rat atrial myocardium displays a temporary shortening of refractory periods, a progressive slowing of the ROC of action potentials and a persistent shortening of the APD. These changes are similar to those described in various other species and in several other parts of the myocardium (see Introduction).

Hypoxia was the only component of SI which separately caused electrical effects that resembled those produced by the full SI. Clearly, however, other components of SI contributed to the observed electrical effects of this type of injury since hypoxia alone produced less marked effects than those produced by full SI. Many previous investigators have observed a shortening of APD and refractory periods *in vitro* during hypoxia. This was first noted in the rat atrium by Webb & Hollander (1956) and in human papillary muscle by Prasad & Callaghan (1969), the subject having been reviewed by Morena *et al.* (1980) and more recently by Conrad *et al.* (1983).

Lack of glucose, by itself, has been demonstrated to have rather variable electrical effects upon the myocardium. Macloed & Prasad (1969) found shortening of APD and refractory periods in guinea-pig ventricular myocardium, whereas tissues from rabbit and dog hearts were unaffected (Senges *et al.*, 1980). From the present results and those of Webb & Hollander (1956), rat atrium seems to resemble the myocardium of the rabbit and dog more than that of the guinea-pig. The guinea-pig heart is exceptional in other respects, because Kodama *et al.* (1984) found that although SI produced the expected shortening of APD in this species, the associated refractory periods were actually lengthened. Despite the latter results, most previous workers have found that the shortening of APD and refractory periods caused by hypoxia was potentiated by a lack of glucose and overcome by elevating glucose concentrations, particularly elevation to supraphysiological levels (Macloed & Prasad, 1969; Prasad & Callaghan, 1969; Morena *et al.*, 1980; Ballantyne & Davis, 1983; Conrad *et al.*, 1983; Cobb *et al.*, 1985). The protective effect of glucose against the shortening of refractory periods caused by SI in the present experiments, therefore, confirms earlier findings.

Maximal shortening of atrial refractory periods in response to SI in the present experiments occurred after 12 min of exposure to injury. This corresponds closely with the time of the greatest shortening of refractory periods and the time of the appearance of the most severe ventricular tachyarrhythmias in the rat heart *in vivo* after coronary artery occlusion (Northover, 1986). The similar timing of the two sets of electrical events suggests that a similar mechanism operates *in vivo* as *in vitro*.

Several previous workers have shown that in dogs, rabbits and guinea-pigs, ischaemia *in vivo* or SI *in vitro* causes a slowing of the ROC of myocardial action potentials which is progressive with time. The present findings indicate that a similar phenomenon occurs in rats. A combination of shortened refractory periods and a slowed ROC of action potentials occurring in a restricted region of the cardiac wall, is powerfully conducive to re-entry arrhythmias (Northover, 1986).

Sulphinpyrazone in the present experiments protected against the shortening of APD and refractory periods and against the slowing of the ROC of action potentials during exposure to SI. This confirms the preliminary results of Iansmith *et al.* (1979) who studied, *in vitro*, the myocardial response to an abnormally low extracellular pH. The protective effect of sulphinpyrazone in the present experiments was graded at concentrations of 1 and 10 $\mu g\,ml^{-1}$. Concentrations of the drug between 0.1 and 1 $\mu g\,ml^{-1}$ have been found by Karmazyn (1984) to protect rat hearts *in vitro* against some of the biochemical consequences of ischaemia. Peak blood plasma concentrations after a 200 mg oral dose to man were shown to reach 13–16 $\mu g\,ml^{-1}$ (Mahony *et al.*, 1983; Pedersen & Fitzgerald, 1985), and doses of 400 mg by mouth gave peak plasma concentrations in excess of 20 $\mu g\,ml^{-1}$

(Rosenkranz *et al.*, 1983). Clearly, therefore, under conditions of ordinary therapeutic use, even allowing for binding of the drug to plasma proteins, an opportunity exists for the drug to exert electrical effects of an anti-arrhythmic type. The same cannot be said for indomethacin, as peak plasma concentrations after a 50 mg oral dose of the drug to man reached only $5\,\mu g\,ml^{-1}$ (Rothermich, 1966), yet the drug was much less effective than sulphinpyrazone in the present experiments in preventing the electrical consequences of SI, with a minimum effective concentration of $10\,\mu g\,ml^{-1}$. Moreover, indomethacin was much less effective than sulphinpyrazone *in vivo* in preventing episodes of ventricular tachyarrhythmia after coronary artery occlusion in the rat and the associated shortening of myocardial refractory periods (Northover, 1986).

Verapamil in the present experiments inhibited both the SI-induced slowing of the ROC of action potentials and the SI-induced shortening of both the APD and the refractory periods. This confirms earlier findings, albeit in other species (Nakaya *et al.*, 1981; 1982; Kimura *et al.*, 1982; 1983; Gettes *et al.*, 1985). Since verapamil blocks the entry of calcium ions via the sarcolemmal slow-channels, this raises the question of the extent to which alterations in myocardial calcium metabolism are responsible for the electrical changes observed during exposure to SI. The shortening of myocardial APD and refractory periods and the slowing of the ROC of action potentials produced by SI in the present experiments were shown to depend upon the concentration of calcium in the superfusate. Previous workers have suggested that calcium ions are involved in the shortening of myocardial APD and refractory periods during both hypoxia and SI (Wojtczak, 1979; Conrad *et al.*, 1983; Kimura *et al.*, 1983; Fitzpatrick & Karmazyn, 1984). Raising the concentration of calcium in the perfusate of isolated hearts from the rat also aggravated the ventricular tachyarrhythmias produced by coronary artery ligation (Daugherty & Woodward, 1981). The mechanisms whereby calcium ions promote shortening of APD and refractory periods, however, are still uncertain. Vleugels *et al.* (1980), using feline voltage-clamped ventricular myocardium, showed that during hypoxia the concentration of calcium ions in the cytoplasm rose. The greater the availability of intracellular calcium ions the greater was the conductance of the sarcolemma to potassium ions. The greater the conductance of the plasma membrane to potassium the earlier the repolarization occurs during an action potential and the shorter the APD. Conrad *et al.* (1983) found evidence that this mechanism operates in the rat heart during hypoxia. At the same time, but independently of effects on potassium ion conductance and APD, a raised concentration of cytoplasmic calcium shortens the time constant for re-activation of the sodium ion channels in the sarcolemma, and thus shortens refractory periods. So far, the latter process has not been studied in rat hearts, but it is well established in the hearts of several other species (Gettes & Reuter, 1974).

The slowing of the ROC of myocardial action potentials during ischaemia *in vivo* and during SI *in vitro* has been attributed, by many previous workers, to alterations in cellular calcium metabolism (Wojtczak, 1979; Nakaya *et al.*, 1980; 1981; 1982; Kimura *et al.*, 1982; 1983; Gettes *et al.*, 1985). It is well established that conduction of action potentials through the myocardium depends upon a selective flow of ionic current through specialised regions of low electrical resistance between adjacent cells (DeMello, 1982). These specialized regions of low electrical resistance are disrupted, lost or occluded when the cytoplasmic calcium concentration rises (DeMello, 1975), as it does during ischaemia, thus slowing the ROC of the action potential. Several groups of workers have demonstrated that slow channel calcium entry blockers of various chemical types (Nakaya *et al.*, 1980; 1981; 1982; Kimura *et al.*, 1982; 1983; Gettes *et al.*, 1985), as well as certain intracellular calcium antagonists (Anno *et al.*, 1986), inhibit the slowing of the ROC of action potentials during exposure of the myocardium to ischaemia or SI. The ability of verapamil in the present experiments, therefore, to inhibit SI-induced slowing of the ROC extends to the rat the findings in other species.

In conclusion, the rat atrium exposed *in vitro* to SI offers an opportunity to study the mechanism of action of anti-arrhythmic drugs. The extent to which these drugs exert their protective effects by altering cellular calcium ion metabolism warrants further study.

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Triazolodiazepines: dissociation of their Paf (platelet activating factor) antagonistic and CNS activity

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1 The relationship between the activity of thieno- or benzo-triazolodiazepines on platelet-activating factor (Paf)-induced effects and on the CNS (central nervous system) was studied *in vitro* and *in vivo*.

2 Brotizolam and triazolam inhibited Paf-induced human platelet aggregation. The IC_{50} - values were 0.54 and 7.6 μM, respectively. This inhibitory effect was not blocked by the specific central-type benzodiazepine (BDZ) antagonist, Ro 15–1788, or the specific peripheral-type BDZ ligand, Ro 5–4846. These BDZ ligands also showed an inhibitory effect on Paf-induced platelet aggregation (IC_{50} = 200 and 560 μM, respectively). Ro 15–1788 or Ro 5–4846 in combination with brotizolam or triazolam enhanced the Paf inhibitory effect of these triazolodiazepines.

3 In guinea-pigs, Ro 15–1788, 100 mg kg^{-1} p.o. and 10 mg kg^{-1} i.v. completely inhibited the hypnogenic effect of 10 mg kg^{-1} p.o. and 1 mg kg^{-1} i.v. of brotizolam, respectively. Similar results were obtained with triazolam but at higher doses.

4 In anaesthetized guinea-pigs, a dose of 100 mg kg^{-1} p.o. of Ro 15–1788 did not inhibit bronchoconstriction and hypotension caused by Paf (30 ng kg^{-1} min^{-1} i.v.). The combination of brotizolam (10 mg kg^{-1} p.o.) or triazolam (200 mg kg^{-1} p.o.) with this BDZ antagonist (100 and 400 mg kg^{-1} p.o., respectively) did not affect the Paf inhibitory activity of these triazolodiazepines.

5 These results show that the Paf antagonistic properties of the triazolodiazepines can be dissociated from their CNS activity. It is conceivable that compounds of this structural type could be the forerunners of a novel series of potent Paf antagonists.

Introduction

Platelet activating factor (Paf; PAF-acether; 1-O-alkyl-2-acetyl-sn-glycero-phosphorylcholine) is a phospholipid mediator generated in inflammatory and allergic responses (Vargaftig *et al.*, 1981a). *In vitro*, Paf induces aggregation of human platelets or neutrophil leukocytes (Vargaftig *et al.*, 1981b; Camussi *et al.*, 1981). *In vivo*, when injected intravenously into guinea-pigs this substance provokes thrombocytopenia, leukopenia, systemic hypotension and respiratory alterations characterized by bronchoconstriction and oedema of the lungs (Vargaftig *et al.*, 1980).

Benzodiazepines (BDZ) are polycyclic compounds which have been proved clinically effective as anxiolytics, anti-convulsants, hypnotics or sedatives and muscle relaxants (Haefely *et al.*, 1981). These pharmacological activities are mediated by BDZ receptors in the central nervous system ('central type' binding sites) and can be antagonized by the specific BDZ antagonist Ro 15–1788 (Hunkeler *et al.*, 1981). Binding sites for benzodiazepines have also been described in a number of peripheral tissues e.g. lung, kidney, liver, heart (Gehlert *et al.*, 1985) and brain (Marangos *et al.*, 1982) and proinflammatory cells such as human and rat platelets (Moingeon *et al.*, 1984; Wang *et al.*, 1980), macrophages (Zavala *et al.*, 1985), lymphocytes (Moingeon *et al.*, 1983) and mast cells (Taniguchi *et al.*, 1980). The physiological significance of the peripheral-type binding site for BDZ is not yet known.

In a recent study, specific inhibition of Paf-induced activation of human platelets by triazolobenzodiazepines was demonstrated (Kornecki *et al.*, 1984). Alprazolam and triazolam inhibited Paf-induced changes in shape, aggregation, and secretion by platelets. In our laboratory, the inhibitory properties of a triazolothieno-diazepine, brotizolam, on Paf-induced effects *in vitro* and *in vivo* has also been shown (Casals-Stenzel, 1986). Because of the structural dif-

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ference between benzo- and thieno-diazepines, we use the expression 'triazolodiazepines' to cover both types of compounds.

The purpose of this study was to investigate the correlation between the activity of BDZ in general, and triazolodiazepines in particular, in the CNS and on Paf-induced effects. Using the central and peripheral-type BDZ receptor ligands, Ro 15–1788 (an antagonist) and Ro 5–4884, respectively, we have demonstrated that there is no correlation between the activity of these drugs in the two systems.

Methods

In vitro *platelet aggregation*

Human venous blood was collected and transferred into a tube containing 3.8% w/v trisodium citrate solution, and centrifuged at 150 *g* for 20 min at room temperature. The upper layer of platelet rich plasma (PRP) was collected and stored at room temperature in a plastic container for no longer than 2 h before use.

Platelet aggregation was determined according to a modification of the method of Born & Cross (1963). Aliquots, 0.8 ml of PRP were diluted with 0.2 ml modified Tyrode solution (composition, mM:NaCl 137, KCl 2.7, $MgCl_2$ 0.5, $CaCl_2$ 1.8, NaH_2PO_4 0.4, $NaHCO_3$ 11.9, glucose 5.5). The diluted PRP was transferred to 1 ml plastic cuvettes and stirred continuously (1000 r.p.m.) at 37°C. Aggregation was induced by addition of either Paf, adenosine 5'-pyrophosphate (ADP), adrenaline, 5-hydroxytryptamine (5-HT), arachidonic acid (AA) or collagen in a volume of 10 μl to achieve final concentrations of 0.05, 1, 10, 100, 500 μM and 0.1 μg ml^{-1}, respectively.

The reaction was followed by using a LAbor-Aggregometer. The inhibitory effect of the substances was tested by adding them (dissolved in a DMSO-water mixture) in a volume of 10 μl 3 min before the aggregating agent.

IC_{50} values were calculated by linear regression analysis of log concentration versus % inhibition values. For statistical evaluation, one way analysis of variance and paired comparison of mean values by the Dunnett test was employed.

In vivo *experiments*

Evaluation of the hypnotic effect in guinea-pigs The hypnotic effect of brotizolam and triazolam was evaluated by observation of orally pretreated freely-moving animals. The guinea-pigs (Pirbright white, 300–400 g body weight) were observed for 0.5 h before, and 2 h after drug administration. Several behavioural parameters including sedation, escape reactions, and onset of 'sleep' (closed eyes) were monitored and recorded.

Evaluation of respiratory function and arterial pressure Pirbright white guinea-pigs (400 to 500 g) were anaesthetized with urethane (0.6–1.8 g kg^{-1} i.p.) and

Table 1 Inhibition of human platelet aggregation by triazolodiazepines, benzodiazepines (BDZ) and BDZ receptor ligands *in vitro*

	Aggregating agent					
	Paf 0.05 μM	*ADP* 1 μM	*Adrenaline* 10 μM	*5-HT*†	*Collagen* 10 μM	*AA* 500 μM
Brotizolam	0.54 (0.45–0.71)	> 100	> 1000	320 (*)	> 1000	290 (*)
Triazolam	7.6 (4.9–12.3)	> 1000	> 1000	> 1000	> 1000	> 1000
Alprazolam	13.7 (8.8–20.8)	> 100	> 1000	280 (*)	450 (*)	250
Flunitrazepam	42.0 (32.4–59.4)	77 46.2–201.4	64 (*)	9.9 (2.5–17.5)	16 (2.1–31.5)	15 (*)
Diazepam	260.0 (*)	> 1000	> 1000	300 (*)	300 (*)	26 (15.4–37.2)
Ro 15–1788	183.0 (59.3–312.8)	> 1000	> 1000	470 (*)	> 1000	> 1000
Ro 5–4846	560.0 (*)	> 1000	> 1000	> 1000	> 1000	25 (18.1–35.4)
PK 11195	260.0 (*)	> 1000	> 1000	> 1000	> 1000	26 (17.8–37.3)

Data presented are IC_{50} values (μM) and 95% confidence limits (in parentheses); $n = 4$ + 0.1 μg ml^{-1}; *cannot be estimated; †5-HT = 5-hydroxytryptamine; AA = arachidonic acid.

prepared for recording of pulmonary function (intratracheal cannula) and mean arterial pressure (MAP; catheter in the left carotid artery). Tidal volume (TV) was measured with a Buxco pulmonary mechanics analyzer in spontaneously breathing animals and used as an index of pulmonary function. MAP was measured continuously through the implanted arterial catheter connected to a Statham PD 23 pressure transducer.

Brotizolam and triazolam (in a volume of 0.5 ml kg^{-1}) were administered orally 30 to 60 min, or intravenously (into the right jugular vein) 10 min before an i.v. infusion of Paf (30 ng kg^{-1} min^{-1}). The amount of urethane was varied depending on the oral pretreatment with brotizolam or triazolam.

For statistical analysis of the *in vivo* experiments, one way analysis of variance and paired comparison of mean values by the Newman-Keuls method was used at the first, second, third and fourth time of registration.

Drugs and materials

The following drugs and chemicals were used: brotizolam, Ro 5–4864 (7 - chloro - 5 - (4 - chlorophenyl) - 1,3 - dihydro - 1 - methyl - 2H - 1,4 - benzodiazepine - 2 - one) and PK 11195 (1 - (2 - chlorophenyl) - N - (1 - methylpropyl) - 3 - isoquinoline - carboxamide) from Boehringer Ingelheim, triazolam from Upjohn, Ro 15–1788 (ethyl 8 - fluoro - 5 - methyl - 5,6 - dihydro - 6 - oxo - 4H - imidazo [1,5 - a] [1,4] benzodiazepine - 3 - carboxylate) from Boehrigner Ingelheim or from Hoffmann-La Roche, Basle. Fresh solutions of these compounds were prepared daily for the *in vivo* experiments in a mixture of 1, 2-propylene-glycol and saline (1:1 v/v). Dilutions were made with saline.

Other compounds used included: Paf (Bachem Feinchemikalien AG, Switzerland), collagen (Hormon Chemie, Munich), (−)-adrenaline and 5-HT (Fluka), ADP (Serva), arachidonic acid and bovine serum albumin (BSA) (Sigma).

Stock solutions of Paf were prepared in absolute ethanol and subsequent dilutions were made with Tyrode solution containing 0.25% BSA.

Results

In vitro *platelet aggregation*

The triazolodiazepines and flunitrazepam inhibited the Paf-induced platelet aggregation in a concentration-dependent manner, but in different concentration ranges. Brotizolam, triazolam and alprazolam showed no ($IC_{50} > 1000\ \mu M$) or little effect ($IC_{50} > 100\ \mu M$) on platelet aggregation induced by other aggregating agents and therefore a specific action against Paf. Flunitrazepam showed a comparable inhibitory effect on the aggregation induced by Paf and the other agents (Table 1).

Ro 15–1788, Ro 5–4846 and PK 11195, all benzodiazepine (BDZ) receptor ligands, inhibited the Paf-induced aggregation only slightly (Table 1). Ro 15–1788 and PK 11195 (a peripheral-type BDZ receptor ligand) showed a certain specificity towards Paf, while Ro 5–4864 showed clear antiaggregatory activity against the arachidonic acid-induced aggregation only. A combination of either brotizolam or triazolam with Ro 15–1788 not only did not inhibit, but actually increased the Paf antagonistic activity of both triazolodiazepines (Table 2).

A similar result was obtained with the combination of brotizolam and Ro 5–4864. The Paf antagonistic effect of brotizolam was slightly increased by Ro–4864 (Table 3).

In vivo *experiments*

In two series of experiments *in vivo*, brotizolam and triazolam, and a combination of either compound

Table 2 Effect of brotizolam and triazolam in combination with the benzodiazepine receptor antagonist Ro 15–1788 on Paf-induced human platelet aggregation *in vitro*

	Ro 15–1788 (μM)	n	*% inhibition of aggregation*
Brotizolam (1 μM) +	0	8	65.3 ± 7.9
	10	8	74.9 ± 5.7*
	100	8	74.9 ± 4.3*
	1000	8	99.7 ± 0.3*
Triazolam (20 μM) +	0	6	72.4 ± 2.8
	10	6	72.6 ± 2.3
	100	6	68.1 ± 3.3
	1000	6	97.3 ± 1.7*

Data shown are mean values ± s.e.mean. * Significant difference from brotizolam or triazolam alone, $P < 0.05$; $n = 4$.

Table 3 Effect of brotizolam in combination with the peripheral-type benzodiazepine ligand Ro 5-4864 on Paf-induced human platelet aggregation *in vitro*

	Ro 5-4864 (μM)	n	*% inhibition of aggregation*
Brotizolam (1 μM) +	0	4	48.5 ± 3.3
	10	4	52.2 ± 9.2
	100	4	61.7 ± 5.0
	1000	4	86.9 ± 2.8*

Data shown are mean values ± s.e.mean. * Significant difference from brotizolam alone, $P < 0.05$; $n = 4$.

with Ro 15-1788 were compared in conscious, as well as in Paf-treated, anaesthetized guinea-pigs.

The first series of experiments in conscious animals examined the antagonistic activity of Ro 15-1788 against brotizolam or triazolam in relation to their sedative and hypnotic activity, i.e. the BDZ like activity. Brotizolam and triazolam were combined with Ro 15-1788 and administered orally. Animals treated only with brotizolam (10 mg kg^{-1} p.o.) or triazolam (200 mg kg^{-1} p.o.) appeared sedated and slept within the first 10 to 15 min after administration. But no hypnotic effect appeared during the observation period (120 min) in the guinea-pigs which received brotizolam (10 mg kg^{-1} p.o.) combined with Ro 15-1788 (100 mg kg^{-1} p.o.). When agonist and antagonist were combined in a ratio of 1:2 as was done with triazolam and with one group of brotizolam, sedation and sleep was generally inhibited during the first 45 min after administration (Table 4).

In the second series of experiments, anaesthetized guinea-pigs were pretreated with brotizolam (p.o. or i.v.), triazolam (p.o. only) or a combination of either substance with Ro 15-1788. Thirty to 60 min after oral administration, depending on the dose of Ro 15-1788, or 10 min after i.v. injection they received a 30 min i.v. infusion of Paf (30 ng kg^{-1} min^{-1}), a dose lethal to control animals within 15-20 min.

The doses of brotizolam (10 mg kg^{-1} p.o. and 0.5 mg kg^{-1} i.v.) and triazolam (200 mg kg^{-1} p.o.) used were the maximum doses which protected the animals from the action of the i.v. infusion of Paf described. A high oral dose of Ro 15-1788 (100 mg kg^{-1} p.o.) neither prevented death (Table 5) nor the decrease in tidal volume and mean blood pressure (Figures 1, 2 and 3) induced by the Paf infusion. Similarly, a high intravenous dose (10 mg kg^{-1}) of Ro 15-1788 did not prevent death but in contrast it blunted the decrease in tidal volume and significantly reduced the Paf action on blood pressure. On average the animals died 20 min after the beginning of the Paf infusion.

The oral combination of brotizolam or triazolam with Ro 15-1788 did not affect their Paf antagonistic activity at all. When brotizolam was injected intraven-

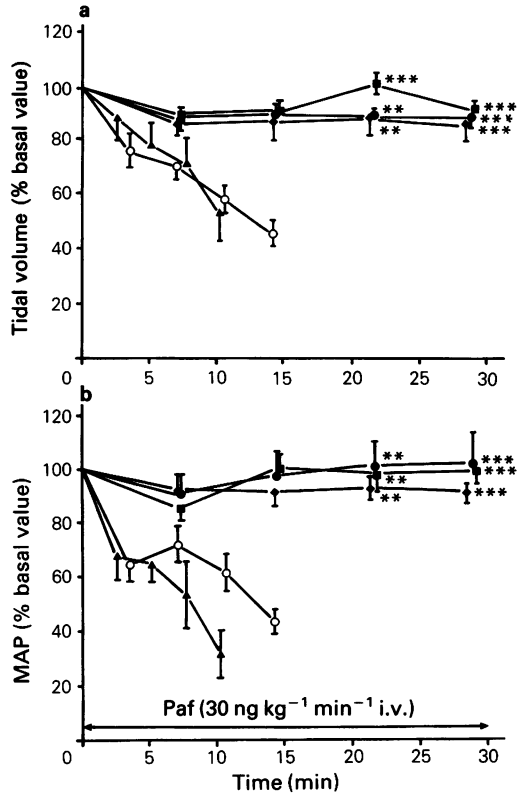

Figure 1 Inhibition of Paf-induced (a) bronchoconstriction and (b) hypotension in guinea-pigs by a combination of brotizolam and the benzodiazepine receptor antagonist Ro 15-1788. Both compounds were administered orally 60 min before an i.v. infusion of Paf (30 ng kg^{-1} min^{-1}). MAP: mean arterial pressure. (○) Control ($n = 7$); (●) brotizolam 10 mg kg^{-1} ($n = 8$); (▲) Ro 15-1788 100 mg kg^{-1} ($n = 6$); (■) brotizolam 10 mg kg^{-1} + Ro 15-1788 100 mg kg^{-1} ($n = 6$); (◆) brotizolam 10 mg kg^{-1} + Ro 15-1788 20 mg kg^{-1} ($n = 6$). Each point represents the mean and vertical lines show s.e.mean. $^{**}P < 0.01$, $^{***}P < 0.001$; significantly different from control.

Table 4 Effect of brotizolam or triazolam in combination with the benzodiazepine receptor antagonist Ro 15–1788 on behavioural changes in guinea-pigs

	Time after oral administration (min)	*Sedation* (%)	*Sleep* (%)
Control	—	No changes during the 2 h observation period	
Brotizolam (10 mg kg^{-1})	10	0	0
	15		0
	30		
	45	100	100
	60		
	90		50
	120		
Brotizolam (10 mg kg^{-1}) + Ro 15–1788 (20 mg kg^{-1})	10		
	15	No sedation	
	30		No sleep
	45	16.5	
	60	33	
	90	100	50
	120	100	50
Brotizolam (10 mg kg^{-1} + Ro 15–1788 (100 mg kg^{-1})	10		
	15		
	30	No sedation	
	45		No sleep
	60		
	90	16.5	
	120	50	
Triazolam (200 mg kg^{-1})	10		0
	15		0
	30		50
	45	100	
	60		100
	90		
	120		
Triazolam (200 mg kg^{-1}) + Ro 15–1788 (400 mg kg^{-1})	10		
	15	No sedation	No sleep
	30		
	45		
	60	50	16.5
	90	100	50
	120	100	50

The observaton period lasted 0.5 h before (not shown) and 2 h after oral administration of the drugs. The results are expressed as % of animals showing sedation and/or sleep in groups of 6 guinea-pigs.

ously, the maximum dose tolerated by the urethane anaesthetized guinea-pigs was 0.5 mg kg^{-1}, but the i.v. administration of 1 mg kg^{-1} brotizolam in combination with 2 mg kg^{-1} Ro 15–1788 was always tolerated by all the animals and also protected them totally from the effect of the i.v. Paf infusion (Table 5, and Figures 1 and 2). After the i.v. combination of brotizolam and Ro 15–1788, tidal volume and MAP were even increased in comparison with the basal value, showing an additive effect.

Table 5 Protective effect of brotizolam and triazolam in combination with the benzodiazepine receptor antagonist Ro 15–1788 against a lethal dose of Paf (30 ng kg^{-1} min^{-1} i.v. for 30 min) in guinea-pigs

	Dose (mg kg^{-1})	*Route of administration*	*Protective effect* (%)
Ro 15–1788	100	p.o.	0
	10	i.v.	0
Brotizolam	10	p.o.	100
	1	i.v.	100
Brotizolam + Ro 15–1788	10 + 20	p.o.	100
	100		100
Brotizolam + Ro 15–1788	1 + 2	i.v.	100
Triazolam	200	p.o.	100
Triazolam + Ro 15–1788	200 + 400	p.o.	100

The protective activity against Paf is expressed as % survival in groups of 6 animals.

Discussion

The results of this study show that the Paf antagonistic activity of triazolodiazepines is independent of their sedative and hypnotic effect *in vivo*. The antagonism of the effects of Paf induced by brotizolam and triazolam is not inhibited by a specific antagonist of central-type BDZ receptors *in vitro* or *in vivo*.

A great difference in Paf antagonistic activity exists between BDZ with and without a triazole ring. The normal benzodiazepines generally show a weak and often non-specific antagonism of the effects of Paf, while the triazolodiazepines brotizolam, triazolam and alprazolam are potent and specific Paf antagonists *in vitro*. A similar finding has been recently described by Kornecki *et al.* (1984).

The possibility that the Paf antagonistic effects of thieno- or benzo-triazolodiazepines are mediated by a central- or peripheral-type BDZ receptor, was examined using the specific BDZ ligands Ro 15–1788 (central antagonist) and Ro 5–4864 (peripheral ligand). *In vitro* Ro 15–1788 also inhibits the platelet aggregatory activity of Paf in relatively high concentrations. When combined with brotizolam or

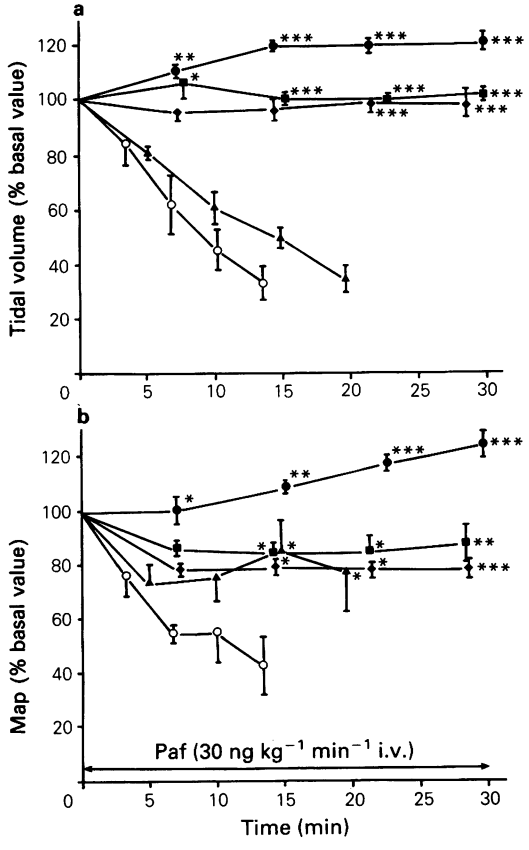

Figure 2 Inhibition of Paf-induced (a) bronchoconstriction and (b) hypotension in guinea-pigs by a combination of brotizolam and the benzodiazepine receptor antagonist Ro 15–1788. Both compounds were administered intravenously 10 min before an i.v. infusion of Paf (30 ng kg^{-1} mg^{-1}). MAP: mean arterial pressure. (○) Control ($n = 7$); (●) brotizolam 1 mg kg^{-1} + Ro 15–1788 2 mg kg^{-1} (a) or 10 mg kg^{-1} (b) ($n = 6$); (■) brotizolam 1 mg kg^{-1} + Ro 15–1788 10 mg kg^{-1} (a) or 2 mg kg^{-1} (b) ($n = 6$); (◆) brotizolam 0.5 mg kg^{-1} ($n = 6$); (▲) Ro 15–1788 10 mg kg^{-1} ($n = 8$). Each point represents the mean and vertical lines show s.e.mean. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$; significantly different from control.

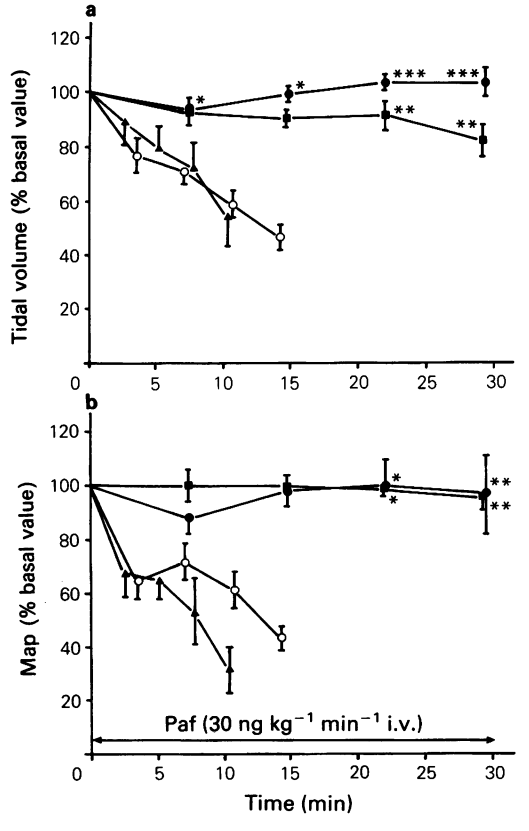

Figure 3 Inhibition of Paf-induced (a) bronchoconstriction and (b) hypotension in guinea-pigs by a combination of triazolam and the benzodiazepine receptor antagonist Ro 15–1788. Both compounds were administered orally 45 min before an i.v. infusion of Paf (30 ng kg^{-1} min^{-1}). MAP: mean arterial pressure. (○) Control ($n = 7$); (▲) Ro 15–1788 100 mg kg^{-1} ($n = 6$); (■) triazolam 200 mg kg^{-1} ($n = 6$); (●) triazolam 200 mg kg^{-1} + Ro 15–1788 400 mg kg^{-1} ($n = 5$). Each point represents the mean and vertical lines show s.e.mean. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$; significantly different from control.

triazolam the Paf antagonistic activity of either triazolobenzodiazepines is not only not inhibited but is actually increased.

These findings were confirmed by *in vivo* experiments in guinea-pigs. On the one hand, the Paf antagonistic activity of the triazolodiazepines brotizolam (p.o. or i.v.) and triazolam (p.o.) in combination with Ro 15–1788 in a ratio of 1:2 or 1:10 (agonist:antagonist) was not affected but actually (numerically) enhanced. On the other hand, the BDZ antagonist Ro 15–1788 clearly blocked the motor depressant and hypnotic effect of brotizolam and triazolam in guinea-pigs. The ratio of triazolodiazepine:BDZ antagonist necessary for complete inhibition over 2 h was 1:10.

Ro 15–1788 itself given at high doses (100 mg kg^{-1} p.o. or 10 mg kg^{-1} i.v.) did not prevent the lethal effect of Paf. This result was expected because of the *in*

vitro studies. In comparison with brotizolam or triazolam, the concentrations of Ro 15–1788 needed to achieve a Paf antagonistic effect *in vitro* were approximately 100 to 1000 times higher. Extrapolating these results to an *in vivo* situation, it is obvious that a very high dose would be required to obtain similar antagonism of the effects of Paf with this BDZ antagonist. Nevertheless, when Ro 15–1788 was given intravenously its weak Paf antagonistic activity became quite clear. Moreover, in combination with brotizolam an additive effect was observed. This phenomenon could be due to inhibition of endogenous Paf by brotizolam and Ro 15–1788.

Ro 15–1788, 10 mg kg^{-1} i.v., blocked significantly the Paf-induced hypotension, but only slightly influenced changes in tidal volume and did not prevent the death of the animals during the whole 30 min period of the experiment. In comparison, brotizolam 0.5 mg kg^{-1} i.v. was able to inhibit the effects of Paf on the lung and blood pressure and to protect the guinea-pigs from death over a period which exceeded 30 min. The early death of the Ro 15–1788 treated animals was mainly caused by asphyxia, while the controls probably died from asphyxia and hypotension. Such a separation of both Paf effects can also be achieved by low doses of brotizolam (not shown).

Peripheral-type BDZ receptors have been described in human platelets (Moingeon *et al.*, 1984). Therefore, it was also of interest to search for a relationship between these receptors and the activity of Paf on platelets. It was conceivable that peripheral-type BDZ receptors and Paf binding sites are the same or similar for this type of compound. This idea was not confirmed by the *in vitro* studies performed with Ro 5–4864. This peripheral-type BDZ receptor ligand had virtually no effect on Paf-induced platelet aggregation (IC_{50} = 500 μM) and did not counteract the Paf antagonistic activity of brotizolam. Similarly, another peripheral ligand, PK 11195, revealed a very weak Paf antagonistic effect *in vitro* (IC_{50} = 260 μM), and did not inhibit the Paf antagonistic activity of brotizolam (result not shown).

In conclusion, no relationship exists between the Paf antagonistic activity of triazolodiazepines and their activity on the central nervous system, or at their peripheral binding sites. This clear dissociation of antagonism of Paf-induced effects and activity in the CNS opens up the possibility of developing new triazolodiazepine derivatives with potent Paf antagonistic activity but lacking the characteristic CNS side-effects of benzodiazepines.

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Decreased responsiveness of the aortae of hypertensive rats to acetylcholine, histamine and noradrenaline

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1 The responses to noradrenaline (NA) of the aortae of various hypertensive rats, namely the spontaneously hypertensive rat (SHR), the low blood pressure SHR (LBP-SHR), and the left renal artery stenosed LBP-SHR (LRAS-LBR-SHR), were compared to those of the normotensive Wistar-Kyoto rats (WKY).

2 The aortae of the hypertensive rats were significantly more responsive ($P < 0.05$) to 10^{-8} M NA. However, the reverse was true for higher doses of NA. The ED_{50} values for the aortae of WKY, LBP-SHR, SHR and LRAS-LBP-SHR were 20, 8.5, 7.8 and 8 nM respectively.

3 The NA-contracted aortae of the LRAS-LBP-SHR were significantly less responsive ($P < 0.05$) to the relaxant action of histamine and acetylcholine (ACh) compared to those of the WKY. This observation was not made in the aortae of the LBP-SHR. The maximal relaxation (% of the maximal contraction induced by 10^{-8} M NA) observed in the aortae of WKY, LBP-SHR and LRAS-LBP-SHR were, respectively, 72 ± 2, 66 ± 6, 39 ± 7 for ACh and 50 ± 3, 36 ± 4, 27 ± 3 for histamine.

4 In aortae where the endothelium had been removed by collagenase treatment, histamine induced a dose-related contraction. The rank order of this dose-related contraction was WKY > LBP-SHR > SHR > LRAS-LBP-SHR with the corresponding maximal tension (g) 0.89 ± 0.04, 0.59 ± 0.04, 0.36 ± 0.04, 0.19 ± 0.05.

5 The results suggested that elevation of blood pressure above the normal (due either to intrinsic or extrinsic factors), as seen in SHR and LRAS-LBP-SHR, results in a decreased response of the aortae to ACh and histamine. This effect was seen in both the endothelium mediated relaxation and the non-endothelium mediated contraction.

Introduction

The blood vessels of spontaneously hypertensive rats (SHR) have been found to differ from those of normotensive Wistar-Kyoto rats (WKY) in their relaxation responses to acetylcholine (ACh), adenosine and isoprenaline (Triner *et al.*, 1975; Cohen & Berkowitz, 1976; Shibata & Cheng, 1978). In a recent study, we also showed that the noradrenaline (NA)-contracted aortae of renal hypertensive rats and SHR were hyporesponsive to the relaxant action of histamine, but differed significantly in their relaxation response to ACh, i.e. the aortae of the former animals were hyporesponsive whilst the aortae of the latter animals were not (Sim & Chua, 1985). In an attempt to identify which of the endothelium, smooth muscle or genetic factors was a possible cause for this difference, we studied: (1) the relaxant actions of histamine and ACh on NA-contracted aortae of SHR whose blood pressures were below 150 mmHg (LBP-SHR), on the same type of animals whose left renal arteries had been stenosed for a period of 4 weeks (LRAS-LBP-SHR), and on normotensive WKY; (2) the action of NA on the aortae of the same 3 types of animal and SHR whose blood pressures were above 150 mmHg; (3) the action of histamine on the collagenase-treated aortae of the same types of animal as in (2).

Methods

The rats used were 4–6 months old and of the following strains, systolic blood pressure and heart rate: (i) normotensive WKY, 122 ± 9 mmHg, 273 ± 15 beats min^{-1} ($n = 31$); (ii) SHR, 180 ± 20 mmHg, 337 ± 14 beats min^{-1} ($n = 21$); (iii) LBP-SHR, 133 ± 12 mmHg, 287 ± 84 beats min^{-1} ($n = 32$); (iv) LRAS-LBP-SHR, 230 ± 50 mmHg, 328 ± 19 beats min^{-1} ($n = 36$). SHR stock was obtained from Professor Y. Yamori, Shimane Medical University, Japan. The rats were bred in the depart-

ment by sibling mating, of which 10–15% of the offspring had a blood pressure of less than 150 mmHg (LBP-SHR). Systolic blood pressure and heart rate were measured in the conscious rats by tail plethysmography. The operation to stenose the left renal artery was carried out under methoxyhexital sodium anaesthesia (4 mg 100 g^{-1}, i.p.) A flank incision was made on the left side of the animal and a silver clip with a 0.2 mm clearance between the two arms was placed over the renal artery. After the stenosis operation, the blood pressure of the LRAS-LBP-SHR rose rapidly in the first 10 days and reached a maximal value within a month.

Rats were killed by a blow on the head and two thoracic aortic rings of 3 mm per rat were prepared and fixed isometrically in a 10 ml muscle chamber containing Krebs-Ringer bicarbonate solution (composition, mM: NaCl 135, KCl 5, $NaHCO_3$ 20, glucose 10, $CaCl_2$ 2.5, $MgSO_4$ 7 H_2O, 1.3, KH_2PO_4 1.2, EDTA 0.026) kept at 37°C and bubbled with a mixture of 95% O_2 and 5% CO_2. Each ring was stretched with a tension of 1 g for 2 h. Care was exercised to preserve the integrity of the endothelium throughout the preparation. The first ring was then contracted with 10^{-8} M NA and, 8 min later (at maximal contraction), increasing doses of ACh were cumulatively added, at 2 min intervals, to produce a graded relaxation. The drugs were then washed out and, after a 60 min rest period, the experiment was repeated with histamine instead of ACh. For the second ring, histamine was used to produce relaxation before ACh.

The second part of the study was carried out to investigate the responses of the aortae to increasing doses (10^{-9}–10^{-5} M) of NA. After a period of 6 min to allow for maximal contraction, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} M NA were added cumulatively with an interval of 6 min between each addition. The tension developed with the cumulative doses of NA was expressed in g.

The third part of the study was carried out with the collagenase-treated aortae. Cannulated aortae were filled with 0.2% collagenase Type 1 (Sigma) and incubated at 37°C in Krebs-Ringer solution for 20 min. After rinsing, a section of each aorta was then set aside for histological examination and two 3 mm rings were similarly prepared and contracted with 10^{-8} M NA. Failure of 10^{-6} M ACh to relax the NA-contracted aortae was taken to indicate that the endothelium had been removed. This was confirmed histologically by the paraffin wax method where the cut sections were stained with haematoxylin and eosin. Each ring was then contracted with cumulative doses of histamine (10^{-6}–10^{-3} M) as described for NA. There was no change in pH of the Krebs-Ringer bicarbonate solution in the 10 ml muscle chamber after a maximal volume of 100 µl of the various drug solutions had been added.

Drugs

NA (arterenol, Sigma) was dissolved in 1% ascorbic acid. Acetylcholine chloride (Sigma) was prepared as a stock solution of 1 M in 0.5 M NaH_2PO_4. Histamine (Sigma) was prepared as a stock solution of 1 M in distilled water. Collagenase Type I (Sigma) was prepared in phosphate buffered saline.

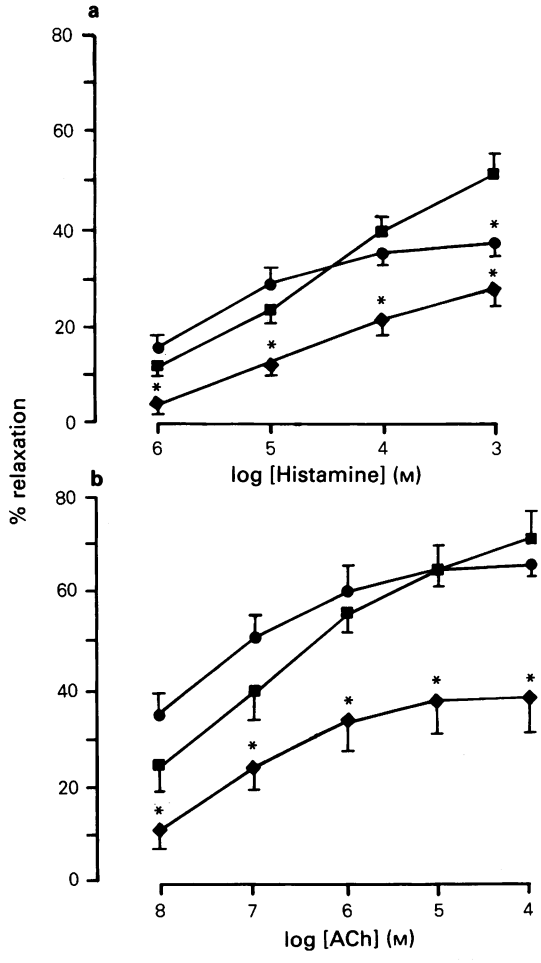

Figure 1 Concentration-response curves for the relaxation of the noradrenaline-contracted aortae of WKY (■), LBP-SHR (●), and LRAS-LBP-SHR (◆) to various doses of histamine (a) and acetylcholine (b). Responses are expressed as % relaxation of the maximal contraction induced by 10^{-8} M noradrenaline. *Indicates significant difference ($P < 0.05$) between WKY and the other animals. Each point represents the mean and vertical lines show s.e.mean; $n = 10$–13.

Statistical analysis

The data are expressed as mean ± s.e.mean. Tests of significance were performed by use of 2-way analysis of variance and Student's t test. P values of less than 0.05 were considered significant.

Results

Relaxation responses

Figure 1 shows that histamine and ACh relaxed the NA-contracted aortae in a dose-dependent manner with ACh being a more potent vasodilator of the blood vessel. The responses of the aortae of WKY and LBP-SHR to the two vasodilators (except to 10^{-3} M histamine) were not significantly different ($P > 0.05$) from each other. However, the aortae of LRAS-LBP-SHR, compared to the aortae of WKY, were significantly less responsive ($P < 0.05$) to doses of histamine and ACh.

Responses to noradrenaline

Figure 2 shows that the aortae of the 4 types of rat contracted to increasing doses of NA (10^{-8}–10^{-5} M) in a dose-dependent manner; 10^{-9} M NA produced no appreciable contraction in any of the aortae tested. At 10^{-8} M, the responses (742 ± 31 mg) of the aortae of the WKY were significantly less ($P < 0.05$) than those of the LBP-SHR, SHR and LRAS-LBP-SHR which were, respectively, 879 ± 44, 946 ± 55, 832 ± 59 mg. However, at higher doses of NA (10^{-7}–10^{-5} M) the aortae of the hypertensive rats were significantly less responsive ($P < 0.05$), compared to those of the normotensive WKY. Of the hypertensive rats studied, the aortae of the LRAS-LBP-SHR were found to be the least responsive ($P < 0.05$) to all the doses (except 10^{-8} M) of NA tested, whilst the aortae of the LBP-SHR and SHR responded similarly ($P > 0.05$), except to 10^{-8} M NA. The maximal tension (g) of the responses developed in the aortae of WKY, LBP-SHR, SHR and LRAS-LBP-SHR was 1.8 ± 0.09, 1.5 ± 0.07, 1.5 ± 0.05 and 1.3 ± 0.08, respectively.

Responses to histamine

Figure 3 shows that all the aortae with the endothelium removed contracted in a dose-dependent manner to histamine. The aortae of hypertensive rats were found to be significantly less responsive ($P < 0.05$), to all six doses of histamine used, compared to those of normotensive WKY. Of the hypertensive rats, the

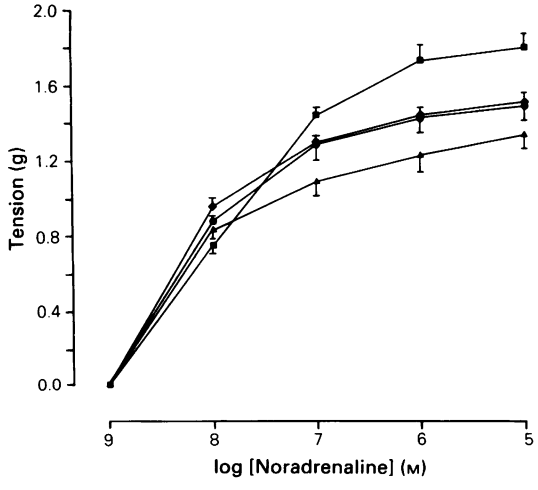

Figure 2 Concentration-response curves for the contraction (developed tension, g) of the aortae of WKY (■), LBP-SHR (●), SHR (◆) and LRAS-LBP-SHR (▲) to 10^{-9}–10^{-5} M noradrenaline. The tension of the responses developed in all the aortae of hypertensive animals was significantly lower than that obtained for WKY for all the concentrations tested ($P < 0.05$), except 10^{-8} M NA. Each point represents the mean and vertical lines show s.e.mean; $n = 13$.

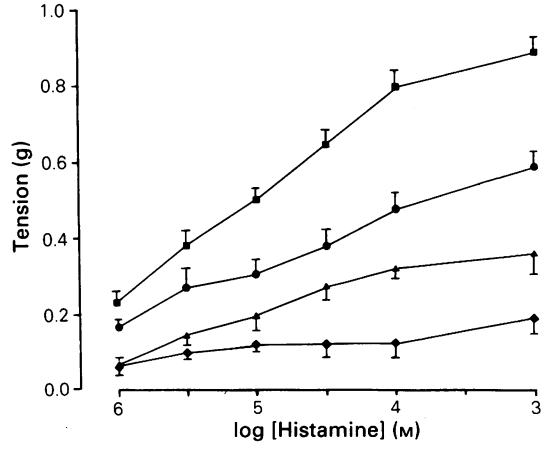

Figure 3 Concentration-response curves for the contraction (developed tension, g) of the collagenase-treated aortae of WKY (■), LBP-SHR (●), SHR (▲) and LRAS-LBP-SHR (◆) to various doses of histamine. The tension of the responses developed in all the hypertensive animals was significantly lower than that obtained for WKY for all the concentrations tested ($P < 0.05$). Each point represents the mean and vertical lines show s.e.mean; $n = 7$–10.

rank order of responsiveness to histamine with respect to tension developed was LBP-SHR>SHR->LRAS-LBP-SHR. The maximal tension (g) of the responses developed in aortae of WKY, LBP-SHR, SHR and LRAS-LBP-SHR was 0.89 ± 0.04, 0.59 ± 0.04, 0.36 ± 0.04 and 0.19 ± 0.05, respectively. The corresponding ED_{50}s for WKY, LBP-SHR, SHR and LRAS-LBP-SHR were, 7.5, 8.5, 8.5 and 4 μM respectively.

Discussion

The results indicate that the decreases in the responses of aortae of hypertensive rats to various vasoactive agents tend to be related to blood pressure. In the LRAS-LBP-SHR, where the blood pressure rose rapidly to a mean of 230 mmHg, the relaxant responses of the aortae to histamine and ACh were significantly smaller ($P<0.05$) than those exhibited by the control LBP-SHR and WKY. However in an earlier study (Sim & Chua, 1985), we found that the aortae of SHR and stroke prone SHR, compared to the aortae of WKY, were not significantly different in their responses to ACh, despite the fact that the former two strains of animals were hypertensive. Thus, it is possible that, besides the magnitude of the blood pressure, the onset and cause of hypertension can also affect the sensitivity of the aortae to ACh. This hypothesis is supported by the rapid development of hypertension in our LRAS-LBP-SHR and the reported slower rise of blood pressure in SHR (Lias *et al.*, 1977), and the renal-induced hypertension in the LRAS-LBP-SHR, which is probably different from the underlying causes of hypertension in the SHR. Since the LRAS-LBP-SHR, LBP-SHR and SHR are genetically of the same strain, the possibility of genetic factors contributing to this difference is minimal.

Konishi & Su (1983) found that the intact aortae of SHR showed a decreased responsiveness to various doses of NA (10^{-8}–10^{-5} M) compared to those of the WKY. We also observed a similar but significant ($P<0.05$) decreased responsiveness to 10^{-7}–10^{-5} M NA (see Figure 2). The different magnitude of this decrease is possibly due to the fact that the blood pressure of the WKY used by Konishi & Su was in the blood pressure range of our LBP-SHR. We found no significant differences between the aortae of our LBP-SHR and SHR in their responses to 10^{-7}–10^{-5} M NA.

The collagenase-treated aortae contracted to histamine in a dose-dependent manner (see Figure 3), which agrees with the observations of Van de Voorde & Leusen (1983). The magnitude of this contraction was also related to blood pressure, as the hypertensive animals were found to be significantly less responsive ($P<0.05$) to increasing doses of histamine. The degree of hyporesponsiveness was found to be more pronounced with increasing blood pressure as seen in the LRAS-LBP-SHR, indicating again that hypertension, *per se*, reduces the sensitivity of the arterial smooth muscle to directly acting vasoactive agents.

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Influence of 5-hydroxytryptamine uptake on the apparent 5-hydroxytryptamine antagonist potency of metoclopramide in the rat isolated superior cervical ganglion

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1 Metoclopramide, 1×10^{-6}–1×10^{-4} M, was found to behave as a reversible, competitive antagonist of 5-hydroxytryptamine (5-HT)-induced depolarization of the rat isolated vagus nerve (VN) and superior cervical ganglion (SCG). The pK_B values were 6.60 (± 0.04) and 5.74 (± 0.07), respectively. The possibility that this apparent difference in potency was due to saturable 5-HT uptake was investigated.

2 The SCG, but not the VN, accumulated tritium-labelled 5-HT via a saturable, sodium- and temperature-dependent mechanism. Ganglionic 5-HT uptake was blocked by desmethylimipramine (IC_{50} 1.4×10^{-6} M), chlorimipramine (8.7×10^{-9} M), zimelidine (1.5×10^{-7} M), paroxetine (4.3×10^{-8} M) and citalopram (6.2×10^{-8} M).

3 The 5-HT uptake inhibitor paroxetine, 1×10^{-6} M, did not modify the apparent 5-HT antagonist potency of metoclopramide on the VN, but raised the pK_B obtained against 5-HT on the SCG from 5.74 (± 0.07) to 6.25 (± 0.03).

4 It is suggested that the observed difference in the potency of metoclopramide as a 5-HT antagonist on the rat VN and SCG was due to saturable 5-HT uptake in the latter preparation. The results do not support a difference in the 5-HT receptors mediating depolarization on the VN and SCG.

Introduction

The potencies of certain antagonists, selective against the actions of 5-hydroxytryptamine (5-HT) on mammalian peripheral neurones, appear to depend upon the preparation used. Such observations support the existence of sub-types of peripheral neuronal 5-HT receptors (see Fozard, 1984; Richardson *et al.*, 1985). 5-HT depolarizes both the rat isolated superior cervical ganglion (SCG) and vagus nerve (VN) (Watson, 1970; Ireland *et al.*, 1982). However, it is not known whether the receptors in these preparations are the same. In order to address this problem, we have compared the actions of metoclopramide against these 5-HT-induced responses on the two tissues.

In a previous study (Ireland *et al.*, 1982), we described how metoclopramide behaves as a reversible competitive antagonist of 5-HT-induced depolarization of the VN for which the negative log of the apparent equilibrium dissociation constant (pK_B) was 6.60 (± 0.04). In contrast, in preliminary studies on the SCG, the pK_B value for metoclopramide was 5.74 (± 0.07). This result may indicate that the 5-HT receptors on the rat VN and SCG are different. However, an alternative explanation comes from the work of Langer & Trendelenburg (1969), who proposed that the potency of an antagonist may be underestimated if the agonist against which it is tested is the substrate for a saturable uptake process.

The existence of an uptake process that specifically accumulates 5-HT has been well documented for both rat brain and blood platelets (see review by Ross, 1982). 5-HT may also be a substrate for noradrenaline uptake processes (Burgen & Iversen, 1965; Shaskan & Snyder, 1970). The rat isolated SCG has been found to accumulate tritium-labelled noradrenaline (Brown & Caulfield, 1979), but the potential of this tissue, or of the rat VN, to accumulate 5-HT has not been des-

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cribed previously. We have examined both tissues for their abilities to accumulate radiolabelled 5-HT and have attempted to characterize any processes involved. We have also sought to quantify the possible influence of 5-HT uptake on the 5-HT antagonist activity of metoclopramide by the use of 5-HT uptake inhibitors, and to compare the results obtained with the quantitative prediction of the model of Furchgott (1972).

A preliminary account of this work has been presented to the British Pharmacological Society (Ireland *et al.*, 1983).

Methods

Preparation of tissues

All experiments were performed on freshly-dissected superior cervical ganglia (SCG), or 10–20 mm lengths of cervical vagus nerve (VN) (minus the nodose ganglion), excised from male Lister-hooded rats (01ac) anaesthetized with chloral hydrate (300 mg kg^{-1} i.p.). Animals weighed between 250 and 350 g.

The connective tissue capsule around each isolated VN or SCG was removed; tissues were maintained in Krebs-Henseleit medium (> 25 ml per tissue) at room temperature (approximately 21°C), gassed with 95% O_2 and 5% CO_2, until use. For uptake experiments only, all nerve trunks were removed from each SCG and the medium contained nialamide, 1×10^{-5} M, unless otherwise stated.

Extracellular recording

5-HT-induced depolarization was recorded extracellularly from VN or SCG preparations mounted in two-compartment perspex baths, as described previously (Ireland *et al.*, 1982). Briefly, each VN was positioned so that 50 to 70% of the nerve lay in the first compartment, while the remainder projected through a greased slot (Dow-Corning high vacuum grease) into the second. Each SCG was mounted with the ganglion lying in the first compartment and the internal carotid nerve containing postganglionic fibres, projecting through the greased slot into the second. The d.c. potential between the two compartments was recorded via silver-silver chloride electrodes connected to the preparation through agar-saline and filter paper bridges and was displayed on a potentiometric chart recorder (Servoscribe 200). Each compartment was perfused continuously at a constant rate of approximately 1 ml min^{-1} with Krebs-Henseleit medium dripped directly onto the tissue. Drugs were applied at known concentrations via the superfusion stream into the first compartment only.

The temperature of each preparation was maintained at 27 ± 1°C by passing solutions through heat exchangers immediately before applying them to the tissue, and by placing the recording bath and electrodes in a temperature-controlled chamber. This temperature was chosen since in preliminary experiments, recorded base-lines were more stable at 27°C than at 37°C (results not shown).

Measurement of monoamine uptake

Individual VN segments or superior cervical ganglia were pre-incubated in 1.9 ml of Krebs-Henseleit medium, maintained at 27°C, and continuously gassed with 95% O_2 and 5% CO_2 or 100% O_2 as appropriate. Generally, compounds to be examined for ability to inhibit uptake were added at the beginning of this period. A pre-incubation time of 60 min was found to be adequate for the equilibration of uptake inhibitors and was therefore used routinely.

Uptake of [^{3}H]-5-HT was studied over a wide range of concentrations (1×10^{-8}–1×10^{-4} M); in some experiments the accumulation of (−)-[^{3}H]-noradrenaline ((−)-[^{3}H]-NA), rather than 5-HT, was examined. For these latter studies, the incubation medium contained ascorbate, 5×10^{-5} M. Incubation was initiated by adding 100 µl of radioligand solution to each tissue preparation. A standard incubation time of 20 min was used routinely, although uptake occurring during both longer and shorter periods was also measured. Uptake was terminated by filtration under vacuum. Tissues were washed with 5 ml of ice-cold Krebs-Henseleit medium and weighed 0.5 and 1.0 min after exposure to room air. Fresh mass was determined by extrapolation to zero time (Brown *et al.*, 1971). Recorded mass ranged from 0.5–1.1 mg for the SCG, 0.6–1.4 mg for the VN. Tissues were dissolved in 0.5 ml of Soluene-350 (Packard). Radioactivity was determined by liquid scintillation spectrometry. Samples were corrected individually for quenching, using the external standard pulse method. Counting efficiencies ranged from 40–50%.

Quantification of uptake inhibition

In experiments in which compounds or manipulations were used to block uptake, results are expressed as the percentage inhibition of the control uptake, measured in separate tissues, in the same experiment.

The potency of each inhibitor was expressed in terms of an IC_{50} value, which was the concentration of the inhibitor calculated to produce 50% of its own maximum effect.

Radiochemical purity

Radiochemical purity was checked by thin-layer chromatography on Avicel plates (Anachem Ltd),

using two solvent systems (ethanol:ethanoic acid: water, 25:4:10 by volume, and *n*-butanol:ethanoic acid:water, 6:3:1 by volume).

In the absence of a monoamine oxidase inhibitor, significant amounts of a total radioactivity recovered from homogenates of ganglia previously incubated with [^{3}H]-5-HT, 1×10^{-8}–1×10^{-5} M for 60 min, co-migrated with 5-hydroxyindoleacetic acid (5-HIAA). This amounted to 31% after exposure to [^{3}H]-5-HT, 1×10^{-8}LH M and 55% after exposure to [^{3}H]-5-HT, 1×10^{-5} M. The conversion of [^{3}H]-5-HT to [^{3}H]-5-HIAA was unlikely to have been an artefact of the preparative procedure. Thus, in control experiments in which ganglia, not previously exposed to [^{3}H]-5-HT, were homogenized in the presence of the radioligand, less than 5% of the total radioactivity co-migrated with 5-HIAA. In ganglia treated with the monoamine oxidase inhibitor nialamide, 1×10^{-5} M, at least 90% of the radioactivity accumulated during incubation with [^{3}H]-5-HT, 1×10^{-8} M for 60 min, co-migrated with 5-HT. After incubation with [^{3}H]-5-HT, 1×10^{-5} M for 60 min, the proportion was 83%. The corresponding estimates for [^{3}H]-5-HIAA were 4% and 13%, respectively. Nialamide, 1×10^{-5} M, was used routinely in all further studies.

Brown & Caulfield (1979) found that 97% of the radioactivity extracted from rat SCG after incubation with (−)-[^{3}H]-NA for 2 h in the presence of nialamide, 1×10^{-5} M, was unchanged amine. This was not checked in the present experiments.

Drugs and solutions

The composition of the normal Krebs-Henseleit medium used in the present study was (in mmol l^{-1}): NaCl 118, $NaHCO_3$ 25, KH_2PO_4 1.18, KCl 4.7, $MgSO_4$ $7H_2O$ 1.18, $CaCl_2$ 2.5, glucose 11. Na^+-free medium was prepared by substitution of LiCl (118 mM) for NaCl (118 mM) and Tris HCl (pH 7.4, 25 mM, Sigma) in place of $NaHCO_3$ (25 mM). This solution was gassed with 100% O_2 rather than 95% O_2 and CO_2. The media were prepared in glass-distilled water and reagents, which were all AR-grade, were obtained from commercial sources.

The drugs used were: 5-HT creatinine sulphate (Sigma), 1-phenylbiguanide (Aldrich), (−)-noradrenaline bitartrate (Sigma), metoclopramide hydrochloride (Beecham), ouabain (strophanthin G, Sigma), desmethylimipramine hydrochloride and chlorimipramine hydrochloride (Ciba), zimelidine dihydrochloride (Astra), citalopram hydrobromide (Lundbeck), paroxetine hydrochloride (Ferrosan), nialamide (Sigma), and 5-hydroxyindoleacetic acid (Sigma).

Tritium-labelled 5-HT creatinine sulphate (15–30 Ci $mmol^{-1}$), and (−)-noradrenaline (47.7 Ci $mmol^{-1}$) were purchased from New England Nuclear.

Curve fitting procedures

The methods used for fitting hyperbolic and logistic curves were based on those of Parker & Waud (1971), which were in turn specializations of the general method of Snedecor & Cochran (1968). The computer programs used for curve fitting in the present study were written by Miss F.J. Illingworth, Department of Computer Science, Glaxo Group Research Ltd, Greenford, Middx.

Results

Antagonism of 5-HT-induced depolarizations of the vagus nerve and superior cervical ganglion in the absence of uptake inhibitors

On the VN, 5-HT, 1×10^{-7}–3×10^{-5} M, caused rapid concentration-dependent depolarization responses with a maximum amplitude for a given tissue of between 300 and 600 μV. Similar depolarization responses were obtained on the SCG, using 5-HT concentrations ranging from 1×10^{-6} to 3×10^{-4} M. On both preparations, repolarization following 5-HT wash-out took 10–20 min; the time-course sometimes appeared biphasic, with a prolonged late component. In the present study, VN and SCG preparations were always allowed to repolarize fully between agonist applications. Agonists were left in contact with the tissues until apparent equilibrium was reached–this usually took 3 min or less. Two sequential control concentration-response curves obtained on the same VN or SCG at 1–2 h intervals were virtually superimposable. Therefore, a standard procedure was used for the measurement of the potency of antagonists. Only one concentration was applied to each VN or SCG; its effects were measured only once it had achieved apparent equilibrium. This was taken to have occurred when repeated application of an approximate EC_{50} of the agonist gave responses equal to within ± 10% of each other. Lateral displacements of concentration-depolarization response curves were measured at the control half-maximal response level. pK_B values were calculated as the mean (± s.e.mean) of the individual results: $pK_B = \log (\text{dose-ratio} - 1) - \log (\text{antagonist concentration})$.

As previously found (Ireland *et al.*, 1982), metoclopramide, 3×10^{-6}–1×10^{-4} M, caused parallel rightward shifts of the 5-HT concentration-response curve on the VN with no significant change in the maximum response (Figure 1). A plot of these data according to the method of Arunlakshana & Schild (1959), and a gradient of 0.98 (± 0.07) (Figure 2). The pK_B value was 6.60 (± 0.04, $n = 16$). On the SCG, metoclopramide, 3×10^{-6}–1×10^{-4} M, also caused parallel rightward displacements of the 5-HT concen-

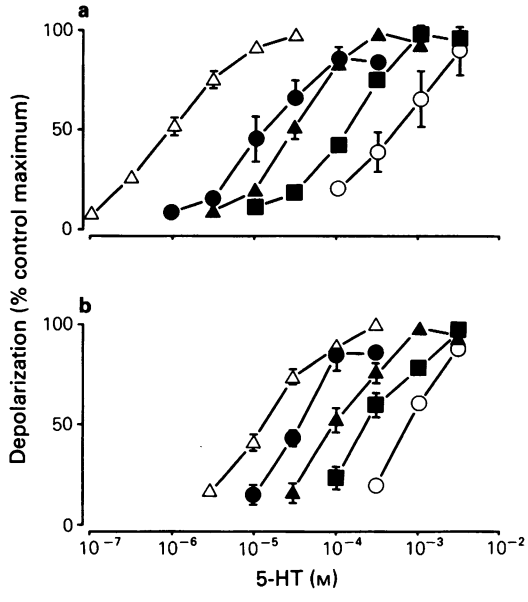

Figure 1 Antagonism by metoclopramide of 5-hydroxytryptamine (5-HT)-induced depolarization of the rat isolated vagus nerve (a) and superior cervical ganglion (b). Symbols indicate controls (△), and the presence of metoclopramide 3×10^{-6} M (●), 1×10^{-5} M (▲), 3×10^{-5} M (■) and 1×10^{-4} M (○). Each point is the mean, with vertical lines indicating s.e.mean, of single determinations from at least 4 separate tissue preparations, each obtained from a different rat.

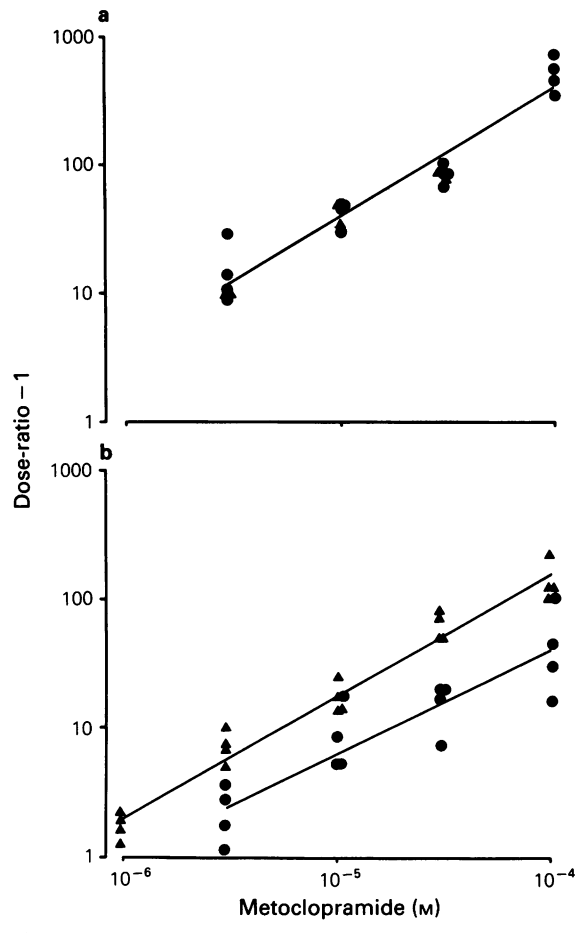

Figure 2 Schild plots of (dose-ratio − 1) against antagonist concentration for metoclopramide antagonism of 5-hydroxytryptamine (5-HT)-induced depolarization of the rat isolated vagus nerve (a) and superior cervical ganglion (b). Each point represents the result obtained on a separate tissue. (●) Data obtained in the absence of a 5-HT uptake inhibitor, (▲) results obtained in the presence of paroxetine, 1×10^{-6} M. Lines were fitted by linear regression.

tration-depolarization curve (Figure 1). However, the Schild plot constructed with these data had a gradient of 0.82 (± 0.12), although this was not significantly different from unity ($P > 0.05$, Students t test) (Figure 2). The pK_B value was 5.74 (± 0.07, $n = 16$).

Accumulation of radiolabelled monoamines by the superior cervical ganglion and vagus nerve

[^{3}H]-5-hydroxytryptamine After incubation with [^{3}H]-5-HT, 1×10^{-8}–1×10^{-5} M, ganglia were found to have accumulated radioactivity against a concentration gradient. The accumulation was linear with time for at least 60 min. The mean tissue-to-medium ratio achieved in ganglia incubated with [^{3}H]-5-HT, 1×10^{-8} M, for 60 min was 26.9 ± 2.4 ($n = 3$). The attained tissue-to-medium ratios were observed to decrease with increased substrate concentration. This effect was significant ($P < 0.001$, analysis of variance on $\log_{10}$ transformed data) at all incubation times from 1 to 60 min. A standard incubation time of 20 min was used subsequently to estimate initial uptake rates.

Vagus nerves incubated with [^{3}H]-5-HT, 1×10^{-8} – 1×10^{-4} M, were also found to accumulate radioactivity. However, the tissue-to-medium ratios achieved were much smaller than those observed in ganglia – the maximum attained in any preparation was 4.1. The rate of accumulation appeared to decrease after 2 min exposure to the radioligand. Tissue-to-medium ratios measured after incubations of 2 min or less did not change significantly with substrate concentration ($P > 0.05$, analysis of variance on $\log_{10}$ transformed data). Therefore, in contrast to the SCG, the VN did not appear to possess a saturable 5-HT uptake system.

Table 1 Effects of various procedures producing inhibition of [^{3}H]-5-hydroxytryptamine ([^{3}H]-5-HT) uptake in the rat superior cervical ganglion

	Inhibition of [^{3}H]-5-HT uptake (mean % ± s.e.mean)			
Treatment	*[^{3}H]-5-HT 1 × 10^{-8} M*	(n)	*[^{3}H]-5-HT 1 × 10^{-5} M*	(n)
Cold (4°C)	79.5 ± 1.0	(6)	77.3 ± 1.1	(6)
Ouabain, 1 × 10^{-3} M	61.2 ± 2.2	(5)	56.6 ± 1.7	(6)
Na^+-free Krebs	68.2 ± 2.1	(5)	52.7 ± 3.0	(6)

Tissues were pre-incubated in the test medium for the 60 min immediately before, and during incubation with [^{3}H]-5-HT. In the Na^+-free medium, LiCl (118 mM), and Tris HCl (25 mM, pH 7.4) substituted for NaCl (118 mM) and $NaHCO_3$ (25 mM) respectively.

(−)-[^{3}H]-noradrenaline SCG preparations incubated with (−)-[^{3}H]-NA, 1 × 10^{-8} M, accumulated radioactivity against a concentration gradient. Uptake was linear with time for at least 80 min (result not shown).

Inhibition of ganglionic monoamine uptake

The accumulation of radiolabel by ganglia during incubation with [^{3}H]-5-HT, 1 × 10^{-8} M and 1 × 10^{-5} M, was found to be temperature-sensitive and Na^+-dependent. The uptake was also inhibited by ouabain at 1 × 10^{-3} M (Table 1), but not 1 × 10^{-4} M. This observation accords with the relative insensitivity of the rat SCG to this cardiac glycoside (see Brown & Scholfield, 1974).

The accumulation of radioactivity during incubation with [^{3}H]-5-HT, 1 × 10^{-8} M, was also reduced in ganglia exposed to the uptake inhibitors chlorimipramine, 1 × 10^{-9}–1 × 10^{-4} M, desmethylimipramine, 3 × 10^{-7}–1 × 10^{-4} M, zimelidine, 3 × 10^{-8}–1 × 10^{-4} M, paroxetine 1 × 10^{-8}–1 × 10^{-4} M. and citalopram, 1 × 10^{-8}–1 × 10^{-4} M (Table 2). The accumulation of radioactivity by ganglia during incubation with (−)-[^{3}H]-NA, 1 × 10^{-8} M, was inhibited by desmethylimipramine, 1 × 10^{-9} –3 × 10^{-5} M, and paroxetine, 1 × 10^{-7}–1 × 10^{-4} M.

Ganglionic monoamine uptake was also susceptible to inhibition by agonists. In contrast to the other inhibitors, these were added to the incubation medium concurrently with the radioligand. [^{3}H]-5-HT accumulation was inhibited by 5-HT, 1 × 10^{-8} –1 × 10^{-4} M, but not by phenylbiguanide, 1 × 10^{-8}–1 × 10^{-4} M, or (−)-NA, 1 × 10^{-8} –1 × 10^{-4} M. In contrast, (−)-[^{3}H]-NA uptake was inhibited by both (−)-NA, 1 × 10^{-8}–1 × 10^{-4} M, and 5-HT, 1 × 10^{-8}–1 × 10^{-4} M.

In all the above cases, inhibition was concentration-related. It was found empirically that the logistic curve was a good fit to the experimental data, all deviations

Table 2 Effects of uptake inhibitors on monamine uptake in rat superior cervical ganglia

	Inhibition of 5-HT uptake			*Inhibition of NA uptake*
Compound	*IC_{50}* (μM)	*Maximum* (mean % ± s.e.)	(n)	*IC_{50}* (μM)
Chlorimipramine	0.0087	81.1 ± 0.8	(5)	–
Desmethylimipramine	1.44	82.6 ± 0.9	(5)	0.0025
Zimelidine	0.15	81.0 ± 0.6	(5)	–
Paroxetine	0.043	84.4 ± 1.0	(5)	0.37
Citalopram	0.062	80.2 ± 1.2	(5)	–
5-HT	4.8	–		33.0
(−)-NA	> 100	–		3.2

Ganglia were incubated with [^{3}H]-5-hydroxytryptamine ([^{3}H]-5-HT) 1 × 10^{-8} M, or (−)-[^{3}H]-noradrenaline, 1 × 10^{-8} M. IC_{50} values were determined by direct fit of the logistic curve to the concentration-inhibition curve for each compound; estimated standard errors were less than 10%. Values for the maximum inhibition of [^{3}H]-5-HT uptake were determined in the presence of chlorimipramine, 1 × 10^{-5} M, or desmethylimipramine, zimelidine, paroxetine, or citalopram at 1 × 10^{-4} M, all in the same experiment.

being non-significant ($P > 0.05$, analysis of variance). Values for IC_{50} were determined from the fitted curves; supra-maximal concentrations of the inhibitors all produced approximately similar inhibition of ganglionic [^{3}H]-5-HT uptake (Table 2).

Metoclopramide caused significant inhibition ($P < 0.05$) of the accumulation of radioactivity by ganglia incubated with [^{3}H]-5-HT, 1×10^{-8} M, only at concentrations of 3×10^{-5} M or greater.

Kinetics of the uptake of [^{3}H]-5-HT by the rat superior cervical ganglion

A plot of the rate of total 5-HT uptake against [^{3}H]-5-HT concentration over the range 1×10^{-8} -1×10^{-5} M, shows some deviation from linearity, but no appreciable saturation (Figure 3). It is possible that this uptake may have comprised more than one component, since it was not completely blocked by any of the inhibitory procedures tested (see Tables 1 and 2). Therefore an analysis was made of the kinetics of those elements of [^{3}H]-5-HT uptake sensitive to inhibition by ouabain, low temperature or a supra-maximal concentration of a 5-HT uptake inhibitor. Figure 3 shows the chlorimipramine-sensitive component of total 5-HT uptake determined by subtraction of the element resistant to chlorimipramine, 1×10^{-5} M. Chlorimipramine-sensitive uptake exhibited marked saturation; kinetic constants were determined by direct fit of a hyperbola to the data. The ouabain-sensitive and cold-sensitive components of total 5-HT uptake were determined similarly. The derived kinetic constants are shown in Table 3.

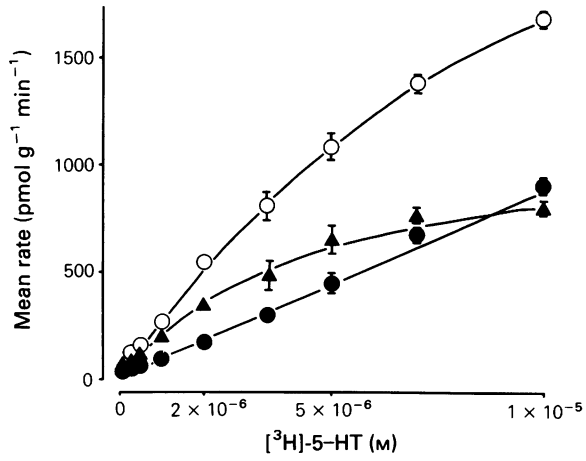

Figure 3 Kinetics of the accumulation of [^{3}H]-5-hydroxytryptamine ([^{3}H]-5-HT) by rat superior cervical ganglion. Points show experimentally determined total (○), and chlorimipramine-resistant (●) accumulation. Chlorimipramine-sensitive uptake (▲) was calculated from individual values of the total accumulation by subtraction of the appropriate mean chlorimipramine-resistant rate. The latter was determined in separate ganglia incubated with chorimipramine 1×10^{-5} M. Points are means, with vertical lines indicating s.e.mean, of results from at least 4 individual ganglia. The straight line and the hyperbola were each fitted using a computer program.

The effects of uptake inhibition on the antagonist potency of metoclopramide.

The 5-HT uptake inhibitor paroxetine was used in studies on the possible effects of uptake inhibition on 5-HT-induced depolarization of the VN and SCG. This compound was chosen in preference to the other uptake inhibitors since, in preliminary studies, it appeared the least prone to antagonize 5-HT-induced responses at concentrations producing significant inhibition of 5-HT uptake (results not shown). The effects of uptake inhibitors on 5-HT-induced depolarization were measured after a minimum of 60 min exposure to the drug.

On the VN, paroxetine, $3 \times 10^{-7} - 1 \times 10^{-6}$ M, had no significant effect on 5-HT-induced depolarization responses, although at 3×10^{-6} M it caused attenuation. The principal effect was a reduction in the maximum response. On the SCG, paroxetine, 1×10^{-6} M, caused a leftward shift of the 5-HT concen-

Table 3 Kinetic constants for [^{3}H]-5-hydroxytryptamine ([^{3}H]-5-HT) uptake in rat superior cervical ganglion

	[^{3}H]-5-HT uptake	
Uptake component	K_M (μM)	V_{max} (pmol g^{-1} min^{-1})
Chlorimipramine-sensitive	4.7	1187
Ouabain-sensitive	3.9	926
Cold-sensitive	6.7	1848

Values were determined by direct fit of a hyperbola to the calculated chlorimipramine-sensitive, ouabain-sensitive, or cold-sensitive components of total 5-HT uptake. Standard errors were approximately 5%, but these do not include the error induced by calculating the inhibition-sensitive components of total 5-HT uptake.

tration-depolarization curve with no change in the maximum response; in the presence of paroxetine, 1×10^{-6} M, the mean EC_{50} ($\pm$ s.e.mean) for 5-HT was $2.7 \pm 0.2 \times 10^{-6}$ M ($n = 40$) compared to $1.4 \pm 0.1 \times 10^{-5}$ M ($n = 36$) in the absence of the uptake inhibitor. However, at a concentration of 3×10^{-6} M, which is only slightly higher than that found to have this potentiating effect, paroxetine inhibited 5-HT-induced depolarization. As on the VN the principal effect was a reduction in the maximum response. Paroxetine, 3×10^{-7} M, had no significant effect on 5-HT-induced depolarization of the SCG.

The NA-uptake inhibitor desmethylimipramine, 1×10^{-7} M, had no significant effect on 5-HT-induced depolarization of the VN or SCG (results not shown). In the SCG, this concentration of desmethylimipramine caused inhibition of (−)-[^{3}H]-NA but not of [^{3}H]-5-HT accumulation.

The effects of metoclopramide against 5-HT-induced depolarization were examined in VN and SCG preparations exposed to paroxetine, 1×10^{-6} M. On the VN the uptake inhibitor did not modify the potency of metoclopramide (Figure 2). On the SCG, in the presence of paroxetine, metoclopramide, 1×10^{-6}–1×10^{-4} M, produced parallel rightward shifts of the 5-HT concentration-response curve. However, the gradient of the Schild plot was 0.93 ($\pm$ 0.04), compared to the value of 0.82 ($\pm$ 0.12) obtained in the absence of the uptake inhibitor. Neither value was significantly different from unity ($P > 0.05$) (Figure 2). The corresponding pK_B values were 6.25 ($\pm$ 0.03, $n = 20$) and 5.74 ($\pm$ 0.07, $n = 16$), respectively. The apparent potency of metoclopramide as an antagonist of phenylbiguanide-in-

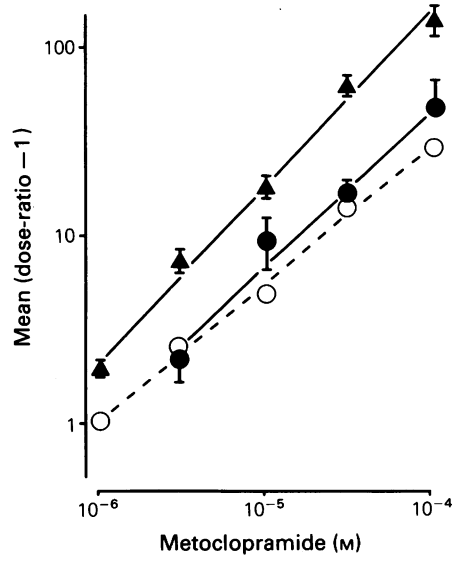

Figure 4 Use of Furchgott's (1972) model to predict the effect of 5-hydroxytryptamine (5-HT) uptake on the apparent 5-HT antagonist potency of metoclopramide on the rat superior cervical ganglion (SCG). Filled symbols and solid lines represent observed results; each symbol indicates the mean, with vertical lines representing s.e.mean, of single determinations on 4 separate tissues. (▲) Data recorded from ganglia exposed to paroxetine, 1×10^{-6} M. (●) Results obtained in the absence of the 5-HT uptake inhibitor. Furchgott's model was used to predict the influence of chlorimipramine-sensitive 5-HT uptake by the SCG, on the data recorded in the presence of paroxetine. These calculated results are indicated by (O - - - O).

Table 4 The observed and predicted effects of 5-hydroxytryptamine (5-HT) uptake on the apparent 5-HT-antagonist potency of metoclopramide on the rat superior cervical ganglion (SCG)

Source of result	Apparent 5-HT-antagonist potency of metoclopramide on rat SCG $pK_B \pm s.e.$	(n)	*slope* $\pm$ *s.e.*
A Observed in presence of paroxetine 1×10^{-6}M	6.25 ± 0.03	(20)	0.93 ± 0.04
B_1 Calculated from A for CMI-sensitive uptake	5.73		0.73
B_2 Calculated from A for cold-sensitive uptake	5.77		0.71
B_3 Calculated from A for ouabain-sensitive uptake	5.80		0.76
C Observed in absence of paroxetine	5.74 ± 0.07	(16)	0.82 ± 0.12

A and C show the data observed in the presence and absence of paroxetine, 1×10^{-6} M, respectively. The slope values are the gradients of plots of log (dose-ratio − 1) against log (antagonist concentration). The predicted effects on the results shown in A, of chlorimipramine (CMI)-sensitive, cold-sensitive, and ouabain-sensitive ganglionic 5-HT uptake, were estimated using Furchgott's (1972) model (see text and Figure 4).

duced depolarization of the SCG, was unaffected by paroxetine (results not shown).

The selective NA-uptake inhibitor desmethylimipramine, 1×10^{-7} M, did not alter the apparent potency of metoclopramide as an antagonist of depolarization responses induced by 5-HT on the SCG: the Schild plot slope and pK_B value were 0.83 (± 0.09) and 5.76 (± 0.05, $n = 16$), respectively.

Comparison of the experimental results with those predicted by Furchgott's (1972) model

Furchgott (1972) devised the following equation to simulate the influence of saturable agonist uptake on the relationship between the concentration of agonist in the external medium ([Aa]) and that at equilibrium with the receptors ([Ab]), in the presence of various concentrations of a reversible competitive antagonist ([B]):

$$[Aa] = [Ab]_o(1 + [B]/K_B)\left[1 + \frac{\frac{V_{max}}{kK_M}}{1 + \frac{[Ab]_o(1 + [B]/K_B)}{K_M}}\right]$$

where k is a constant for movement of the agonist into the region of the receptors. We have used this relationship to predict the effect of 5-HT uptake on the 5-HT antagonist activity of metoclopramide on the SCG, using the maximum rate (V_{max}) and half-maximal rate constant (K_M) values for uptake, determined in the present study. It was assumed that the 5-HT uptake system was inactive in the presence of paroxetine, 1×10^{-6} M.

Figure 4 and Table 4 reveal good agreement between the predicted and observed data.

Discussion

Metoclopramide caused parallel rightward displacements of the 5-HT concentration-depolarization curves on both the rat VN and SCG. The pK_B values calculated from the effects of this antagonist measured in the absence of a 5-HT uptake inhibitor were 6.60 (± 0.04) and 5.74 (± 0.07), respectively. These differed sufficiently to suggest that the 5-HT receptors that mediate depolarization of the rat VN may not be identical to those on the SCG. We have previously shown that depolarization induced by even high concentrations of 5-HT applied to the VN and SCG both in the presence and absence of metoclopramide, 1×10^{-4} M, was resistant to blockade by a variety of non-5-HT antagonists (see Ireland *et al.*, 1982; Fortune *et al.*, 1983). This makes it unlikely that the relatively low potency of metoclopramide as a 5-HT antagonist on the SCG was due to activation of non-5-HT receptors by elevated concentrations of the agonist.

In this paper, we have examined an alternative suggestion prompted by the work of Langer & Trendelenburg (1969), who proposed that the potency of a competitive antagonist may be underestimated if the agonist against which it is tested is also the substrate for a saturable uptake system within the test tissue.

It was found that both whole SCG preparations and VN segments accumulated tritium during incubation with a wide range of [^{3}H]-5-HT concentrations. With the VN, no evidence was obtained to suggest that this accumulation was saturable. In contrast, with the SCG, the elements of total [^{3}H]-5-HT uptake sensitive to inhibition by chlorimipramine, ouabain, or low temperature appeared saturable: each could be adequately described by a single hyperbola. The possibility that these apparently saturable elements comprised heterogeneous transport systems was not investigated. The value of the half-maximal rate constant (K_M) for saturable ganglionic [^{3}H]-5-HT uptake was close to the threshold concentration of 5-HT required to depolarize the tissue. However, it is unlikely that the apparent saturation of uptake was due to depolarization, since phenylbiguanide did not affect the accumulation of [^{3}H]-5-HT by the SCG, even though it mimics the depolarizing activity of 5-HT in this preparation (Fortune *et al.*, 1983).

Results obtained with a range of uptake inhibitors against the accumulation of radiolabel by ganglia incubated with a low concentration of [^{3}H]-5-HT, suggested a similarity between 5-HT uptake systems in the rat SCG and brain (see Ross, 1982). The former appeared distinct from (−)-[^{3}H]-NA accumulation (Table 2). 5-HT did inhibit (−)-[^{3}H]-NA accumulation, although the concentrations required were greater than those needed to block [^{3}H]-5-HT uptake.

In the present study, no attempt was made to compare the location of the 5-HT uptake processes within the SCG with the sites at which the agonist acted to cause depolarization. It is also unclear to what extent the apparently saturable accumulation of [^{3}H]-5-HT by this tissue indicated a net uptake of 5-HT, as opposed to exchange of radiolabelled for endogenous material (see Cerrito & Raiteri, 1979). The latter is possible since the 5-HT-synthetic enzyme tryptophan hydroxylase, and cells specifically containing 5-HT-like immunoreactivity have been found in the rat SCG (Saavedra & Luizzi, 1978; Verhofstadt *et al.*, 1981). However, evidence was obtained that 5-HT uptake can influence the effect of 5-HT on the rat isolated SCG preparation. Thus, when 5-HT-induced depolarization responses were recorded from SCG preparations exposed to the 5-HT uptake inhibitor paroxetine, 1×10^{-6} M, it was found that the 5-HT

concentration-depolarization curve was shifted to the left of the control, and that the apparent potency of metoclopramide as a 5-HT antagonist was increased. It is likely that this effect of paroxetine was due to inhibition of 5-HT uptake *per se*. Thus, paroxetine, 1×10^{-6} M, did not affect the potency of metoclopramide as an antagonist of the depolarization induced by the 5-HT-mimetic phenylbiguanide. This is consistent with the low affinity of phenylbiguanide for the 5-HT uptake process. Further, desmethylimipramine, at a concentration (1×10^{-7} M) that selectively blocks ganglionic NA but not 5-HT uptake, did not modify the effects of 5-HT or metoclopramide on the SCG. Finally, on the VN, paroxetine, 1×10^{-6} M, did not change the apparent potency of either metoclopramide or 5-HT.

The experimental observations of the effects of paroxetine on the 5-HT antagonist potency of metoclopramide on the SCG were compared with those predicted by Furchgott's (1972) model. The form of the model employed assumes that the antagonist has no effect on agonist uptake. This seemed appropriate since metoclopramide was found to reduce [^{3}H]-5-HT uptake only at a concentration at least 30 fold in excess of those required to antagonize 5-HT-induced depolarization. The model requires values for the V_{max} and K_M of the uptake system studied. In the present experiments, these depended on whether the cold-sensitive, ouabain-sensitive or chlorimipramine-sensitive component of total 5-HT uptake was analysed. However, the use of these different estimates did not cause material change to the predicted influence of 5-HT uptake on the apparent potency of metoclopramide as a 5-HT antagonist (see Table 4). This was characterized by a small rightward shift and decline in slope of the plot of log dose-ratio − 1 against log antagonist concentration. In all cases, the predicted plots deviated slightly from linearity (Figure 4). There was close agreement between the predicted and observed data for antagonism by metoclopramide of 5-HT-induced depolarization of the SCG in the absence of an uptake inhibitor.

In conclusion, the results are consistent with the suggestion that the saturable uptake of [^{3}H]-5-HT by the rat SCG reduced the apparent potency of metoclopramide as a 5-HT antagonist on this tissue. In contrast to the SCG, the VN was shown not to accumulate [^{3}H]-5-HT via a saturable mechanism. Therefore, it is likely that the observed difference in the potency of metoclopramide as a 5-HT antagonist on these two tissues was due to the influence of 5-HT uptake, rather than the existence of sub-types of 5-HT receptors. The influence of 5-HT uptake on the apparent potency of other 5-HT antagonists on the rat SCG has yet to be tested. It is interesting to note that Round & Wallis (1986) found almost identical pA_2 values for the antagonism by ICS 205-930 of 5-HT-induced depolarization of the rabbit vagal and SCG neurones. These results were obtained in the absence of a 5-HT uptake inhibitor since in the rabbit SCG, the effects of 5-HT do not seem to be influenced by 5-HT uptake (Round & Wallis, 1986).

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Suppression and potentiation of 5-hydroxytryptophan-induced hypoglycaemia by α-monofluoromethyldopa: correlation with the accumulation of 5-hydroxytryptamine in the liver

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1 Experiments were done to examine whether the accumulation of 5-hydroxytryptamine (5-HT) in the liver is responsible for the hypoglycaemia induced in mice by 5-hydroxytryptophan (5-HTP) and lipopolysaccharides (LPS).

2 (±)-α-Monofluoromethyldopa (FMD), a potent irreversible inhibitor of aromatic amino acid decarboxylase, suppressed the 5-HTP-induced accumulation of 5-HT in the liver at a dose of 2 mg kg^{-1} or more, but potentiated the accumulation at a lower dose of 0.4 mg kg^{-1}. Corresponding to these effects, the hypoglycaemic response was prevented by the higher doses of FMD and potentiated by the lower dose. These contrasting effects of FMD were explicable by the amounts of 5-HTP entering the liver.

3 In contrast, FMD did not prevent either the hypoglycaemia or the accumulation of 5-HT in the liver induced by LPS.

4 These results further support the hypothesis that the accumulation of 5-HT in the liver is causally related to the hypoglycaemia induced by 5-HTP and indicate that the LPS-induced 5-HT accumulation in the liver is not derived from stimulation of 5-HT synthesis. It is still not clear whether the accumulation of 5-HT in the liver is involved in the hypoglycaemic response to LPS.

Introduction

On the basis of experiments using carbidopa, it was supposed that the accumulation of 5-hydroxytryptamine (5-HT) in the liver may be a cause of the hypoglycaemia induced by 5-hydroxytryptophan (5-HTP) (Endo, 1985a). Carbidopa is a competitive inhibitor of aromatic amino acid decarboxylase and can suppress the formation of 5-HT from 5-HTP. As shown in a previous study, however, a large dose of the agent (50 to 100 mg kg^{-1}) was required to suppress the formation of 5-HT in the liver from 5-HTP administered to mice (Endo, 1985a). Additionally, the agent has been shown not to be a specific inhibitor, because it also inhibits other enzymes in the tryptophan oxidative pathway at concentrations that are likely to be encountered *in vivo* after administration to experimental animals (Bender, 1980; Smith & Pogson, 1981). In fact, in spite of the protective effect of carbidopa on the tryptophan-induced hypoglycaemia, it has been suggested that 5-HT is unlikely to mediate the hypoglycaemic response to tryptophan (Smith *et al.*, 1980; Lloyd *et al.*, 1982a,b). Therefore, the possibility that metabolites of 5-HTP other than 5-HT may contribute to the development of 5-HTP-induced hypoglycaemia cannot be excluded. In the present study, I tested the effect of α-monofluoromethyldopa (FMD), a potent irreversible inhibitor of aromatic amino acid decarboxylase (Kollonitsch *et al.*, 1978; Jung *et al.*, 1979), on 5-HTP-induced hypoglycaemia.

Various mitogenic substances, including lipopolysaccharides (LPS, endotoxin), cause accumulation of 5-HT predominantly in the liver, and these substances can also produce hypoglycaemia (Endo, 1983; 1984; Endo *et al.*, 1985b). Development of the hypoglycaemic response and the accumulation of 5-HT in the liver correlate well with each other in terms of time course and dose-response. In the present study, therefore, the effects of carbidopa and FMD on these responses induced by LPS were also examined.

Methods

Determination of 5-hydroxytryptamine and blood glucose

Male ddI mice were obtained from the Mouse Centre of this university. The mice were kept under fixed conditions of light and dark (1900 h to 0700 h) and fed *ad libitum*. All injections were made to fed mice (6 to 7 weeks old, 24 to 27 g body weight) between 0900 h to 1200 h. Sampling of tissues and blood and determination of 5-HT and blood glucose were the same as described previously (Endo, 1984).

Assay of 5-hydroxytryptophan decarboxylase activity

Tissues were homogenized in 10 volumes of 0.1 M sodium phosphate buffer (pH 7.0) containing 0.1 mM pyridoxal-5'-phosphate by using an Ultra Turrax homogenizer (Janke & Kunkel Co., West Germany). The supernatant obtained by centrifugation (20,000 *g*, 20 min at 4°C) was used as the enzyme solution. The enzymatic reaction was carried out in 1 ml of 0.04 M sodium phosphate buffer (pH 7.0) containing pyridoxal-5'-phosphate (0.04 mM), dithiothreitol (0.5 mM), pargyline (1 mM), 5-HTP (1 mM) and the enzyme solution (0.05 or 0.1 ml). After incubation at 37°C for 1 h, the reaction was terminated by the addition of 2 ml of 0.4 M $HClO_4$ containing 2 mM EDTA 2Na and 0.1% cysteine HCl. The reaction was linear during the incubation period. The 5-HT generated in the reaction mixture was determined as described previously (Tadano *et al.*, 1980).

Chemicals

5-Hydroxy-L-tryptophan (5-HTP) was purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.). (±)-α-Monofluoromethyldopa (FMD) was a gift from Merrel Dow Research Institute (Strasburg, France). A lipopolysaccharide (LPS) derived from *Escherichia coli* 055:B5, prepared by the Boivin method, was obtained from Difco, Lab. (Detroit, MI U.S.A.). Other agents were from Wako Chemical Ind. (Osaka, Japan). Agents were dissolved in 0.9% w/v NaCl solution (saline) and injected into mice intraperitoneally (5-HTP and FMD) or intravenously through tail vein (LPS). In combined injections of these agents, the total volume was limited to less than 0.4 ml per mouse.

Results

Effects of α-fluoromethyldopa on 5-hydroxytryptophan decarboxylase activity

To determine appropriate doses of FMD, its effect on the decarboxylation of 5-HTP was examined as follows: various doses of FMD were injected into mice, tissues were removed 3 h later, and 5-HTP decarboxylase activity in the tissues was determined (Table 1). In this experiment, FMD did not show any significant effect on the level of blood glucose. The activity of the enzyme was highest in the liver, and its activity was inhibited almost completely by 2 mg kg^{-1} FMD. On the other hand, the activity in the brain was less affected by the FMD treatment, i.e. about 80% of the activity remained after the treatment with 2 mg kg^{-1} FMD. In order to inhibit the formation of 5-HT selectively in the liver, therefore, this dose was used in the next experiment.

Effects of α-fluoromethyldopa on 5-hydroxytryptophan-induced hypoglycaemia and on accumulation of 5-hydroxytryptamine

As shown in a previous study (Endo, 1985a), 250 mg kg^{-1} 5-HTP produced a profound hypoglycaemia in our mice without death. The treatment of the mice with 2 mg kg^{-1} FMD before the administration of 5-HTP prevented completely the hy-

Table 1 5-Hydroxytryptophan (5-HTP) decarboxylase activity in the tissues of mice treated with α-monofluoromethyldopa (FMD)

FMD (mg kg^{-1})	*5-HTP decarboxylase activity* (nmol h^{-1} g^{-1})				
	Brain	*Liver*	*Kidney*	*Intestine*	*Spleen*
0	435 ± 27 (100)	5544 ± 198 (100)	1200 ± 87 (100)	132 ± 27 (100)	21 ± 2 (100)
0.4	387 ± 24 (89)	669 ± 150* (12)	81 ± 21* (7)	22 ± 2* (16)	17 ± 4 (80)
2	354 ± 18*(81)	90 ± 18*(2)	22 ± 3* (2)	10 ± 4* (7)	11 ± 2* (52)
10	189 ± 21*(43)	<30	<8	6 ± 3 (4)	<3
50	33 ± 18*(8)	<30	<8	<3	<3

Mice were killed at 3 h after the injection of FMD, and 5-HTP decarboxylase activities were determined. Each value is the mean ± s.d. of 3 mice. Values in parentheses are % activity. Significantly different from control, $*P < 0.05$ (Student's *t* test).

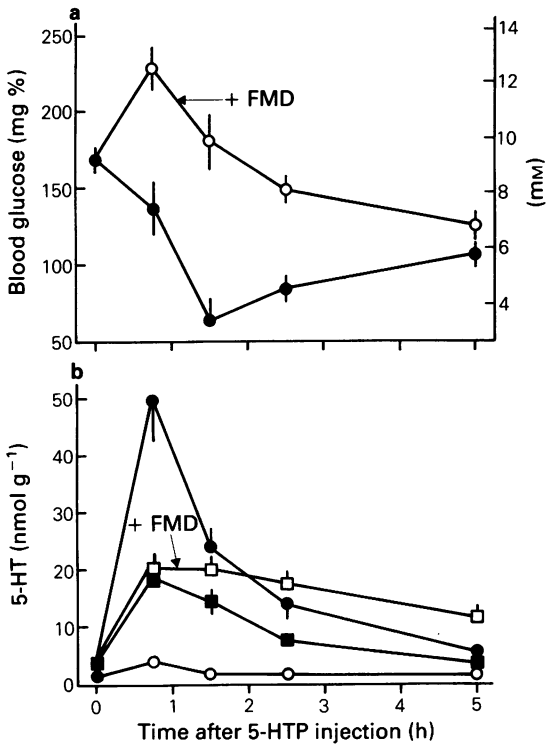

Figure 1 Effects of α-monofluoromethyldopa (FMD) on (a) the hypoglycaemic response to 5-hydroxytryptophan (5-HTP) and (b) on the accumulation of 5-hydroxytryptamine (5-HT) in the liver and brain. FMD (2 mg kg^{-1}) was injected into mice 1.5 h before the injection of 5-HTP (250 mg kg^{-1}). (a) (○) FMD-treated and (●) control (saline injected) mice. (b) Accumulation of 5-HT in the liver, in control (●) and FMD-treated (○) mice, and in the brain in control (■) and FMD-treated (□) mice. Each value is the mean from 4 mice; s.d. shown by vertical lines.

poglycaemia, or rather, produced hyperglycaemia at an early period after the 5-HTP injection (Figure 1). Control mice given saline or FMD alone did not show such a hyperglycaemic response (data not shown). Although the reason is not clear, a similar initial hyperglycaemic effect was also observed in the experiments using carbidopa (Endo, 1985a).

The accumulation of 5-HT in the liver was also entirely suppressed by the FMD treatment throughout the experimental period (Figure 1). In contrast, there was no inhibition of 5-HT formation in the brain, or rather, its elevated level of 5-HT was retained for a longer period, as has been observed in a previous study using carbidopa (Endo, 1985a).

Potentiation of 5-hydroxytryptophan-induced hypoglycaemia by α-fluoromethyldopa

This study produced the unexpected finding that a low dose of FMD sometimes killed the mice and potentiated the hypoglycaemic response to 5-HTP. To investigate this effect, mice were treated with various doses of FMD and given 5-HTP at a dose producing a mild degree of hypoglycaemia (150 mg kg^{-1}, Endo, 1985a). As shown in Figure 2, the treatment with

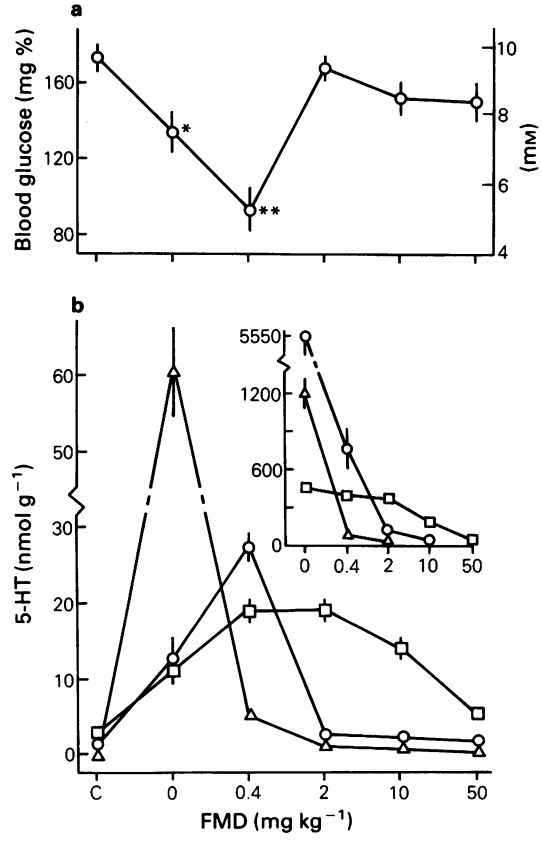

Figure 2 (a) The hypoglycaemic response to 5-hydroxytryptophan (5-HTP). (b) The accumulation of 5-hydroxytryptamine (5-HT) in the brain (□), liver (○) and kidney (△) of mice treated with various doses of α-monofluoromethyldopa (FMD). The FMD was injected into mice 1.5 h before an injection of 5-HTP (150 mg kg^{-1}), and the mice were killed 1.5 h after the 5-HTP injection. C, values from control (saline-injected) mice. Each value is the mean from 4 mice; s.d. shown by vertical lines. *$P < 0.05$ vs control, **$P < 0.05$ vs *. The insert in (b) shows the 5-HTP decarboxylase activities in the brain (□), liver (○) and kidney (△) presented in Table 1.

0.4 mg kg^{-1} FMD potentiated the hypoglycaemic response to 5-HTP, but FMD 2 mg kg^{-1} or more prevented completely the fall in blood glucose.

The levels of 5-HT in the liver, brain and kidney in this experiment are also shown in Figure 2 together with the data on 5-HTP decarboxylase presented in Table 1 for convenience. The accumulation of 5-HT in the liver was potentiated by 0.4 mg kg^{-1} FMD, but it was inhibited completely by 2 mg kg^{-1} or more of FMD. These effects corresponded well to those on blood glucose. In the brain, the elevation of 5-HT was potentiated by FMD in a range 0.4 to 10 mg kg^{-1}.

It is noticeable that in the absence of FMD, 5-HT formation in the kidney was higher than in other tissues, in spite of a lower 5-HTP decarboxylase activity than that in the liver. Both 5-HT formation and 5-HTP decarboxylase activity in the kidney was inhibited completely by 0.4 mg kg^{-1} FMD.

Ineffectiveness of α-fluoromethyldopa on lipopolysaccharide-induced hypoglycaemia and on 5-hydroxytryptamine accumulation in the liver

LPS produces an accumulation of 5-HT in the liver but not in the brain (Endo, 1983). After injection of LPS into mice, the 5-HT level in the liver was elevated within 2 h and reached its maximum level within 3 to 5 h. Corresponding to the 5-HT increase, blood glucose declined (Endo, 1984). However, treatment of mice with FMD, as well as with carbidopa, failed to prevent either the 5-HT increase or the hypoglycaemia induced by LPS, i.e. about a 5 fold increase in 5-HT and a 45% decline in blood glucose at 4.5 h after LPS injection (0.5 mg kg^{-1}) were not affected by treatment with FMD (10, 20 and 40 mg kg^{-1}, 0.5 h before LPS) or carbidopa (90 mg kg^{-1}, 0.5 h after LPS).

Discussion

In previous experiments using carbidopa, a competitive inhibitor of aromatic amino acid decarboxylase, the hypoglycaemic response to 5-HTP corresponded well to the accumulation of 5-HT in the liver but not in the brain (Endo, 1985a). In the present study, the suppressive effects of FMD, a potent irreversible inhibitor of the enzyme, on the accumulation of 5-HT and hypoglycaemia were clearly shown and were essentially the same as those of carbidopa. That is, a close correlation was again observed between the accumulation of 5-HT in the liver and the hypoglycaemic response to 5-HTP.

Additionally and interestingly, a low dose of FMD (0.4 mg kg^{-1}) potentiated both the accumulation of 5-HT in the liver and the hypoglycaemic response to 5-HTP (Figure 2). These effects of FMD can be explained as follows. In the absence of FMD, the kidney was the most active organ for the formation of 5-HT from the 5-HTP administered, in spite of there being lower 5-HTP decarboxylase activity (1200 nmol h^{-1} g^{-1}) in this organ than in the liver (5500 nmol h^{-1} g^{-1}). The low dose of FMD (0.4 mg kg^{-1}) inhibited almost completely the activity in the kidney (only 81 nmol h^{-1} g^{-1} remained) and inhibited entirely the formation of 5-HT in this organ. This inhibitory effect in the kidney may result in an increased amount of 5-HTP entering the liver (and also the brain) which retains a high level of decarboxylase activity (670 nmol h^{-1} g^{-1}) leading to an accelerated production of 5-HT. Therefore, these observations also support the idea that the accumulation of 5-HT in the liver is involved in the hypoglycaemic response to 5-HTP.

It has been suggested that various metabolites of tryptophan, including 5-HT, inhibit hepatic gluconeogenesis at the step catalysed by phosphoenolpyruvate carboxykinase (Smith *et al.*, 1979). In addition, 3-mercaptopicolinic acid, an analogue of quinolinate, has been shown to be a potent inhibitor of the enzyme and to produce hypoglycaemia (DiTullio *et al.*, 1974; Jomain-Baum *et al.*, 1976). Therefore, it remains to be clarified whether the active component of 5-HTP metabolites capable of inducing hypoglycaemia is 5-HT itself or metabolites of 5-HT and/or both. In the case of the metabolites, products from the monoamine oxidase pathway seem to be unlikely candidates, because monoamine oxidase inhibitors markedly potentiate the hypoglycaemic response to 5-HTP (Endo, 1985a).

As described in the Introduction, LPS causes an accumulation of 5-HT in the liver and produces hypoglycaemia. In addition, it has been suggested that a major mechanism underlying LPS-induced hypoglycaemia may be an impairment of hepatic gluconeogenesis (McCallum & Berry, 1973; Filkins & Cornell, 1974). Therefore, it is expected that the accumulation of 5-HT in the liver may be causally related to the hypoglycaemic response to LPS as in the case of 5-HTP. However, neither the accumulation of 5-HT nor the hypoglycaemic response to LPS was prevented by the treatment with FMD or carbidopa. These results indicate that the LPS-induced 5-HT accumulation in the liver is not due to the stimulation of 5-HT synthesis via the decarboxylase of 5-HTP. Results from preliminary experiments using mice treated with reserpine, which depletes amines from platelets and the nervous system, have suggested that the accumulation of 5-HT may be derived from the transfer of 5-HT and/or platelets into the liver from other sites through the circulation. The 5-HT accumulation induced by LPS was markedly diminished in the mice treated with reserpine (2 days before LPS), even though reserpine itself has been shown to be hypoglycaemic (Lernmark,

1971). The causal relationship between the accumulation of 5-HT in the liver and the hypoglycaemic response to LPS is still not clear.

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The effect of nicotine on motoneurones of the immature rat spinal cord *in vitro*

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1 Nicotine [(−)-nicotine di(+)-tartrate 1–50 μM] produced depolarization of motoneurones as recorded from ventral roots of immature (1–5 day old) rat hemisected spinal cord preparations. This action of nicotine was accompanied by marked desensitization which persisted for at least 2 h following a 2 min application.

2 Ventral roots sectioned from the spinal cord, which were sensitive to glycine, failed to respond to nicotine.

3 Blockade of regenerative electrical activity with tetrodotoxin produced a mean reduction of 39% in the response to 10 μM nicotine.

4 In order to avoid desensitization, blocking agents were tested for their ability to suppress the initial response to a 2 min application of 10 μM nicotine (0.58 mV ± 0.07 s.e.mean, 21 preparations) in 4 or more naïve preparations. Responses to nicotine (10 μM) were significantly reduced by 10 μM hexamethonium and were abolished by 250 μM hexamethonium but were resistant to the following antagonists:- atropine (1 μM), phentolamine (2 μM), strychnine (10 μM), kynurenic acid (2 mM) and a mixture of bicuculline (50 μM) and picrotoxin (50 μM).

5 It is concluded that the depolarizing responses to nicotine may be due to the presence of nicotinic receptors either on the motoneurone membrane or on nerve terminals adjacent to motoneurones which release an unidentified neurotransmitter.

Introduction

Muscarinic sensitivity of immature rat motoneurones *in vitro* has been described previously (Evans, 1977) in agreement with an earlier finding that electrophoretically applied acetylcholine produces an atropine-sensitive response of cat motoneurones *in vivo* (Zieglgansberger & Bayerl, 1976). Nicotinic activity of cholinomimetics was not observed in either of these studies.

However, the chance observation of a depolarizing action of nicotine on the immature rat spinal cord preparation has led to the present study. Resistance of this effect to atropine and other antagonists and its sensitivity to hexamethonium suggests that immature motoneurones may possess cholinoceptors of the nicotinic type as well as the muscarinic receptors described previously.

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Methods

Hemisected spinal cords or ventral roots were taken from rats 1–6 days after birth (body weight 6–11 g). The preparations were maintained at 25°C and they were set up for recording of d.c. levels from ventral roots (L5) with superfusion of Ringer solution and drugs as previously described (Evans, 1978). Some preparations were electrically stimulated by means of a stainless steel wire in contact with the L5 dorsal root.

Tetrodotoxin was used in many of the experiments to block regenerative activity. As an economy measure the toxin was applied initially at 1–2.5 μM for 2 min and superfused thereafter in the bathing medium at 0.1 μM. As previously observed (Evans & Watkins, 1978) this procedure produced complete blockade of dorsal root evoked and spontaneous synaptic activity recorded in ventral roots.

Superior cervical ganglia were taken from rats of 150 to 250 g body weight. Ganglia were desheathed. Superfusion of ganglia with Ringer solution and drugs

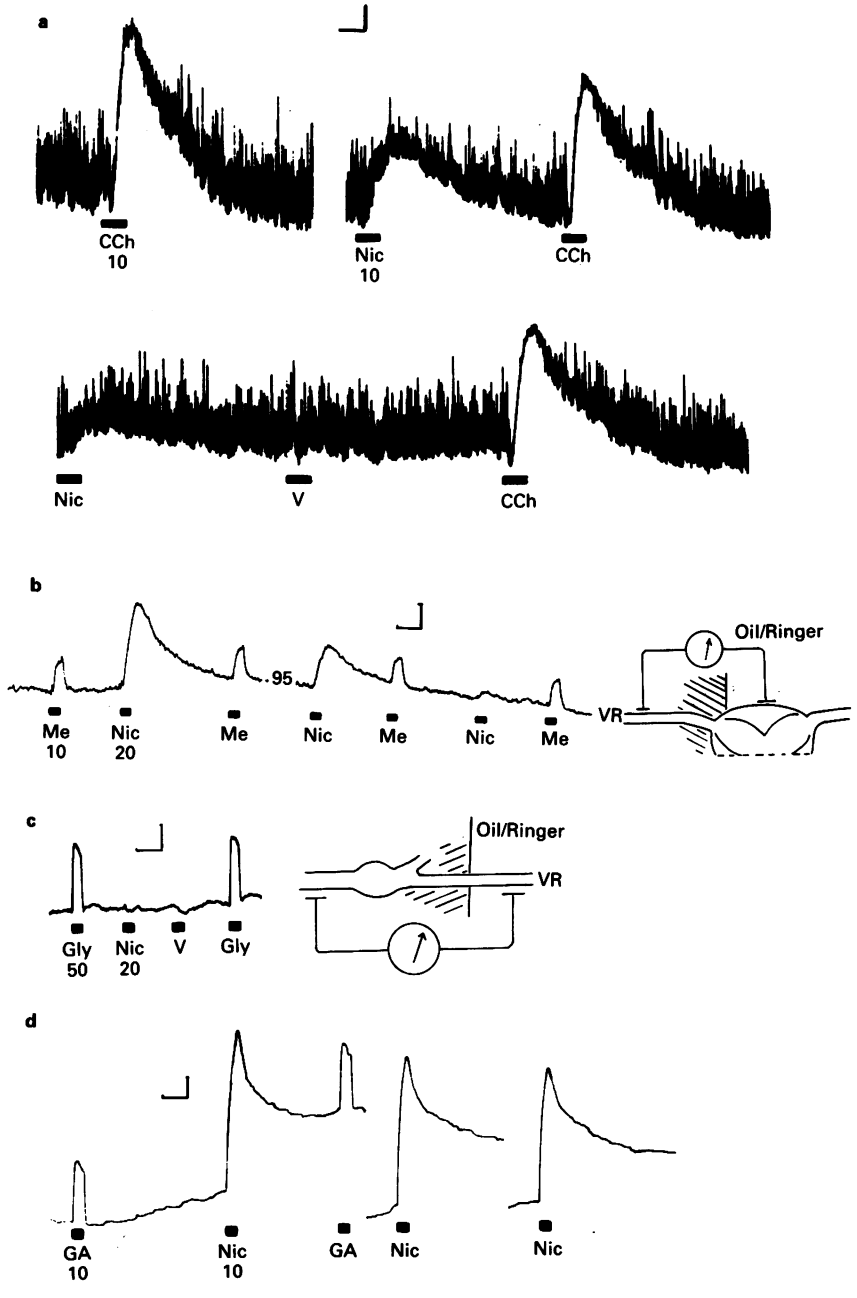

Figure 1 (a) Depolarizing action of carbachol (CCh) and nicotine (Nic) measured in ventral root of hemisected spinal cord. (b) A different preparation bathed in tetrodotoxin (0.1 μM). Note the absence of spontaneous synaptic activity. Methacholine was applied at Me. (c) Effect of glycine (Gly) and nicotine on an isolated ventral root preparation. (d) Effect of nicotine and GABA (GA) on a superior cervical ganglion preparation. Sham doses consisting of bathing medium containing a volume of distilled water equivalent to the volume of stock agonist solution were applied at V. Agonist concentrations indicated in μM. Method of recording in (a) and (b) as indicated in (b), and (c) as shown to the right. Vertical calibration 0.2 mV, horizontal 2 min in (a) and 5 min in (b), (c) and (d).

and recording from the postganglionic nerve were carried out with the same apparatus and methods used for the spinal cord preparations. Ganglia were only used for experiment if they gave a postganglionic response to electrical stimulation of the preganglionic nerve.

All chemicals were obtained from well known commercial sources.

Statistical analysis of results

A runs test, for crossings of the median, on the 21 control depolarizations presented in Figure 4 yielded $P > 0.13$ suggesting non-random grouping of values within the series to be insignificant. Application of the Rankit graphic method for small samples showed that this series of 21 control values was not distributed normally about the mean. Therefore significance of differences between control and drug-treated groups (Figure 4) was tested for, using the non-parametric (Kruskal-Wallis) one way analysis of variance.

The Kruskal-Wallis test is unable to indicate which of the several groups tested are different from each other. Unfortunately, tables which allow such distinction from a single rank order analysis on groups of unequal size are, to our knowledge, not available. However, it was found that a significant difference between groups ($P < 0.05$) only occurred when either of the groups treated with hexamethonium was included within the ranked variates. The group of four preparations treated with strychnine in the presence of tetrodotoxin, being too small, was not included in this analysis.

The Spearman rank coefficient, for the 37 data points of Figure 2, was used to show the correlation of dose with response.

Results

Nicotine (1–50 μM) produced depolarizations recorded in ventral roots which were comparable in time course and amplitude to those produced by carbachol. However, whereas the repetitive application of carbachol produced consistent responses, nicotine-induced responses were subject to marked desensitization (Figure 1a). Such desensitization, produced by 10 μM nicotine, persisted for at least 2 h in the 23 preparations which were tested over this period. The muscarinic character of responses induced by carbachol has been described previously (Evans, 1977). Fading of responses induced by carbachol was not observed either in the present or in previous experiments (Evans, 1977), probably because nicotinic effects are manifest at higher concentrations than those used to induce the muscarinic actions (Dale, 1914) reported in these studies. However, because it seemed likely, from the behaviour of nicotine-induced responses, that any nicotinic component of the effect of carbachol would cause desensitization it was decided to use methacholine in subsequent experiments for comparative purposes.

To see if the time course of desensitization to nicotine observed in spinal cord preparations was different from that known to occur at peripheral sites, nicotine was applied to four isolated superior cervical ganglion preparations under the same experimental conditions used for spinal cord preparations. Ganglionic sensitivity and the time course of responses to nicotine were similar to those of the spinal cord but desensitization was insignificant providing intervals between doses were greater than 20 min (Figure 1d). Sensitivity of spinal cord preparations to nicotine persisted in the presence of tetrodotoxin (0.1 μM) as did the prolonged desensitization (Figure 1b). In 21 preparations treated with tetrodotoxin the application of 10 μM nicotine produced a mean depolarization (± s.e.mean) of 0.58 mV ± 0.07. In the absence of tetrodotoxin a mean depolarization of 0.95 ± 0.22 mV was obtained from seven preparations (Figure 4). Since this indicated the possibility of some indirect action of nicotine it was decided to compare the effects

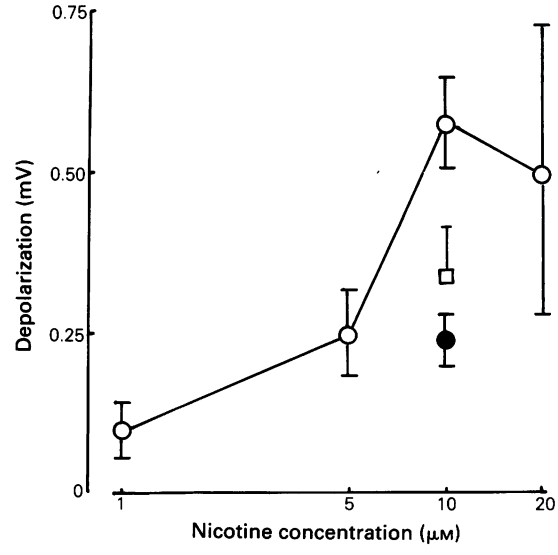

Figure 2 Dose-response plot comprised of mean depolarization produced by first dose of nicotine in at least 5 preparations in the presence of tetrodotoxin (○); vertical bars show s.e.mean. (□) The increased mean response produced by a second dose of 10 μM nicotine applied 30 min after the 1 μM dose; (●) the mean response in the presence of 10 μM hexamethonium (data from Figure 4).

of subsequent antagonists in the presence of this concentration of tetrodotoxin.

Because of the problem of desensitization a useful dose-response plot from a single preparation (Evans, 1977) could not be constructed. Since the response to the first dose of nicotine applied to a preparation is not subject to the conditioning effect of a previous dose it should, in theory, have been possible to construct a dose-response plot using the first responses to nicotine obtained from a number of preparations. However,

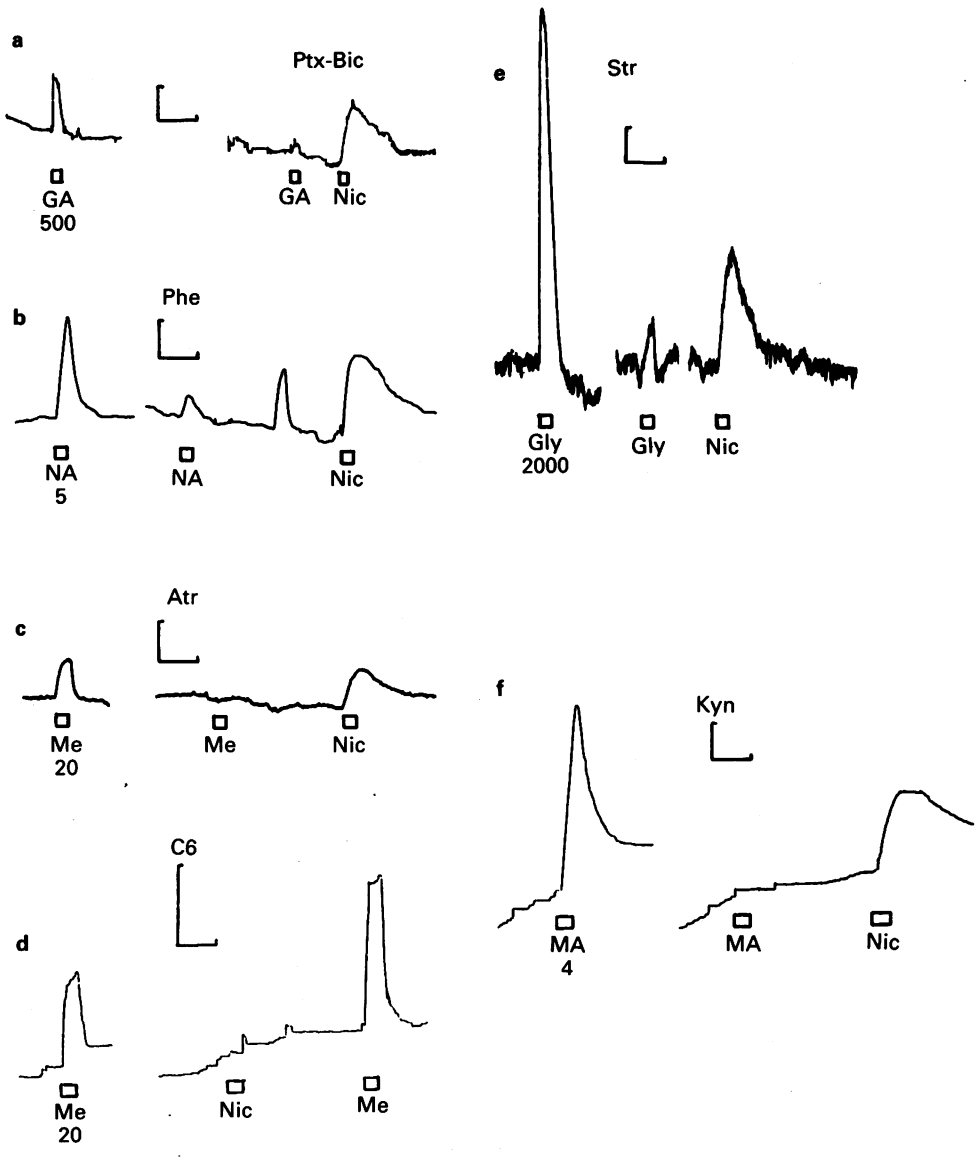

Figure 3 Effect of six different antagonist substances on depolarization induced by nicotine (10 μM) in preparations (a) to (f). Ptx-Bic, mixture of picrotoxin (50 μM) and bicuculline (50 μM); Phe, phentolamine (2 μM); Atr, atropine (1 μM), C6, hexamethonium (250 μM); Str, strychnine (10 μM); Kyn, kynurenic acid 2 mM. Antagonists were applied 15 to 30 min before application of nicotine. Tetrodotoxin was present throughout. Control agonist responses are shown before introduction of antagonist. NA, noradrenaline; Me methacholine, MA N-methyl-D-aspartate; other abbreviations for agonists as for Figure 1. Concentrations of agonist (μM) are indicated below each record. Calibration 0.25 mV and 5 min. Other details as for Figure 1. Method of recording as for Figure 1a and b.

the amplitudes of such responses from naïve preparations were too variable to provide a practicable method of assessing displacement by antagonists (see Figure 2). Thus various parameters were investigated, including the amplitudes of synaptic responses and of responses evoked by methacholine, N-methylaspartate or potassium chloride, as a means of normalizing response amplitudes from different preparations. None of these parameters was found to vary in consistent proportion to the amplitude of responses of naïve preparations to nicotine. Figure 2 shows depolarizations induced in several preparations treated with a first dose of 1 to 20 μM nicotine. Despite the large variance, dose-dependence of the effect of nicotine is evident. The correlation of concentration of nicotine with depolarization produced was significant at the $P<0.01$ level (Spearman rank correlation coefficient 0.457, $t=3.042$, 36 d.f.). Application of 10 μM nicotine to each of the five preparations already treated with 1 μM nicotine, 30 min previously, caused a greater effect in every case (mean depolarization 0.34 mV $\pm$ 0.04, Figure 2 open square). This increased response produced by 10 μM nicotine was significant at the $P<0.01$ level (single tailed, Mann-Whitney U test). This further illustrates the dose-dependence of the effect of nicotine. The mean depolarization caused by this second dose of 10 μM nicotine, although lower, was found to be not significantly different from the mean first-dose control responses in Figure 4 (Mann-Whitney U test). This suggests that the desensitizing effect of 1 μM nicotine was not so great as that which occurred with 10 μM nicotine (Figure 1).

Since the amplitude of depolarizations produced by repeated doses of nicotine declined, as illustrated in Figure 1a and b, antagonists were tested for their ability to abolish responses of naïve preparations to the first application of 10 μM nicotine. The probability that naïve preparations would fail to respond to the application of 10 μM nicotine with a depolarization of at least 0.1 mV was very low since none of the 26 preparations tested with 5 or 10 μM nicotine failed to respond with a depolarization of less than 0.1 mV.

Five preparations treated with hexamethonium (250 μM), prior to application of nicotine, failed to respond to nicotine whereas a similar treatment of five preparations with atropine (1 μM) suppressed respon-

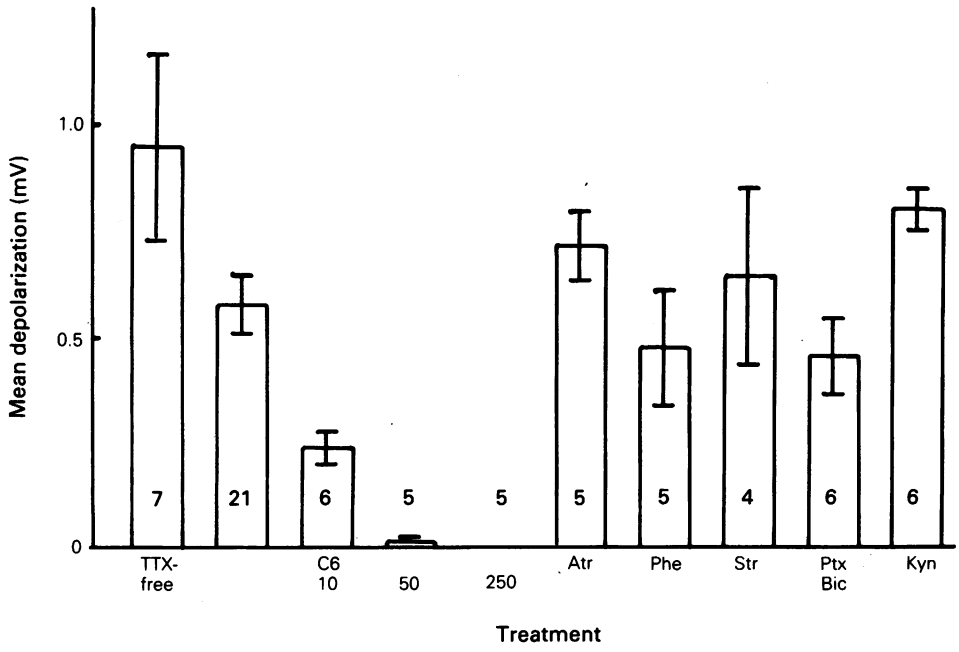

Figure 4 Effects of antagonists on mean depolarization evoked by first dose of nicotine (10 μM) applied to the number of preparations indicated within the column; vertical bars show s.e.mean. Tetrodotoxin (TTX) (0.1 μM) was present in all experiments excepting those represented by the left hand column. The concentrations (μM) of hexamethonium are shown below the columns. Other abbreviations and concentrations of antagonists are as for Figure 3. Only the means obtained in the presence of hexamethonium were significantly different (for the 10 μM hexamethonium data compared to all non-hexamethonium data $P<0.05$, Kruskal-Wallis test) from the mean control response measured in the presence of tetrodotoxin (second column from left).

ses to methacholine but not to nicotine. To guard against the possibility that a weak nicotinic action of methacholine could have desensitized preparations to nicotine, methacholine was applied to only four of the hexamethonium-treated preparations before the application of nicotine. The mean depolarization produced by 10 μM nicotine was markedly depressed by 50 or 10 μM hexamethonium (Figure 4). The effect of 50 μM hexamethonium on one of these preparations is illustrated in Figure 3d.

Treatment of five preparations with 2 μM phentolamine, which produced a marked depression of noradrenaline-induced responses, did not significantly alter the mean response to 10 μM nicotine (Figure 3b and 4).

The depressant amino acids γ-aminobutyrate (GABA) and glycine are known to produce depolarization recorded in isolated ventral roots of the present preparations (Evans, 1980). It was possible therefore that the depolarizing action of nicotine could have been mediated through release of either of these amino acids from the terminals of inhibitory interneurones. However, nicotine (10 μM) depolarized five preparations (one of these was tested in the absence of tetrodotoxin) in the presence of 10 μM strychnine which produced marked antagonism of depolarizations induced by glycine (Figures 3e and 4). Nicotine (10 μM) still produced marked depolarization in the presence of a mixture of bicuculline (50 μM) and picrotoxin (50 μM) which produced dose-ratios for antagonism of GABA responses of at least 20 (Figures 3a and 4).

The effect of antagonists of excitant amino acids on synaptic transmission in the spinal cord indicates that there are large numbers of excitatory terminals which release such amino acids onto motoneurones (see Watkins & Evans, 1981). Such terminals are possible candidates for the site of action of nicotine. Thus six preparations were treated with kynurenic acid (2 mM) which is an antagonist at the three major receptor types for excitant amino acids (Perkins & Stone, 1982). A mean dose-ratio of 119 ± 25 s.d. was obtained for the antagonism of depolarizations induced by N-methyl-D-aspartate in these kynurenate-treated preparations. Nicotine (10 μM) produced marked depolarization of all six preparations (Figures 3f and 4).

The mean depolarizations evoked by nicotine, in the presence of each antagonist, are presented in Figure 4. The data for each antagonist were compared with the mean depolarization induced by 10 μM nicotine on 21 control preparations. Only in the case of hexamethonium were the differences significant.

Discussion

The results show that application of nicotine produces depolarization as recorded in ventral roots of immature spinal cord preparations. This action of nicotine was subject to marked desensitization. In the present experiments responses to nicotine recorded from sympathetic ganglia were similar in time course to those from spinal cord and to those previously reported in ganglia from kittens (Brown, 1966a,b) and were, unlike the responses of spinal preparations, not subject to long lasting desensitization. Such long lasting desensitization does not appear to be a general property of central nicotinic receptors since it was not observed in previous studies on spinal cord (Curtis & Ryall, 1966) or brain (Clarke *et al.*, 1985). The desensitization of nicotinic responses would explain why such effects were not detected in previous experiments (Evans, 1977).

Unfortunately, the slow recovery from desensitization prevented construction of dose-response plots and consequently the direct comparison of the action of pharmacological agents on responses to nicotine within the same preparation. However, of the eight blocking agents tested, only hexamethonium produced a significant change in the sensitivity of naïve preparations to application of a first dose of nicotine (Figure 4). Tetrodotoxin, at a concentration sufficient to block dorsal root evoked and spontaneous electrical activity of the preparations, reduced the mean response to 10 μM nicotine by approximately 40%. This difference, although not significant by the statistical test employed, may reflect an action of nicotine on interneurones and/or primary afferent terminals relayed by synaptic transmission to motoneurones. Such a component would be analogous to nicotine-induced contractures of the gut which are mediated through the activation of neurones in autonomic ganglia (Feldberg, 1951). Since ventral root fibres were insensitive to nicotine (Figure 1c) the tetrodotoxin-resistant component of nicotine-induced responses may reflect an action on the motoneurone soma or dendrites. However it is possible also that nicotine caused release of transmitter(s) from synaptic terminals juxtaposed to motoneurones analogous to the nicotine-induced release of catecholamines from chromaffin cells (Burn *et al.*, 1959).

There are many possible candidates for such mediators but those for which sufficiently selective antagonists are available are relatively few in number. The depressant amino acids have been shown to depolarize ventral root fibres in these preparations (Evans, 1980); thus it was considered possible, although unlikely, that release of such amino acids could induce depolarization at the somata or dendrites of motoneurones. Also since a strong noradrenergic innervation of the spinal cord has been demonstrated (Nygren & Olsen, 1977), corresponding to a phentolamine-sensitive depolarizing action of noradrenaline in the present preparation (Ault & Evans, 1978),

it was thought possible that release of noradrenaline also could have explained the nicotine-induced responses. Acetylcholine acting at muscarinic sites would also be a candidate for the role of an endogenous depolarizing substance although previous experiments have shown the depolarization produced by application of neostigmine, presumably due to endogenous acetylcholine, to be abolished by tetrodotoxin (Evans, 1977). Perhaps the most likely agents to mediate depolarization of motoneurones are excitant amino acids since spontaneous and reflex activity in the present preparations is depressed markedly by antagonists of excitant amino acids (Evans *et al.*, 1982; Ganong *et al.*, 1983).

Because of these possibilities, sensitivity to nicotine was tested in the presence of bicuculline, picrotoxin, strychnine, phentolamine, atropine and kynurenic acid at levels which produced marked depression of depolarizing responses induced by appropriate agonists. Examples of typical tests are shown in Figure 3 but the probability of antagonism having occurred can be judged only from the significance of the differences shown in Figure 4. Thus the only antagonist shown to depress nicotine-induced responses was hexamethonium which had a significant effect on responses to nicotine at 10 μM and abolished them at 250 μM. If it is assumed that the effect of hexamethonium was competitive in the present experiments then comparison of the mean depolarization induced by 10 μM nicotine in the presence of 10 μM hexamethonium with the dose-response plot in Figure 2 (filled circle) is consistent with the reported affinity constant of hexamethonium at ganglionic sites in the gut (Barlow & Franks, 1971).

Therefore it would appear that, in these immature preparations, nicotinic receptors are present either on motoneurones or on terminals juxtaposed to motoneurones which release an unidentified agent. It remains to be seen whether these receptors are present in the mature spinal cord and have a functional role there or whether they are unique to the immature spinal cord.

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Differential effect of temperature on histamine- and carbachol-stimulated inositol phospholipid breakdown in slices of guinea-pig cerebral cortex

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1 Slices of guinea-pig cerebral cortex were incubated with [^{3}H]-inositol at 37°C before exposure to histamine or carbachol at 37°C or 25°C. Histamine-stimulated accumulation of [^{3}H]-inositol 1-phosphate ([^{3}H]-IP_1) at 25°C was only 5–7% of that at 37°C, whereas for carbachol the response at 25°C was 45–49% of that at 37°C.

2 The affinity of benzilylcholine, obtained from inhibition of carbachol-induced accumulation of [^{3}H]-IP_1 was similar at 25°C and 37°C, but the EC_{50} for carbachol was lower at 25°C (20 ± 2 μM) than at 37°C (42 ± 2 μM).

3 The IC_{50} for histamine inhibition of [^{3}H]-mepyramine binding to homogenates of guinea-pig cerebral cortex did not differ significantly at 25°C and 37°C.

4 Histamine-induced accumulations of [^{3}H]-IP_2 and [^{3}H]-IP_3 at 25°C, expressed as a percentage of the accumulation at 37°C, were also much less than the corresponding value for carbachol.

5 These observations imply that the locus or pathway(s) of agonist-induced formation of [^{3}H]-IP_1 are not the same for histamine and carbachol.

Introduction

There is now a wealth of evidence that receptor-coupled inositol phospholipid breakdown, may be an early step in the chain of events which leads to the cellular response to 'calcium-mobilising' agonists (Michell *et al.*, 1981; Berridge, 1984; Berridge & Irvine, 1984). In most tissues the agonist-catalysed step appears to be the hydrolysis of phosphatidylinositol 4,5-bisphosphate, but in some cells, such as thrombin-stimulated platelets (Wilson *et al.*, 1985), other mechanisms may contribute to the formation of inositol 1-phosphate (IP_1). Whether such secondary mechanisms also operate in cells of the mammalian CNS is not established, but there are features of the accumulation of IP_1 induced by histamine in lithium-treated slices of guinea-pig brain which suggest that the actions of histamine may be more complex than the simple reaction schemes would suggest (Carswell *et al.*, 1985; Carswell & Young, 1985). The same may be true in slices of guinea-pig intestinal smooth muscle, where the response to histamine is relatively insensitive to histamine H_1-antagonists (Donaldson & Hill, 1985).

In the course of an investigation of the properties of histamine-induced accumulation of IP_1 in slices of guinea-pig cerebral cortex, we observed that lowering the temperature to 25°C appeared to have a much greater depressive effect on the response than would have been expected from measurements on other systems, such as thyrotropin releasing hormone (TRH)-stimulated GH_3 pituitary tumour cells (Drummond *et al.*, 1984). To try and establish whether the sensitivity of the histamine response is unusual, we have compared the effect of temperature on the responses to histamine and to the muscarinic agonist carbachol in slices of guinea-pig cerebral cortex. Some of these results have been presented in preliminary form to the British Pharmacological Society (Carswell *et al.*, 1986).

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Methods

Agonist-induced accumulation of [^{3}H]-inositol phosphates

Cross-chopped slices (350 × 350 μm, McIlwain tissue chopper) of guinea-pig (Dunkin-Hartley strain, males) cerebral cortex were washed three times and then incubated at 37°C for 60 min in Kreb-Henseleit medium (in mM: NaCl 116, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $HaHCO_3$ 25, $CaCl_2$ 2.5 and D-glucose 11) with three further changes of medium. The medium was bubbled throughout with O_2/CO_2 (95:5, vol/vol). The slices were then transferred to Krebs-Henseleit solution (8.4 ml per cortex) containing 0.33 μM *myo*-[^{3}H]-inositol and incubated for a further 30 min at 37°C. The slices were washed three times and then transferred to a flat-bottomed vial (Hughes & Hughes Ltd, scintillation vial insert) and allowed to settle under gravity. Aliquots of the slices (40 μl) were added to 200 μl Krebs-Henseleit medium containing 10 mM LiCl and 1 mM unlabelled *myo*-inositol and incubated for 15 min at 25°C or 37°C. At the end of this time histamine or carbachol (10 μl of the appropriate solution) was added and the incubation continued for the desired period before termination by addition of 0.94 ml chloroform/methanol (1:2, vol/vol) or 200 μl ice-cold 15% trichloroacetic acid.

This procedure, in which the [^{3}H]-inositol is removed before addition of the agonist + excess unlabelled inositol, is termed 'pulse-labelling'. In a few experiments, denoted as 'continuous labelling', the [^{3}H]-inositol was not removed following the 30 min incubation at 37°C and no unlabelled inositol was present during the period of exposure to the agonist. Otherwise the procedure was the same as described above.

In incubations terminated by addition of chloroform/methanol, [^{3}H]-inositol phosphates were extracted and separated essentially as described by Berridge *et al.* (1982). Chloroform (0.31 ml) and 0.31 ml water were added and the phases separated by centrifugation at 950 *g* for 5 min. A portion of the upper phase (0.8 ml) was applied to a column containing 2 ml of an approximately 1:1 slurry of Dowex-1 anion-exchange resin (formate form) and distilled water. The column was then washed with 5 ml water to remove any [^{3}H]-inositol, followed by 8 ml 60 mM ammonium formate/5 mM sodium tetraborate to remove [^{3}H]-glycerophosphoinositol. [^{3}H]-IP_1 was then eluted with 8 ml 200 mM ammonium formate/100 mM formic acid. In some experiments [^{3}H]-IP_2 and [^{3}H]-IP_3 were eluted with 8 ml 400 mM ammonium formate/100 mM formic acid and 8 ml 1 M ammonium formate/100 mM formic acid, respectively. Aquasol-2 (8 ml) was added to each fraction and tritium determined by scintillation counting.

In incubations terminated by addition of trichloroacetic acid the mixture was allowed to stand on ice for 15 min before centrifugation at 950 *g* for 5 min. A sample of the supernatant (250 μl) was extracted 5 times with ether to remove trichloroacetic acid and the aqueous layer then neutralised with Na tetraborate before separation of the [^{3}H]-inositol phosphates as described above.

Inhibition of [^{3}H]-mepyramine and [^{3}H]-methylscopolamine binding

Preparation of a membrance fraction from guinea-pig cerebral cortex and measurement of histamine inhibition of [^{3}H]-mepyramine binding in 50 mM Na-K phosphate buffer, pH 7.5, was carried out essentially as described previously (Aceves *et al.*, 1985), except that incubations were for 30 min at 37°C or 60 min at 25°C. The concentration of [^{3}H]-mepyramine was 0.37–0.57 nM and non-specific binding was defined with 2 μM promethazine. Pentuplicate measurements were made at 12–14 histamine concentrations.

Measurements of benzilylcholine inhibition of [^{3}H]-N-methylscopolamine binding were made similarly, with non-specific binding defined by 1 μM N-methylatropine.

Analysis of data

Concentration-response curves for histamine- and carbachol-induced accumulation of [^{3}H]-IP_1 following 60 min incubation with the agonist were constructed by combining the data (after subtraction of the accumulation in the absence of agonist) from two or more experiments. The response to 200 μM histamine or 1 mM carbachol was measured in all experiments and was used to correct for differences in the absolute level of accumulated [^{3}H]-IP_1 between slice preparations. The concentration-response curves were fitted to a Hill equation (logistic equation) using the Harwell library non-linear regression programme VB01A. The actual equation fitted was: [^{3}H]-IP_1 accumulated = $Resp_{max} \times D^n/(D^n + EC_{50}^n)$, where D is the agonist concentration, n is the Hill coefficient, EC_{50} is the concentration giving half-maximal response and $Resp_{max}$ is the maximum response. Each point was weighted according to the reciprocal of the variance associated with it. Repeated trials were made with different initial parameter estimates and the final best-fit values defined as those that were associated with the lowest residual.

The affinity constant of benzilylcholine against carbachol-induced [^{3}H]-IP_1 accumulation was obtained from parallel shifts of the concentration-response curve to carbachol, using the relationship: Concentration-ratio = $[A] \times K_A + 1$, where [A] is the concentration of benzilylcholine and K_a the affinity

constant. The concentration-ratio is the concentration of carbachol required for a given response in the presence of antagonist divided by the concentration required in the absence of antagonist.

Curves of the inhibition by histamine of [^{3}H]-mepyramine binding were fitted as described previously (Aceves *et al.*, 1985) using weighted non-linear regression analysis with the Hill coefficient, the IC_{50} (the concentration of histamine required for 50% inhibition of the histamine-sensitive component of the response) and the percentage of the response insensitive to inhibition by histamine as unknowns.

Drugs

Myo-[2-^{3}H]-inositol (16.3 Ci mmol^{-1}) and Aquasol-2 were purchased from New England Nuclear and [pyridinyl-5-^{3}H]-mepyramine (26 Ci mmol^{-1}) and (−)-[N-methyl-^{3}H]-N-methylscopolamine chloride (76 Ci mmol^{-1}) from Amersham International. Histamine dihydrochloride and carbachol (carbamylcholine chloride) were obtained from Sigma. Benzilylcholine chloride was prepared by the method of Ford-Moore & Ing (1947).

Results

Time-course and characteristics of histamine- and carbachol-stimulated [^{3}H]-inositol 1-phosphate accumulation

The time courses of histamine- and carbachol-stimulated accumulation of [^{3}H]-IP_1 in cerebral cortical slices prelabelled with [^{3}H]-inositol were similar to those described previously for histamine using the continuous labelling protocol (Daum *et al.*, 1984; Carswell *et al.*, 1985). The agonist-induced [^{3}H]-IP_1 accumulation increased approximately linearly with time, while the basal accumulation showed no significant change over the same period (5–90 min). The level of the basal accumulation was somewhat lower in experiments using the pulse-labelling protocol, 772 ± 61 d.p.m. (mean ± s.e.mean from 11 determinations), than in experiments carried out under continuous labelling conditions, 1032 ± 171 d.p.m. (34 experiments).

The EC_{50} and Hill coefficients for concentration-response curves for the accumulation of [^{3}H]-IP_1 induced by histamine and carbachol at 37°C were closely similar for both experimental protocols (Table 1). The pulse-labelling protocol was used in all subsequent experiments.

Agonist-induced [^{3}H]-inositol 1-phosphate formation at 37°C and 25°C

The response to carbachol was decreased when cerebral cortical slices, prelabelled at 37°C, were incubated with the agonist at 25°C rather than 37°C. The time-course of the response to 0.1 mM carbachol at 25°C is shown in Figure 1(a) and comparison made with the basal and agonist-stimulated levels measured at 45 min at 37°C on the same slice preparation in the same experiment. Essentially similiar results were obtained in a second independent experiment. The basal accumulation was not significantly reduced at the lower temperature, 578 ± 126 d.p.m. at 25°C (mean ± s.e.mean of 5 determinations) and 772 ± 61 at 37°C (11 determinations).

The response to 0.2 mM histamine was reduced to a much greater extent than that to carbachol on lower-

Table 1 Parameters of concentration-response curves for histamine and carbachol-induced accumulation of [^{3}H]-inositol 1-phosphate ([^{3}H]-IP_1)

[^{3}H]-inositol labelling	*Histamine* EC_{50} (μM)	n_H	*Carbachol* EC_{50} (μM)	n_H
37°C				
Continuous	16 ± 1	1.24 ± 0.03 (20)	42 ± 4	0.95 ± 0.05 (3)
Pulse	17 ± 1	1.21 ± 0.07 (2)	42 ± 4	1.02 ± 0.04 (2)
25°C				
Continuous		—	25 ± 2	0.91 ± 0.03 (2)
Pulse		—	20 ± 2	0.99 ± 0.06 (2)

Values are the best-fit parameters ± estimated s.e. obtained from non-linear regression analysis of concentration-response curves measured using pulse- or continuous-labelling conditions as described under Methods. The points fitted were the weighted means at each concentration from the number of independent experiments given in parentheses. n_H is the Hill coefficient. The values for histamine at 37°C under continuous-labelling conditions are taken from a series of experiments using a simplified protocol in which the labelling with [^{3}H]-inositol and incubation with histamine took place in the final incubation vial (Daum *et al.*, 1984; Carswell *et al.*, 1985).

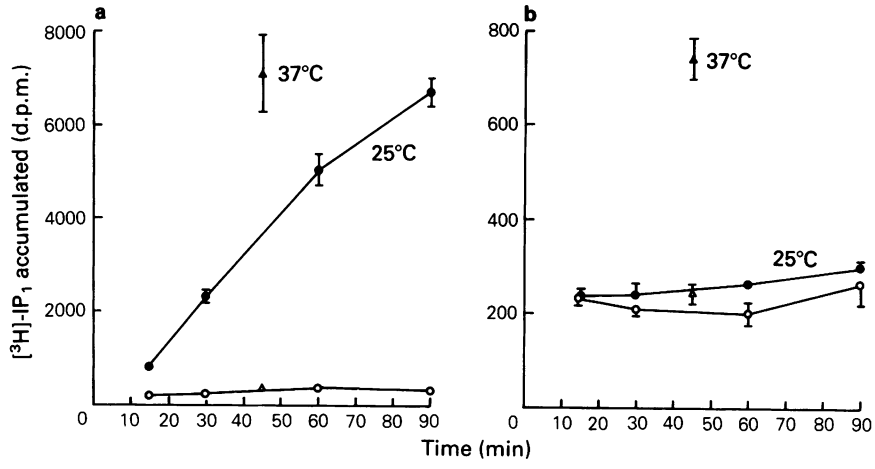

Figure 1 Time-course of carbachol- and histamine-induced accumulation of [^{3}H]-inositol 1-phosphate ([^{3}H]-IP$_1$) at 25°C. (a) Carbachol, (b) histamine. Slices of guinea-pig cerebral cortex were labelled with [^{3}H]-inositol before incubation with 0.1 mM carbachol or 0.2 M histamine at 25°C or 37°C (45 min time point), as described under Methods. The data in (a) and (b) are from independent experiments but for each agonist, measurements were made at 25°C and 37°C with the same slice preparation at the same time. Points are the mean ± s.e.mean of 5 replicate determinations at each time point at 25°C and 6 determinations at 37°C. Where no error bars are shown the error was within the size of the symbol. (●), 25°C in presence of agonist; (○), 25°C no agonist; (▲), 37°C in presence of agonist; (△), 37°C no agonist.

ing the incubation temperature to 25°C. An experiment carried out under the same conditions as for carbachol is shown in Figure 1(b). Even after 90 min exposure to histamine only a very small accumulation of [^{3}H]-IP$_1$ was detected. Similar results were obtained in two further experiments.

The marked difference of the effect of temperature on the response to histamine and carbachol might reflect changes at the level of the agonist-receptor interaction rather than in the receptor-coupled breakdown. The affinity of benzilylcholine, a muscarinic antagonist, deduced from the parallel shift of the concentration-response curve for carbachol-induced [^{3}H]-IP$_1$ accumulation in the presence of 50 nM benzilylcholine, was little altered by the decrease in temperature, K_a 3.3×10^8 M^{-1} at 37°C and 3.7×10^8 M^{-1} at 25°C, in good agreement with the value of $3.4 \pm 0.3 \times 10^8$ M^{-1} obtained from measurements of the inhibition by benzilylcholine of the binding of [^{3}H]-N-methylscopolamine to a homogenate of the same tissue at 30°C. These values are in accord with the affinity reported for inhibition of carbachol-induced contraction of guinea-pig ileum at 37°C, 3.2×10^8 M^{-1} (Abramson *et al.*, 1969). However, in contrast to the apparent lack of change in the affinity of the muscarinic antagonist, the EC_{50} for carbachol-stimulated accumulation of [^{3}H]-IP$_1$ was lower at 25°C than at 37°C (Table 1). A similar value was measured under continuous labelling conditions (Table 1).

The very small accumulation of [^{3}H]-IP$_1$ induced by histamine at 25°C made it impracticable to use this response to test for changes in agonist-receptor interaction on lowering the temparature from 37°C to 25°C. To try and assess the likelihood of such changes, the characteristics of histamine inhibition of the binding of [^{3}H]-mepyramine to homogenates of guinea-pig cerebral cortex and cerebellum were determined at 37°C and 25°C (Table 2). The similarity of the values of the IC_{50}, corrected to allow for competition with the ^{3}H-ligand (apparent K_d values), gives no indication of large changes in the nature of the interaction of histamine with the H$_1$-receptor as the temperature is lowered to 25°C.

Assuming that the affinity for histamine remains unaltered and that the affinity for carbachol increases as indicated by the decrease in the EC_{50} (Table 1), then the maximum response to each agonist at 25°C can be calculated as a percentage of the maximum response at 37°C. The values obtained from experiments such as those shown in Figure 1, in which measurements at both temperatures were made at the same time with the same slice preparation, are set out in Table 3 (Exptl series 1). Each value is from an independent experiment with 0.2 mM histamine or 0.1 mM carbachol and an incubation time of 45 min.

Table 2 Parameters of histamine inhibition of [^{3}H]-mepyramine binding to cerebral cortical homogenates at 37°C and 25°C

37°C		25°C	
IC_{50} corr. (μM)	n_H	IC_{50} corr. (μM)	n_H
25 ± 5	(0.77 ± 0.08)	30 ± 3	(0.97 ± 0.08)
31 ± 2	(0.86 ± 0.05)	29 ± 2	(0.94 ± 0.05)
26 ± 2	(0.75 ± 0.03)	30 ± 2	(0.88 ± 0.05)

Values are best-fit parameters ± estimated s.e. obtained from non-linear regression analysis of curves of inhibition of 0.37–0.57 nM [^{3}H]-mepyramine binding to a membrane fraction from guinea-pig cerebral cortex as described under Methods. IC_{50} corr. is the concentration of histamine required for 50% inhibition of the histamine-sensitive binding, corrected for competition with [^{3}H]-mepyramine, i.e. the apparent equilibrium dissociation constant. n_H is the Hill coefficient. Each pair of values of IC_{50} corr. and n_H is from an independent experiment.

To minimise the apparent change in the occupancy of carbachol between the two temperatures a second series of measurements was made with a higher concentration of carbachol, 1 mM. At this concentration the increase of occupancy at 25°C over that at 37°C is very small (0.98 at 25°C, 0.96 at 37°C). In each experiment measurements were made of the responses to both histamine and carbachol at both 37°C and 25°C. The calculated maximum response to each agonist, expressed as a percentage of the maximum response at 37°C, are set out in Table 3 (Exptl series 2). The much greater reduction of the response to histamine on lowering the temperature is again clear.

Effect of temperature on agonist-induced formation of [^{3}H]-IP_2 and [^{3}H]-IP_3

The much reduced histamine-induced accumulation of [^{3}H]-IP_1 at 25°C does not appear to be a consequence of a shift in the proportions of [^{3}H]-IP_1, [^{3}H]-IP_2 and [^{3}H]-IP_3 produced. The amounts of [^{3}H]-IP_2 and [^{3}H]-IP_3 produced following a 60 min incubation with agonist were much smaller than the amount of [^{3}H]-IP_1, consistent with the lesser inhibitory effect of 10 mM Li^+ on the phosphatases which hydrolyse IP_2 and IP_3. Thus the mean calculated maximum accumulations of [^{3}H]-IP_2 and [^{3}H]-IP_3 induced by carbachol at 37°C in the 5 experiments in which they were measured were 1827 ± 502 and 993 ± 260 d.p.m., respectively (basal levels subtracted). The large errors indicate the appreciable variation between experiments of the absolute amounts of the phosphates

Table 3 Agonist-induced [^{3}H]-inositol 1-phosphate ([^{3}H]-IP_1) accumulation at 25°C as a percentage of that at 37°C

	Max. [^{3}H]-IP_1 accumulation at 25°C × 100	
	Max. [^{3}H]-IP_1 accumulation at 37°C	
	Histamine	*Carbachol*
Exptl series 1		
	6	44
	9	68
	5*	43*
	10*	26*
Mean + s.e.mean	7 ± 1	45 ± 9
Exptl series 2		
	4	44
	8	45
	2*	57*
Mean + s.e.mean	5 ± 2	49 ± 5

The accumulations of [^{3}H]-IP_1 induced by histamine and carbachol were measured as described under Methods. In experimental series 1, incubations were with 0.2 mM histamine or 0.1 mM carbachol for 45 min. In series 2, 0.2 mM histamine and 1 mM carbachol were present for 60 min. For carbachol, maximum accumulations of [^{3}H]-IP_1 at each temperature were calculated from the values measured, taking the ED_{50} to be equal to the equilibrium dissociation constant, i.e. 42 μM at 37°C and 20 μM at 25°C (Table 1). The affinity of histamine was assumed not to change significantly. The accumulation of [^{3}H]-IP_1 induced by histamine or carbachol was always measured at the two temperatures in the same experiment with the same slice preparation. In series 1, the response to histamine and carbachol was measured in independent experiments. In series 2, responses to both agonists were measured in a single experiment, i.e. the values shown are 3 paired determinations. The values marked * were obtained after extraction of [^{3}H]-IP_1 with trichloracetic acid/ether rather than chloroform/methanol (see Methods).

generated, as was also observed for [^{3}H]-IP_1, 17,065 ± 2527 d.p.m. (mean ± s.e.mean from 7 determinations). Thus the amounts of [^{3}H]-IP_2 and [^{3}H]-IP_3 measured at 37°C were 11% and 2%, respectively, of that of [^{3}H]-IP_1. At 25°C the amounts of [^{3}H]-IP_2 and [^{3}H]-IP_3 produced by carbachol were reduced to a similar extent so that the maximum accumulations at 25°C as a percentage of the maximum at 37°C were

60 ± 12% and 59 ± 9%, respectively. These percentages compare with the two values of 45 ± 9% and 49 ± 5% measured for [^{3}H]-IP$_3$ (Table 3). In all of these experiments the responses at 37°C and 25°C were measured in the same experiment with the same slice preparation.

The amounts of [^{3}H]-IP$_2$ and [^{3}H]-IP$_3$ produced by histamine were smaller than those following carbachol, but the percentages of the maximum [^{3}H]-IP$_1$ response to histamine at 37°C (4960 ± 545 d.p.m., 6 determinations) were similar, 14% and 3%, respectively, to those observed with carbachol, 11% and 2%. The formation of both [^{3}H]-IP$_2$ and [^{3}H]-IP$_3$ in response to histamine was markedly depressed at 25°C so that the errors on the measurements were large. The amount of [^{3}H]-IP$_3$ measured at 25°C was too small (52 ± 42 d.p.m., 5 determinations) to allow meaningful comparison with the accumulation at 37°C. The calculated maximum induced accumulation of [^{3}H]-IP$_2$ at 25°C was only 14 ± 6% of that at 37°C (cf. 60 ± 12% for carbachol). The results were similar whether the inositol phosphates were extracted using the chloroform/methanol or trichloracetic acid/ether method.

Discussion

A decrease in the rate of biochemical reactions as the temperature is lowered is the rule. However, the extent of the decrease in histamine-induced accumulation of [^{3}H]-IP$_1$ as the temperature is lowered from 37°C to 25°C is much greater than might have been expected and contrasts with the lesser decrease in the response to carbachol. The latter is more in line with the fall in the response to TRH in GH$_3$ pituitary tumour cells over the same temperature range (Drummond *et al.*, 1984). The apparent implication is that the locus or pathway of the histamine-induced accumulation differs from that induced by carbachol. The other possibility, that there is an effect of temperature at the level of the histamine-receptor interaction, rather than the subsequent biochemical events, looks unlikely, but cannot be ruled out completely.

Possible changes in histamine receptor conformation and function with temperature have been the subject of some debate (summarised by Cook *et al.*, 1985). An earlier suggestion of a temperature-dependent interconversion of H$_1$- and H$_2$-receptors has not been substantiated by subsequent experimental work (Bertaccini & Zappia, 1983; Cook *et al.*, 1985) and there is no marked change in the affinity of [^{3}H]-mepyramine binding to H$_1$-receptors between 37°C and 25°C, although the rate constants do decrease (Wallace and Young, 1983). However, the rate constants for [^{3}H]-quinuclidinyl benzilate, a muscarinic antagonist, also change markedly with temperature (Gorissen *et al.*, 1978; Hurko, 1978) so that an effect on rate constants alone seems unlikely to be the basis of the differential effect of temperature on histamine- and carbachol-induced [^{3}H]-IP$_1$ accumulation. Evidence based on antagonist affinities is in any case limited in value, since antagonist binding is not necessarily a reliable guide to changes in agonist function, as the data for carbachol indicate. The affinity of benzilylcholine does not change significantly between 37°C and 25°C, but the EC$_{50}$ for carbachol is significantly lower ($P < 0.01$) at the lower temperature (Table 1). The very small accumulation of [^{3}H]-IP$_1$ induced by histamine at 25°C has made it impracticable to determine whether there are also changes in the EC$_{50}$ for histamine. However, if the marked temperature sensitivity is to be explained in this way, the change in the EC$_{50}$ for histamine would have to be considerable, from 16 μM at 37°C to around 1.6 mM at 25°C. This is possible, but seems improbable. The corrected IC$_{50}$ (apparent K_d) for histamine inhibition of [^{3}H]-mepyramine binding does not differ significantly at the two temperatures (Table 2), but the difficulty here is that it is uncertain how agonist binding is to be related to agonist function. Indeed, one of the objects of our studies of histamine-induced IP$_1$ formation is to establish whether this simple assay is suitable for correlating agonist binding and agonist function. The lack of any marked change in the contractile response of the guinea-pig ileum and guinea-pig colon to histamine between 37°C and 25°C (Cook *et al.*, 1985) argues against any universal and marked effect of temperature on H$_1$-receptor function. However, it is not clear how histamine-induced inositol phospholipid breakdown in the guinea-pig ileum is to be related to histamine-induced contraction (Donaldson & Hill, 1985) and the possibility must be borne in mind that there could be differences in the coupling of central and peripheral H$_1$-receptors with their effectors (Harrison *et al.*, 1984).

The most likely explanation for the differential temperature sensitivity of the histamine- and carbachol-induced accumulations of [^{3}H]-IP$_1$ is that the locus or pathways of the responses differ. This explanation is supported indirectly by reports of other differences between histamine and carbachol responses in which temperature is not a factor. Histamine (H$_1$), but not carbachol, potentiates the accumulation of cyclic AMP induced by 2-chloroadenosine in guinea-pig cerebral cortical slices (Hollingsworth & Daly, 1985) and, conversely, adenosine analogue have been reported to enhance histamine-induced, but not carbachol-induced, accumulation of inositol phosphates in the same tissues (Hollingsworth *et al.*, 1986). In rat hippocampal slices, K$^+$ facilitates IP$_1$ accumulation induced by carbachol, but not by histamine (Eva & Costa, 1986).

The biochemical basis of these differences is not

known. The magnitude of the response to carbachol at 37°C is greater than that to histamine, but there is no indication that the difference is some way related to this, since the effect of temperature on the carbachol response was very similar in experiments with 0.1 mM (Table 3, Exptl series 1) and 1 mM carbachol (Series 2). Nor does temperature obviously change the relative amounts of IP_1, IP_2 and IP_3, although it must be borne in mind that the assay does not separate the isomers of the bis- or tris-phosphates. However, there are now several pieces of indirect evidence that the histamine-induced accumulation of [^{3}H]-IP_1 may not be a simple sequence $PIP_2 \rightarrow IP_3 \rightarrow IP_2 \rightarrow IP_1$. The time-course of histamine-induced [^{3}H]-IP_1 accumulation seems to be characterized by a lag period (Daum *et al.*, 1984; Carswell *et al.*, 1985), the response is partly Ca^{2+}-dependent (Carswell *et al.*, 1985) and concentration-response curves for histamine consistently have Hill coefficients > 1 (Table 1). This last observation contrasts with the near hyperbolic curves for carbachol (Table 1). None of these observations or the differential temperature sensitivity is alone convincing evidence of a complex response, but put together they are suggestive. What is clear is that the simple assay of histamine-induced [^{3}H]-IP_1 accumulation may not reflect the agonist-receptor interaction as closely as had been hoped. The major assumption is that as long as H_1-agonist-induced phosphoinositide hydrolysis leads eventually to IP_1, the breakdown of which is inhibited by Li^+, then for every molecule of PIP_2 hydrolysed, one molecule of IP_1 will be formed, irrespective of whether 1, 3, 4-trisphosphate is also formed (Irvine *et al.*, 1986), provided that the incubation period is sufficiently long so that the amounts of IP_2 and IP_3 present are relatively small. There must be some doubt whether this assumption is justified. Whether other pathways of IP_1 formation, such as agonist-stimulated hydrolysis of PI itself, are involved will need to be part of a more detailed examination of the response to histamine.

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A 7-phenyl substituted triazolopyridazine has inverse agonist activity at the benzodiazepine receptor site

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1 To investigate further the structural requirements for benzodiazepine (BZD) receptor ligands, we synthesized SR 95195, [7-phenyl-3-methyl-1,2,4-triazolo-(4,3-b) pyridazine], a positional isomer of the 6-phenyl-triazolo-pyridazines, which were the first non-BZD derivatives to exhibit high affinity for the BZD receptor and BZD-like activity *in vivo*.

2 *In vitro*, SR 95195 displaced specifically bound [^{3}H]-flunitrazepam from rat cerebellar and hippocampal membranes with respective IC_{50} values of 4 and 8 μM.

3 *In vivo*, SR 95195 lacked BZD-like activity. At high doses SR 95195 induced clonic seizures in mice (threshold convulsant dose: 150 mg kg^{-1}; CD_{50}: 160 mg kg^{-1} i.p.) which were antagonized by Ro 15–1788. At non-convulsant doses (25 mg kg^{-1} i.p. and 100 mg kg^{-1} i.p.). SR 95195 significantly decreased punished responding in an operant conflict procedure in the rat, suggesting SR 95195 has intrinsic anxiogenic activity.

4 SR 95195, in mice, reversed the anticonvulsant and myorelaxant actions of diazepam 3 mg kg^{-1}, orally (respective ED_{50} values: 45 mg kg^{-1} i.p. and 44 mg kg^{-1} i.p.). In an operant-conflict test in rats, SR 95195 at non-anxiogenic doses, antagonized the disinhibitory action of diazepam 4 mg kg^{-1}, i.p. (ED_{50}: 8.6 mg kg^{-1}, i.p.), but not that of pentobarbitone 15 mg kg^{-1}, i.p.

5 It is concluded that SR 95195 has the pharmacological profile of an inverse BZD agonist and that displacing the phenyl from the 6- to the 7-position in the triazolopyridazine series causes a shift from agonist to inverse agonist type activity at the BZD receptor site.

Introduction

Brain specific benzodiazepine (BZD) binding sites which selectively recognize pharmacologically and clinically active BZDs have been isolated in all vertebrate species including man (Squires & Braestrup, 1977; Möhler & Okada, 1977; Young & Kuhar, 1979; Speth *et al.*, 1978). These receptors also selectively recognize compounds such as Ro 15–1788 (Hunkeler *et al.*, 1981) which reverse all the actions of BZDs or compounds such as CGS 8216 (Boast *et al.*, 1983; File, 1983) which not only antagonize the actions of BZDs, but which exhibit opposite activities.

Early evidence indicated that only a single homogeneous class of BZD binding sites existed. However the discovery of a series of triazolopyridazines, substituted in the 6-position by a phenyl ring, lent strong support to the concept of multiple BZD receptors. CL 218,872, a member of this series, was the first non-BZD reported to displace [^{3}H]-BZDs from their brain-specific binding sites (Lippa *et al.*, 1979). This compound was found to possess anticonflict and anticonvulsant activities in animals and to produce sedation and myorelaxant effects at doses much higher than the anxiolytic doses (Lippa *et al.*, 1979). Biochemical studies indicated that CL 218,872 preferentially displaced [^{3}H]-BZD from a particular BZD receptor class designated as type I BZD receptor (Klepner *et al.*, 1979). Thus it was suggested that type I receptors mediated the anxiolytic and anticonvulsant actions of BZDs and type II receptors the sedative and myorelaxant actions of BZDs. Recently, however CL 218,872 has been shown to possess sedative properties at anxiolytic doses (Oakley *et al.*, 1984; McElroy *et al.*, 1985) and the

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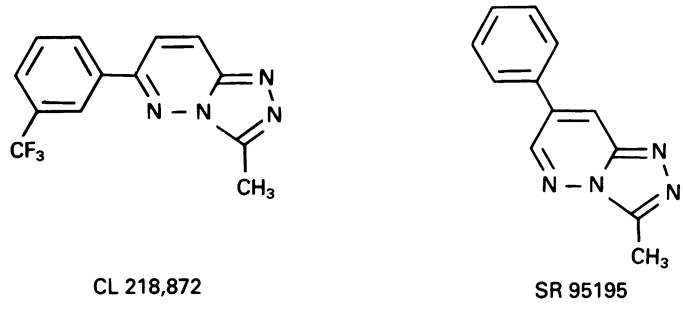

Figure 1 Chemical structures of SR95195 and CL218,872.

initial hypothesis according to which different BZD receptor subtypes could mediate different aspects of BZD activities has been questioned (Oakley *et al.*, 1984). Furthermore CL218,872 has also been shown to reverse diazepam-induced loss of righting reflex (Gee *et al.*, 1983), suggesting together with other results (Gardner, 1984) that this compound may have mixed agonist/antagonist type of activity at the BZD receptor site.

To investigate further the structural prerequisites at the BZD receptor site, we synthesized SR95195 (7-phenyl-3-methyl-1,2,4-triazolo-(4,3-b) pyridazine; Figure 1; Bourguignon *et al.*, 1985). In this compound, the phenyl ring is attached to the 7-position of the pyridazine ring and not to the 6-position, as is the case for CL218,872.

The purpose of this study was to examine the influence of this positional isomery in the 6,7-triazolopyridazine series on BZD receptor affinity and on BZD-like action. Diazepam, Ro 15-1788 and CGS8216 were used as reference benzodiazepine receptor ligands.

Part of this work was presented at the 11th Annual Meeting of the Society for Neuroscience (Chambon *et al.*, 1985).

Methods

Swiss albino CD_1 female mice weighing 18–23 g and male Sprague Dawley rats (200–300 g) were obtained from Charles River Breeding Laboratories (St. Aubin les Elbeuf, France). All animals were weighed, identified one day before the test and housed in groups of 10 (mice) or 5 (rats) in macrolon cages with free access to food and drinking water. A 12 h day/night cycle was maintained in the animal colonies. On the day of the experiment, animals were brought into the laboratory where the test was performed.

For the approach-avoidance conflict experiment rats were housed individually in suspended cages. One week after arrival, they were progressively food deprived until they received a daily diet of 10–12 g of standard laboratory feed (AO4 UAR) except during week-ends when they were fed *ad libitum* on Saturdays, the food being removed on Sunday mornings. Tap-water was always available as drinking fluid. The room temperature was 21°C ± 1, with a relative humidity of 50 ± 10%. The light-dark cycle was 12 L/12 D, lights being on at 07 h 00mm.

In vitro *[^{3}H]-flunitrazepam binding*

In vitro [^{3}H]-flunitrazepam binding was performed according to a slight modification of the method described by Stapleton *et al.* (1982). Rats were stunned and decapitated, their brains were rapidly removed, the cerebella and hippocampi were removed and homogenized in 50 mM Tris-citrate buffer (pH 7.1) with a Brinkman Polytron PT 10 homogenizer. Homogenates were centrifuged at 48,000 *g* for 10 min at 4°C. The pellets were again homogenized, centrifuged twice and frozen at −80°C for at least 24 h. On the day of the experiment the membranes were thawed, homogenized in 100 vol of 25 mM KH_2PO_4 pH 7.1, centrifuged (48,000 *g*, 4°C, 10 min) and resuspended twice in the same buffer. Aliquots of membrane suspensions (0.25 mg protein) were incubated in 1 ml of 25 mM KH_2PO_4 (pH 7.1) with 1.05 nM [^{3}H]-flunitrazepam (80 Ci mmol^{-1}) and various concentrations of potential radioligand displacers for 90 min at 4°C. The assay was terminated by filtration under vacuum on Whatman GF/C filters. Filters were washed with 10 ml of ice-cold buffer, placed in scintillation vials and allowed to dry overnight; 10 ml of scintillation fluid (Biofluor; New England Nuclear, Boston, USA) was then added and radioactivity was counted in a Kontron Betamatic liquid scintillation counter. Specific binding of [^{3}H]-flunitrazepam was defined as the difference between total binding in the presence of radioligand alone and non-specific binding in the presence of 100 μM unlabelled diazepam.

Gross behaviour and convulsant effects in mice

SR 95195, or vehicle, was administered i.p. to groups of 10 mice, or orally to groups of 5 rats. Animals were observed grouped in their treatment cage. The symptomatic scale described by Irwin (1968) was used. This scale provides information on central symptoms such as motor activity, tremor, convulsions, stereotypies and peripheral automatic symptoms such as pupillary diameter, ptosis, salivation, body temperature, respiration. The symptoms were noted as present or absent during the 3 h after drug administration; no scoring system was used and the observer was not blind to treatment conditions. Lethality was recorded up to 72 h after drug administration. The CD_{50} (95% confidence limits), dose which induced clonic seizures in 50% of animals, was calculated by probit analysis (Litchfield & Wilcoxon, 1949).

Antagonism of SR 95195-induced seizures by Ro 15–1788

Groups of 10 mice were used. Ro 15–1788 was injected i.p. at doses ranging from 1.25 mg kg^{-1} to 20 mg kg^{-1}; 5 min later SR 95195 was injected at a dose of 180 mg kg^{-1}, a dose which induced clonic seizures in 90% of animals. The number of mice exhibiting clonic seizures was recorded 1 h after the administration of SR 95195.

Reversal of the anticonvulsant effects of diazepam in mice

Experiments were carried out in groups of 10 mice. Diazepam was administered orally at 3 mg kg^{-1} 30 min before an i.p. injection of test compounds or vehicle and 60 min before pentylenetetrazole (Ptz) 100 mg kg^{-1}, s.c. The number of animals exhibiting seizures in each treatment group was recorded 1 h after the administration of Ptz. All animals pretreated by diazepam alone were protected against seizures. The ED_{50} (95% confidence limits), dose which antagonized the anticonvulsant effect of diazepam in 50% of animals, was calculated for each test compound by probit analysis (Litchfield & Wilcoxon, 1949).

Reversal of the myorelaxant effects of diazepam in mice

Myorelaxation was measured in the Traction test according to a procedure similar to that described by Boissier *et al.* (1961). The test was performed using a metallic wire (diameter 1 mm; length 15 cm) fixed horizontally 20 cm above the experimental table. Groups of 10 mice were used. Diazepam (3 mg kg^{-1}) was injected i.p. 30 min before the i.p. injection of test compounds or vehicle. One hour later animals were suspended by their fore-paws from the metallic wire and the number of mice failing to put one hind-limb on the wire during the following 5 s was counted. All animals pretreated by diazepam alone failed to perform the test. The ED_{50} (95% confidence limits), dose which antagonized the myorelaxant effect of diazepam in 50% of animals, was calculated for each test compound by probit analysis (Litchfield & Wilcoxon, 1949).

Approach-avoidance conflict test in rats

Conditioning was carried out in Skinner boxes (Campden, London, U.K.; ref. 4104) isolated by sound-attenuating chambers. Each Skinner box was supplied with a food tray connected with a pellet dispenser, with one lever above which an electric bulb was located and with a grid floor which was connected to a shock generator and scrambler (Campden, London, U.K. ref. 521C). Operant schedule and animal responding were monitored by a microcomputer (Apple II, Apple Computer Inc., Cupertino, U.S.A).

Experiments were run from 13 h 00 min to 17 h 00 min. Two weeks after arrival, rats were familiarized with the experimental chambers and trained to press the lever in order to obtain one 45 mg food pellet (Bioserv Inc., Frenchtown, U.S.A). When they steadily pressed the lever, they were submitted to a progressively increasing variable interval (VI) schedule, until the final VI 30 s schedule was reached (i.e. a food reinforcement was available at the end of a period of time randomly varying from 5 s to 55 s). The training sessions lasted 30 min. When baseline responding was stable, two fixed ratio 3 (FR 3) segments were included, they were cued by the onset of the lamp above the lever. During the FR3 segments, every third press delivered simultaneously a food pellet and an electric shock. The shock intensity was titrated to each animal's sensitivity so that no more than 6 punished lever presses were made per session. Sessions were organized according to the following sequence: 10 min VI 30 s, 2 min FR3, 10 min VI 30 s, 2 min FR3 and 6 min VI 30 s. Animals were trained 4 days a week. The possible anticonflict effect of drugs was examined when the rats were well trained in the procedure and when steady performances were achieved. They were carried out on two different days a week and were separated by at least one training day.

When studied alone, drugs (i.e. SR 95195 and CL 218,872) were injected i.p. 30 min before testing. When administered in combination with diazepam or pentobarbitone, SR 95195 was injected i.p. 60 min before the beginning of the session and diazepam (4 mg kg^{-1}) or pentobarbitone (15 mg kg^{-1}) were injected i.p. 30 min before testing. Control rats were dosed with the corresponding vehicle of each drug. The order of treatment was randomized for each rat.

The total number of responses during the non-punished and the punished periods of the conflict test were recorded. Punished responses (PR) were submitted to a logarithmic transformation [log (PR + 1)] and non-punished responses (NPR) were expressed as the ratio of response rate on the test day to the response rate on the day preceding drug treatment (NPR/NPR_0). Results were submitted to an ANOVA for repeated measures if variances were assumed to be equal (Levene's test) or to Brown-Forsythe's test otherwise. Comparisons between the means were done by Bonferroni's method. Whenever possible ED_{50} values were calculated by the method of Finney (1971). Rats were considered to be disinhibited when they made more than seven PR per session.

Drugs

Flunitrazepam ([methyl-^{3}H]; 80 Ci mmol^{-1}) was purchased from N.E.N. (Boston, U.S.A.). SR95195 was synthesized at the Laboratoire de Pharmacochimie Moléculaire, UA501, Centre de Neurochimie du CNRS, U.L.P. (Strasbourg, France). CL218,872 was synthesized at the Department of Organic Chemistry of the Centre de Recherches Clin-Midy, Sanofi Recherche (Montpellier, France). Ro 15–1788 (ethyl-8-fluoro-5,6-dihydro-5-methyl-6-oxo-4H-imadazol (1, 5a)-(1,4) benzodiazepine-3-carboxylate) and diazepam were provided by Hoffman-la-Roche (Basel, Switzerland). CGS8216 (2-phenylpyrazolo (4,3-c) quinolin-3 (5H)-one) was provided by Ciba-Geigy (Summit, U.S.A.), pentobarbitone was purchased from Interchim (Montluçon, France) and pentylenetetrazole from Sigma (Saint Louis, U.S.A.).

Mice were dosed orally with diazepam suspended in 5% gum tragacanth (w/v), i.p. with diazepam suspended in 0.5% carboxymethyl cellulose (w/v) or s.c. with pentylenetetrazole dissolved in distilled water. All other drugs were suspended in 0.5% carboxymethyl cellulose (w/v) and injected i.p. In the approach-avoidance conflict test in rats, all the drugs tested were suspended in 0.5% carboxymethyl cellulose (w/v) with two drops of Tween 80 per 10 ml and injected i.p.

Results

In vitro *[^{3}H]-flunitrazepam binding*

SR95195 displaced bound [^{3}H]-flunitrazepam from rat cerebellar and hippocampal membranes with IC_{50} values in the micromolar range (Table 1). SR95195 appeared to be 10 times and 60 times less potent than CL218,872 in hippocampal and cerebellar membranes respectively. It was considerably less potent than diazepam, Ro 15–1788 and CGS8216 in both regions (Table 1). Unlike CL218,872 but like diazepam, Ro 15–1788 and CGS8216, SR95195 exhibited comparable affinities for the cerebellar and hippocampal BZD receptor site (Table 1). Hill plots of the saturation isotherms of [^{3}H]-flunitrazepam binding in cerebellar and hippocampal membranes revealed Hill numbers of 0.85 ± 0.03 and 0.73 ± 0.03 respectively.

Table 1 Inhibition of *in vitro* [^{3}H]-flunitrazepam binding in rat cerebellum and hippocampus by SR95195 and various benzodiazepine receptor ligands

Compounds	*IC_{50} (nM)*	
	Cerebellum	*Hippocampus*
SR95195	4860 ± 580	8500 ± 1300
CL218,872	76 ± 7	866 ± 30
Diazepam	3.3 ± 1.8	3.2 ± 1.9
Ro 15–1788	2.2 ± 1.0	2.3 ± 1.0
CGS8216	0.0063 ± 0.0013	0.0039 ± 0.0013

Data are expressed as the mean ± s.e.mean of 3 individual experiments performed in triplicate on separate days.

Gross behaviour and convulsant effect in mice

When administered i.p. to mice, SR95195 did not induce BZD-like activity; in particular no intoxicated gait and no sedation were observed. At high doses, SR95195 induced clonic seizures (CD_{50} = 160 mg kg^{-1} i.p., 95% confidence limits: 148–175; threshold convulsant dose: 150 mg kg^{-1} i.p.) followed by death (LD_{50} = 240 mg kg^{-1} i.p., 95% confidence limits: 220–261).

Antagonism of SR95195-induced seizures by Ro 15–1788

SR95195 at a dose of 180 mg kg^{-1} induced clonic seizures in 90% of mice, which were antagonized by

Table 2 Reversal by SR95195 and reference compounds of the anticonvulsant and muscle relaxant effects of diazepam (3 mg kg^{-1} orally)

Compounds	*ED_{50} (mg kg^{-1}, i.p.)*	
	Ptz-induced seizures	Traction test
SR95195	45 (30–58)	44 (29–67)
Ro 15–1788	17 (14–21)	3.6 (2.6–4.8)
CGS8216	0.97 (0.20–4.40)	0.87 (0.70–1.04)

Ptz = pentylenetetrazole
Figures in parentheses indicate the 95% confidence limits.

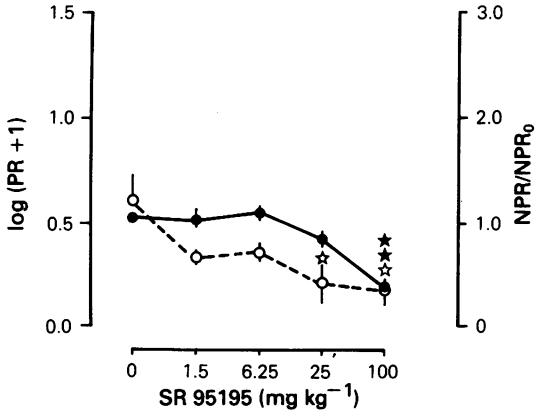

Figure 2 Dose-response curves of SR 95195 on punished (PR ○) and non-punished responding (NPR ●) in the approach-avoidance conflict test. SR 95195 was injected i.p. 30 min before testing. Comparisons between the means were made by Bonferroni's method. Significance is indicated by * for PR and * for NPR (*$P<0.05$; **$P<0.01$).

Ro 15–1788 (ED_{50} = 3.8 mg kg^{-1} (1.3–10.6)). Comparable results were obtained in two different experiments.

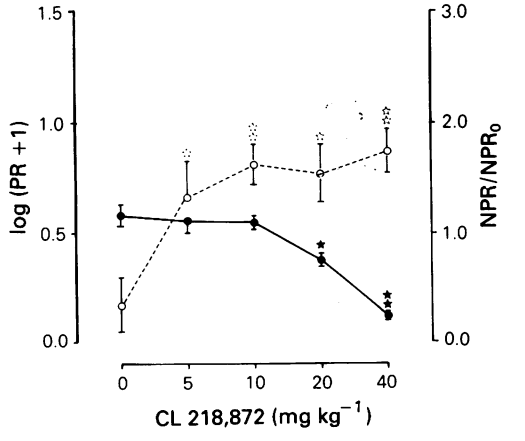

Figure 3 Dose-response curves of CL 218,872 on punished (PR ○) and non-punished responding (NPR ●) in the approach-avoidance conflict test. CL 218,872 was injected i.p. 30 min before testing. Comparisons between the means were made by Bonferroni's method. Significance is indicated by * for PR and * for NPR (*$P<0.05$; **$P<0.01$).

Reversal of the anticonvulsant effects of diazepam in mice

Diazepam at a dose of 3 mg kg^{-1} orally, completely blocked the clonic seizures induced by Ptz (100 mg kg^{-1} s.c.) administered 1 h later. In these conditions, when SR 95195 was administered i.p. at non-convulsant doses (25 to 100 mg kg^{-1}) 30 min after diazepam, it antagonized the protective effect of diazepam against Ptz-induced clonic seizures in a dose-dependent fashion (ED_{50} = 45 mg kg^{-1}). Ro 15–1788 and CGS 8216 also antagonized the anticonvulsant effect of diazepam and were 2.5 times and 50 times more potent respectively than SR 95195 (Table 2).

Reversal of the myorelaxant effect of diazepam in mice

Diazepam at a dose of 3 mg kg^{-1} i.p. induced muscle relaxation in all animals as measured by the traction test, 60 min after injection. When SR 95195 was administered i.p. at various doses, 30 min after

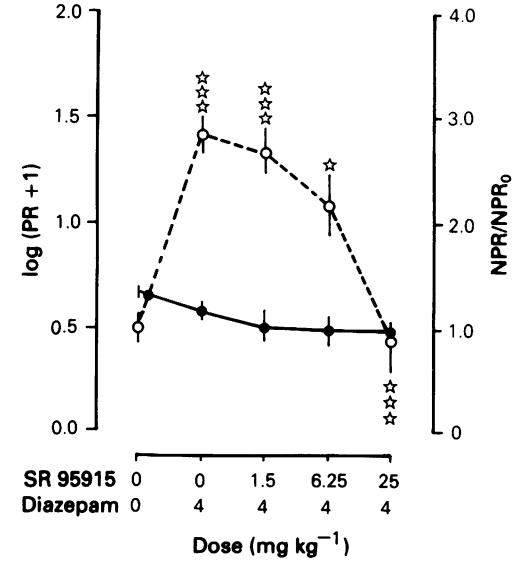

Figure 4 Dose-response curves of SR 95195 on punished (PR ○) and non-punished responding (NPR ●) after injection of diazepam (4 mg kg^{-1}, i.p.) in the approach-avoidance conflict test. SR 95195 was injected i.p. 60 min before testing and 30 min before diazepam. Each point represents the mean with vertical lines showing s.e.mean. Comparisons between the means were made by Bonferroni's method. Significance is indicated by * for PR, they are located above the graph for comparisons with controls and below the graph for comparison with the agonist (*$P<0.05$; ***$P<0.001$).

diazepam, it dose-dependently antagonized the muscle relaxation induced by diazepam (ED_{50} = 44 mg kg^{-1}). In the same experimental conditions, Ro 15–1788 and CGS 8216 also antagonized the myorelaxant effects of diazepam and were 12 times and 50 times more potent respectively than SR95195 (Table 2).

Approach-avoidance conflict test in rats

The activity of SR 95195 in the approach-avoidance conflict test is presented in Figure 2. SR 95195 significantly modified PR (ANOVA: $F(4-25) = 3.6$ $P < 0.05$). Comparisons between the means indicated that PR was reduced at 25 mg kg^{-1} and 100 mg kg^{-1}. SR 95195 also decreased NPR (ANOVA: $F(4-25) = 15.4$, $P < 0.001$); this effect was significant only at 100 mg kg^{-1}. In contrast and as expected, CL 218,872 (Figure 3) increased PR (ANOVA: $F(4-25) = 5.65$, $P < 0.01$). Comparisons between the means showed that the means of all doses were significantly superior to control values. The ED_{50} value for the disinhibitory action of CL 218,872 was 25.3 mg kg^{-1}. CL 218,872 also significantly decreased NPR (Brown-Forsythe's test = 28.0 for 4–17 d.f., $P < 0.001$) at 20 and 40 mg kg^{-1}.

As shown in Figure 4, diazepam (4 mg kg^{-1}) significantly increased PR and this effect was dose-dependently antagonized by SR 95195 (Brown-Forsythe's test = 15.5 for 4–24 d.f., $P < 0.001$; ED_{50} = 8.6 mg kg^{-1}). NPR was not modified by the treatment (Figure 4; ANOVA: $F(4-30) = 0.84$; $P > 0.05$).

Pentobarbitone (15 mg kg^{-1}) increased PR (ANOVA: $F(3-24) = 5.47$; $P < 0.01$), this effect was not antagonized by SR 95195 (6.25 and 25 mg kg^{-1}) and no group injected with SR 95195 differed significantly from the pentobarbitone-treated group (Figure 5). No change in NPR was recorded (ANOVA: $F(3-24) = 0.28$; $P > 0.05$.

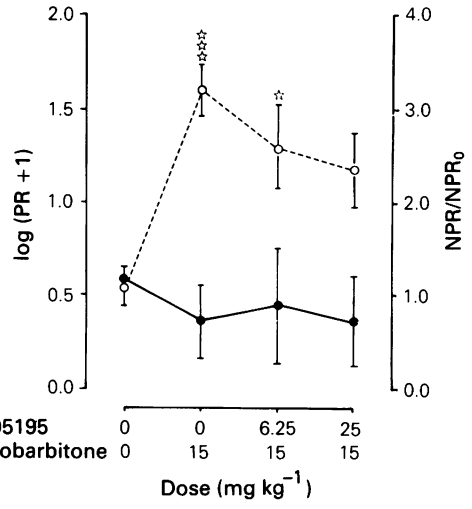

Figure 5 Dose-response curves of SR 95195 on punished (PR ○) and non-punished responding (NPR ●) after injection of pentobarbitone (15 mg kg^{-1}, i.p.) in the approach-avoidance conflict test. SR 95195 was injected i.p. 60 min before testing and 30 min before pentobarbitone. Each point represents mean with vertical lines showing s.e.mean. Comparisons between the means were made by Bonferroni's method. Significance is indicated by * for PR (*$P < 0.05$; ***$P < 0.001$).

Discussion

The results presented in this study clearly showed that SR 95195 has the pharmacological profile of an inverse BZD agonist. *In vitro*, SR 95195 displaced specifically bound [^{3}H]-flunitrazepam from its receptor site. *In vivo*, SR 95195 lacked BZD-like activity, but was able to reverse the anticonvulsant, myorelaxant and anxiolytic actions of diazepam. Moreover SR 95195 appeared to have intrinsic anxiogenic actions and at high doses induced clonic seizures.

The affinity of SR 95195 for the BZD receptor site was considerably lower than that observed with the other reference compounds examined in this study. Moreover, unlike CL 218,872, but like the other reference compounds, SR 95195 displaced [^{3}H]-flunitrazepam from cerebellar and hippocampal membranes with comparable potencies. The chemical structure of SR 95195 differs from that of CL 218,872 not only in the position of the phenyl ring, but also in the absence of a CF_3 substituent on the phenyl. However, in the 6-phenyl-triazolopyridazine series the compound which is not substituted on the phenyl ring has been shown to have the same pharmacological profile as CL 218,872, but to be slightly less potent (Albright *et al.*, 1981). Thus it can be concluded that displacing the phenyl from the 6- to the 7-position in the triazolopyridazine series causes a marked decrease in affinity for the BZD receptor. Moreover, this structural modification also leads to a loss of selectivity for type I BZD receptor sites, as indicated by comparable affinities for the cerebellar and hippocampal BZD receptors.

SR 95195, at doses higher than 150 mg kg^{-1}, induced clonic seizures which were blocked by Ro 15–1788, a BZD receptor antagonist, indicating that seizure activity was probably mediated by the BZD receptor. Inverse BZD agonists have been shown either to induce seizures or to have definite proconvulsant actions (Cowen *et al.*, 1981; Oakley & Jones, 1982; Braestrup *et al.*, 1982; File, 1983) whereas the BZD receptor antagonist, Ro 15–1788 does not lower seizure threshold (Nutt *et al.*, 1982). Thus the induc-

tion of seizures by SR 95195 strongly suggests this compound is an inverse BZD agonist.

Consistent with this hypothesis, in the approach-avoidance conflict test in rats, SR 95195 induced a significant decrease in PR at 25 mg kg^{-1} and 100 mg kg^{-1} suggesting a possible proconflict action. An enhancement of shock-induced suppression has already been described for inverse agonists such as CGS 8216 (Petersen *et al.*, 1983; File & Lister, 1983), β-CCM (De Carvalho *et al.*, 1983) and the endogenous diazepam binding inhibitor (Guidotti *et al.*, 1983). Ro 15–1788, a BZD antagonist, originally considered as devoid of intrinsic pharmacological activity, has also been shown to be anxiogenic when administered alone, however, this effect is weak and disappears at high doses (File *et al.*, 1982).

SR 95195, at non-convulsant doses, reversed the anticonvulsant and myorelaxant effects of diazepam in mice. Similar findings have been reported for other BZD inverse agonists and for the BZD antagonist, Ro 15–1788 (Tenen & Hirsch, 1980; Cowen *et al.*, 1981; Nutt *et al.*, 1982; Oakley & Jones, 1982; Czernik *et al.*, 1982). SR 95195, at low non-anxiogenic doses, also reversed the anticonflict action of diazepam. Again, inverse BZD agonists and BZD antagonists have repeatedly been shown to antagonize the anxiolytic effect of BZD ligands (Bonetti *et al.*, 1982; Patel *et al.*, 1983). SR 95195, unlike CGS 8216 (Mendelson *et al.*, 1983) failed to counteract the disinhibitory effect of pentobarbitone in the approach-avoidance conflict test in rats, suggesting a greater selectivity towards the BZD receptor. Interestingly, the possibility that CGS 8216 may also interact with the picrotoxin site has already been suggested on the basis of its proconvulsant mechanism of action (File, 1983).

In conclusion the results presented in this study have shown that displacing the phenyl from the 6- to the 7-position in the triazolopyridazine series causes a shift from an agonist to an inverse agonist type of activity at the BZD receptor site. Minor structural changes have previously been shown in other BZD receptor ligand series to alter the pharmacological profile of a ligand. Thus, CGS 8216, an inverse agonist at the BZD receptor site, differs from CGS 9896, a full agonist, by an additional chlorine in the 4-position on the phenyl ring (Petrack *et al.*, 1983). In the β-carboline series, addition of a bulky substituent in the 5-position, as is the case for ZK 91296, leads to an anticonvulsant compound (Meldrum *et al.*, 1983). However SR 95195 is the first example in which positional isomery leads to an inversion of activity.

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Differential effects of Ca antagonists on the noradrenaline release and contraction evoked by nerve stimulation in the presence of 4-aminopyridine

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1 We previously reported that verapamil, nicardipine and diltiazem inhibited both neurotransmitter release and contraction evoked by transmural nerve stimulation (TNS) in the canine saphenous vein.

2 To evaluate whether the three Ca antagonists act on the nerve endings by inhibiting Ca^{2+} influx, the effects of the three antagonists were studied in the presence of 4-aminopyridine (4-AP) 3×10^{-4} M on the TNS-evoked tritium overflow and contraction of canine saphenous veins preloaded with [^{3}H]-noradrenaline.

3 4-AP increased both tritium overflow and contraction evoked by TNS, but did not enhance the contraction induced by exogenous noradrenaline (10 nmol). In the veins pretreated with 4-AP, verapamil (3×10^{-5} M) and nicardipine (10^{-5} M and 3×10^{-5} M) caused no significant effects on the TNS-evoked tritium overflow, but they still inhibited the contraction. Diltiazem (10^{-5} M and 3×10^{-5} M) significantly inhibited both responses to TNS in the veins pretreated with 4-AP, the effects being nearly equipotent to those in the absence of 4-AP. The (−)-*cis* isomer of diltiazem (10^{-5} M and 3×10^{-5} M), which is about 100 times less potent than diltiazem in inhibiting Ca^{2+} influx, inhibited both responses to TNS in the presence of 4-AP to almost the same degree as diltiazem.

4 When 4-AP was added after the Ca antagonists, it reversed the TNS-evoked tritium overflow inhibiting actions of verapamil (3×10^{-5} M) and nicardipine (3×10^{-5} M) much more effectively than that of diltiazem (3×10^{-5} M).

5 Tetracaine (4×10^{-6} M) significantly inhibited the TNS-evoked tritium overflow and contraction, which were unaffected by 4-AP.

6 Sodium salicylate (10^{-2} M) failed to modify the inhibition of TNS-evoked tritium overflow following diltiazem (3×10^{-5} M), but it enhanced that of tetracaine (4×10^{-6} M).

7 Verapamil but not diltiazem and nicardipine significantly increased the spontaneous tritium overflow from veins pretreated with 4-AP.

8 The present study together with previous results suggests that diltiazem but not verapamil and nicardipine may inhibit the TNS-evoked neurotransmitter release through an action other than inhibition of Ca^{2+} influx into the adrenergic nerve endings, allowing an inhibition of the resulting contraction.

Introduction

It is known that an increased influx of Ca^{2+} into adrenergic nerve endings triggers the neurotransmitter release evoked by electrical stimulation (Blaustein, 1979). Since Ca antagonists inhibit the transmembrane Ca^{2+} influx into vascular smooth muscle cells (Fleckenstein, 1977), these drugs can be anticipated to suppress the electrically stimulated release of neurotransmitter. Indeed, our previous study showed that verapamil, nicardipine and diltiazem inhibited the neurotransmitter release produced by transmural nerve stimulation (TNS) from isolated canine saphenous veins (Takata & Kato, 1984). Similar results were reported for verapamil in the rabbit isolated heart (Göthert *et al.*, 1979) and for diltiazem in guinea-pig isolated mesenteric arteries (Suzuki *et al.*, 1982). In these studies, however, a concentration range of Ca antagonists required to inhibit the release of neurotransmitter appears to be considerably higher

than that required for the blockade of excitation-contraction coupling in arterial smooth muscle (Fleckenstein, 1977). Furthermore, nifedipine in concentrations that caused a significant inhibition of the contraction induced by nerve stimulation, failed to modify neurotransmitter release from adrenergic nerve endings of the isolated vas deferens of the rat (Hyland *et al.*, 1984). These findings make it doubtful whether Ca antagonists inhibit Ca^{2+} influx into nerve endings, resulting in a decrease in the neurotransmitter release evoked by electrical stimulation. If Ca antagonists inhibit the electrically stimulated release of neurotransmitter as a result of the inhibition of Ca^{2+} influx, then the inhibitory action would be easily antagonized by an enhanced influx of Ca^{2+}, as indicated by Fleckenstein (1977). We have investigated the effects of verapamil, nicardipine and diltiazem on electrically stimulated tritium overflow and the corresponding contraction of canine isolated saphenous veins preloaded with [^{3}H]-noradrenaline, in the presence of 4-aminopyridine (4-AP), a drug that inactivates K conductance, thereby allowing a greater influx of Ca^{2+} into the adrenergic nerve endings to enhance noradrenaline release (Johns *et al.*, 1976; Kirpekar *et al.*, 1977; Glover, 1982); this allowed us to evaluate whether these three Ca antagonists act on the nerve endings by inhibiting Ca^{2+} influx.

Methods

Isotope experiments

Mongrel dogs of either sex, weighing 10–35 kg, were anaesthetized with sodium pentobarbitone (32 mg kg^{-1}, i.v.). The procedures used for obtaining, incubating and superfusing helical strips of the saphenous veins have been described previously in detail (Takata & Kato, 1984). Briefly, the strips (3 × 30 mm) were incubated for 2 h with Krebs-bicarbonate (Krebs) solution containing (−)-[7-^{3}H]-noradrenaline, 8×10^{-8} M (10 μCi in 5 ml of incubation medium) and ascorbic acid 5.7×10^{-5} M, and mounted for superfusion. The Krebs solution had the following composition (in mM); NaCl 118.2, KCl 4.6, $MgSO_4$ 1.2, $CaCl_2$ 2.5, KH_2PO_4 1.2, $NaHCO_3$ 24.8 and glucose 10.0. The strips were then superfused at a rate of 3.6 ml min^{-1} with the Krebs solution maintained at 37°C and bubbled with 95% O_2 and 5% CO_2. After equilibration, TNSs were repeatedly applied 8 times every 16 min by an electric stimulator (Nihon Kohden, SEN-7103) and designated as S_1 to S_8. Stimulation parameters were 0.3 ms duration, supramaximum voltage (20 V) and a frequency of 10 Hz for 15 s. Developed tension was recorded isometrically on an ink-writing oscillograph (Nihon Kohden, WI-640G) through a force-displacement transducer (Nihon Kohden, TB-611T). The superfusate samples were continuously collected every 2 min from 4 min before S_2 throughout the experiment. One ml of each sample was mixed with 10 ml of scintillation mixture (5.5 g DPO, 0.1 g POPOP, 667 ml toluene and 333 ml Triton X-100) and then counted in a liquid scintillation counter (Beckman, LS 9000 or Aloka, LSC-903). The TNS-evoked tritium overflow was estimated by subtracting the radioactivity obtained in the 2 min sample just before TNS from that in the 2 min sample during TNS.

In the first series of experiments, 4-AP (3×10^{-4} M) was superfused for 80 min from 6 min after S_2 to 6 min after S_7, and the three Ca antagonists examined were superfused for 48 min from 6 min after S_3 to 6 min after S_6. The effects of the Ca antagonists on both TNS-evoked tritium overflow and contraction in the presence of 4-AP were estimated from the ratio S_6/S_3 for the reason given in the previous report (Takata & Kato, 1984), and they were compared with the corresponding ratio obtained from the superfusion with 4-AP alone without the Ca antagonists. Radioactivity obtained from the 2 min sample just before S_2-S_6 was considered as the spontaneous overflow of total tritium and designated as Sp_2-Sp_6, respectively. The effects of the Ca antagonists on the spontaneous tritium overflow were estimated from the ratios Sp_4/Sp_2, Sp_5/Sp_2 and Sp_6/Sp_2, and they were compared with the corresponding ratios obtained from the superfusion with 4-AP alone.

In the second series of experiments, the three Ca antagonists were superfused for 48 min from 6 min after S_3 to 6 min after S_6, and 4-AP (3×10^{-4} M) was added to the superfusion medium for 16 min from 6 min after S_5. The effects of 4-AP on the inhibitory actions of the Ca antagonists on both responses to TNS were expressed as the ratio S_6/S_3, and they were estimated by comparison with our previous results obtained with the Ca antagonists alone (Takata & Kato, 1984).

Other experiments

The TNS was applied twice every 16 min under the stimulation conditions mentioned above. Thereafter, noradrenaline (10 nmol) was injected 3 times at 30 min intervals in a volume of 0.1 ml into the superfusion stream (NA_1 to NA_3). The concentration of noradrenaline used was sufficient to cause a contraction similar to that produced by the 2nd TNS. 4-AP (3×10^{-4} M) was superfused 20 min before the 3rd application of noradrenaline (NA_3). The effects of 4-AP on the contraction caused by exogenous noradrenaline were expressed as the ratio NA_3/NA_2 and it was evaluated by comparison with the corresponding ratio obtained by the repeated application of noradrenaline alone.

Statistical analysis

All data are expressed as the mean ± s.e.mean. Statistical analyses were performed using an unpaired Student's two-tailed *t* test for two-sample comparison and an analysis of variance followed by Dunnett test for multiple comparisons. In each case, *P* values less than 0.05 were considered significant.

Drugs

The following drugs were used: (−)-[7-^{3}H(N)]-noradrenaline (specific activity, 22.4 Ci mmol^{-1}, New England Nuclear), noradrenaline bitartrate (Sigma), 4-aminopyridine (Merck), tetrodotoxin (Sankyo), verapamil hydrochloride (Eisai), nicardipine hydrochloride (Yamanouchi), diltiazem hydrochloride (Tanabe), (−)-*cis* isomer of diltiazem hydrochloride (Tanabe), tetracaine hydrochloride (Sigma) and sodium salicylate (Wako Pure Chemicals).

Results

Effects of 4-aminopyridine on TNS-evoked tritium overflow and contraction

4-AP (3×10^{-4} M) enhanced the TNS-evoked tritium overflow and contraction in the veins preloaded with [^{3}H]-noradrenaline. The ratio of the tritium overflow evoked by the first stimulation in the presence of 4-AP (S_3) to that evoked by the stimulation prior to the application of 4-AP (S_2) was 2.71 ± 0.05 ($n = 6$). The corresponding ratio of the contraction was 1.34 ± 0.07. When the veins were repeatedly stimulated 5 times in the presence of 4-AP, the TNS-evoked tritium overflow declined gradually; the ratio S_7/S_2 was 2.35 ± 0.10. On the other hand, the contraction did not decline with repeated stimulation, the ratio S_7/S_2 being 1.37 ± 0.05.

Since the total tritium overflow evoked by TNS is due to a substantial increase in the release of intact [^{3}H]-noradrenaline in the canine saphenous veins (Vanhoutte *et al.*, 1973), we regarded the total evoked tritium overflow as an index of neurotransmitter release.

Effects of tetrodotoxin on the spontaneous and TNS-evoked tritium overflows and contraction in the strips pretreated with 4-aminopyridine

When tetrodotoxin (6×10^{-7} M) was added to the superfusate in the veins pretreated with 4-AP (3×10^{-4} M), it completely inhibited the TNS-evoked overflow and contraction (Figure 1). The inhibitory effects of tetrodotoxin were partially restored by a subsequent resuperfusion with medium containing 4-AP alone. The superfusion with tetrodotoxin in the presence of 4-AP showed no significant effects on the spontaneous tritium overflow, when compared with the spontaneous overflow observed in the superfusion with 4-AP alone.

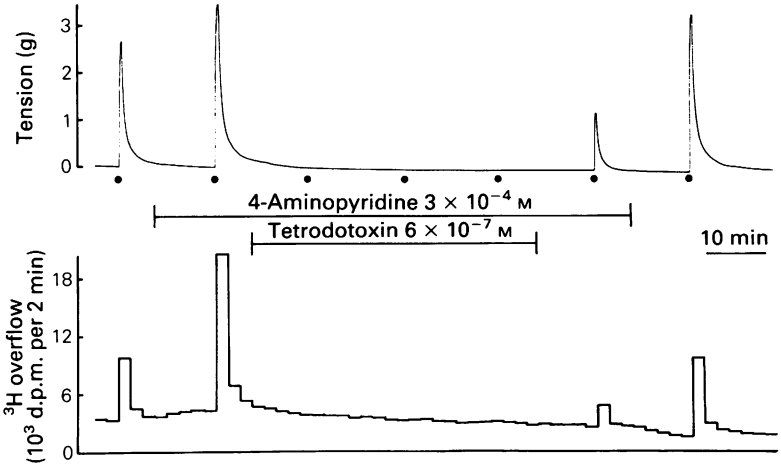

Figure 1 Effects of tetrodotoxin on both tritium overflow and contraction evoked by transmural nerve stimulation in the presence of 4-aminopyridine in a vein preloaded with [^{3}H]-noradrenaline. The responses to the first stimulation are not shown.

Effects of Ca antagonists on the spontaneous and TNS-evoked tritium overflows and contraction in the strips pretreated with 4-aminopyridine

Our previous studies indicate that verapamil (3×10^{-5} M), diltiazem and nicardipine (10^{-5} M and 3×10^{-5} M) significantly inhibited the TNS-evoked tritium overflow and contraction in the canine saphenous veins preloaded with [^{3}H]-noradrenaline (Takata & Kato, 1984).

In the presence of 4-AP (3×10^{-4} M), verapamil (3×10^{-5} M) had no significant effect on the tritium overflow evoked by TNS, but it still produced a significant inhibition of the contraction ($S_6/S_3 = 0.63 \pm 0.05$, $n = 5$, $P < 0.01$), when compared with the corresponding ratio (1.03 ± 0.03, $n = 6$) of the control strips to which TNS was repeatedly applied in the presence of 4-AP alone. The inhibition of the contraction by verapamil in the presence of 4-AP was much smaller than that by verapamil alone. Nicardipine (10^{-5} M and 3×10^{-5} M) also had no significant effects on the TNS-evoked tritium overflow from the strips pretreated with 4-AP. Nicardipine, however, significantly inhibited the corresponding contraction in a concentration-dependent manner; the ratio S_6/S_3 for 3×10^{-5} M was 0.79 ± 0.02 ($n = 6$). This effect was not significantly different from the inhibition by nicardipine alone. Unlike verapamil and nicardipine, diltiazem (10^{-5} M and 3×10^{-5} M) caused a concentration-dependent, significant inhibition of the tritium overflow and contraction produced by TNS in the presence of 4-AP (the ratios S_6/S_3 for 3×10^{-5} M were 0.44 ± 0.04 and 0.64 ± 0.05 respectively, $n = 5$). Figure 2 shows a typical tracing of the effects of 3×10^{-5} M diltiazem on both responses to TNS. The inhibitory actions of diltiazem (10^{-5} M and 3×10^{-5} M) on the evoked tritium overflow and contraction in the veins pretreated with 4-AP were not significantly different from those of diltiazem alone. The inhibition of the contraction by 3×10^{-5} M diltiazem in the strips pretreated with 4-AP was equipotent to that produced by the same concentration of verapamil, but was significantly greater than that by nicardipine ($P < 0.01$).

We further investigated the effects of the (−)-*cis* isomer of diltiazem, a substance which is at least 100 times less potent than diltiazem in inhibiting $CaCl_2$-induced constriction of rabbit ear artery (Nagao *et al.*, 1982), on the TNS-evoked tritium overflow and contraction in the presence or absence of 4-AP. The (−)-*cis* isomer of diltiazem (10^{-5} M and 3×10^{-5} M) caused concentration-dependent and significant inhibition of the TNS-evoked tritium overflow and contraction in the absence of 3×10^{-4} M 4-AP. The inhibition by (−)-*cis* diltiazem was similar to that seen with (+)-*cis* diltiazem. Moreover, pretreatment with 4-AP caused no significant effects on the inhibitory action of the (−)-*cis* isomer of diltiazem on both responses to TNS. Summarized data for verapamil, nicardipine, diltiazem and (−)-*cis* diltiazem are shown in Figure 3.

The inhibitory effects of 3×10^{-5} M verapamil,

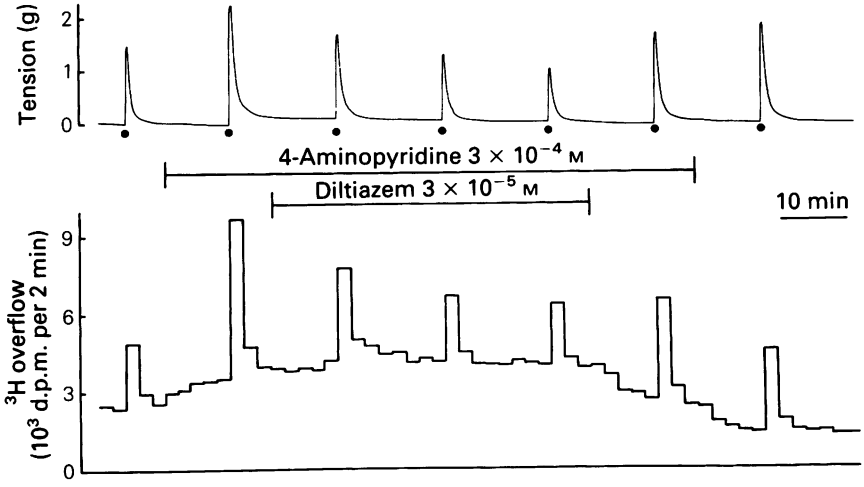

Figure 2 Effects of diltiazem on both tritium overflow and contraction evoked by transmural nerve stimulation in the presence of 4-aminopyridine in a vein preloaded with [^{3}H]-noradrenaline. The responses to the first stimulation are not shown.

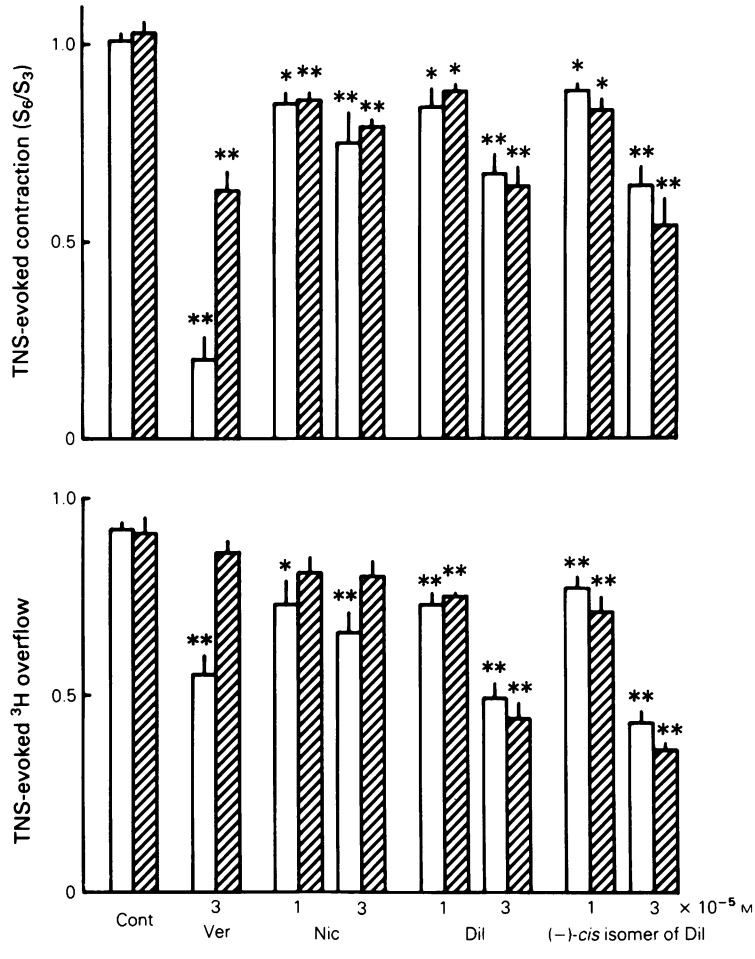

Figure 3 Effects of Ca antagonists on tritium overflow and contraction evoked by transmural nerve stimulation (TNS) in the presence (hatched columns) and absence (open columns) of 4-aminopyridine (4-AP 3×10^{-4} M) in veins preloaded with [^{3}H]-noradrenaline. 4-AP and Ca antagonists were superfused from 10 min before S_3 to 6 min after S_7 and from 10 min before S_4 to 6 min after S_6, respectively. Values are expressed as the mean from 5 to 6 experiments with s.e.mean shown by vertical lines. Cont = control, Ver = verapamil, Nic = nicardipine, Dil = diltiazem. Asterisks indicate significant difference from the corresponding control values: $*P < 0.05$, $**P < 0.01$. The results without 4-AP were taken from our previous paper (1984).

diltiazem and the (−)-*cis* isomer of diltiazem on the contraction were partially restored, when the strips were stimulated 10 min after the onset of the resuperfusion with 4-AP alone, while the inhibition by nicardipine (10^{-5} M and 3×10^{-5} M) was not reversed at all. The inhibition of the TNS-evoked tritium overflow by diltiazem and its (−)-*cis* isomer were partially restored.

Table 1 shows the effects of verapamil, nicardipine, diltiazem and (−)-*cis* isomer of diltiazem on the spontaneous tritium overflow from the veins pretreated with 4-AP. Superfusion with 3×10^{-4} M 4-AP increased the spontaneous tritium overflow; the ratio Sp_3/Sp_2 was 1.36 ± 0.05 ($n = 6$), this value being significantly greater than the corresponding value (0.91 ± 0.05, $n = 5$) in the veins without 4-AP. Thereafter, the spontaneous tritium overflow slowly declined, as shown in the control values of Table 1. Verapamil (3×10^{-5} M) significantly increased the spontaneous tritium overflow, when judged by the ratios Sp_4/Sp_2-Sp_6/Sp_2. Nicardipine, diltiazem and the (−)-*cis* isomer of diltiazem at 3×10^{-5} M also elevated

Table 1 Effects of Ca antagonists on spontaneous tritium overflow from the veins pretreated with 4-aminopyridine

Drugs	*Concentration* (M)	*Spontaneous tritium overflow* Sp_4/Sp_2	Sp_5/Sp_2	Sp_6/Sp_2
Control	—	1.38 ± 0.10	1.30 ± 0.08	1.16 ± 0.09
Verapamil	3×10^{-5}	3.43 ± 0.35**	4.35 ± 0.43**	4.11 ± 0.39**
Nicardipine	10^{-5}	1.26 ± 0.10	1.26 ± 0.08	1.18 ± 0.06
	3×10^{-5}	1.52 ± 0.15	1.48 ± 0.10	1.34 ± 0.09
Diltiazem	10^{-5}	1.21 ± 0.07	1.15 ± 0.07	1.09 ± 0.06
	3×10^{-5}	1.49 ± 0.09	1.50 ± 0.09	1.41 ± 0.10
(−)-*cis* Isomer	10^{-5}	1.43 ± 0.06	1.43 ± 0.08	1.35 ± 0.06
of diltiazem	3×10^{-5}	1.70 ± 0.12	1.83 ± 0.16	1.78 ± 0.16*

The veins were previously incubated with [^{3}H]-noradrenaline. 4-Aminopyridine was present from 10 min before S_3 to 6 min after S_7. Ca antagonists were superfused from 10 min before S_4 to 6 min after S_6. Values are expressed as the mean ± s.e.mean from 5 to 6 experiments. Asterisks indicate significant difference from the control veins superfused with 4-aminopyridine alone: $*P < 0.05$; $**P < 0.01$.

the spontaneous tritium overflow, but these effects did not reach statistical significance, except for the ratio Sp_6/Sp_2 for 3×10^{-5} M (−)-*cis* isomer of diltiazem. The increases in the spontaneous tritium overflow induced by these four drugs were not accompanied by an elevation of basal tension.

Effects of 4-aminopyridine on the inhibitory actions of Ca antagonists on the TNS-evoked tritium overflow and contraction

In this series of experiments, the veins were initially superfused with the medium containing Ca antagonists and then with Ca antagonists plus 3×10^{-4} M

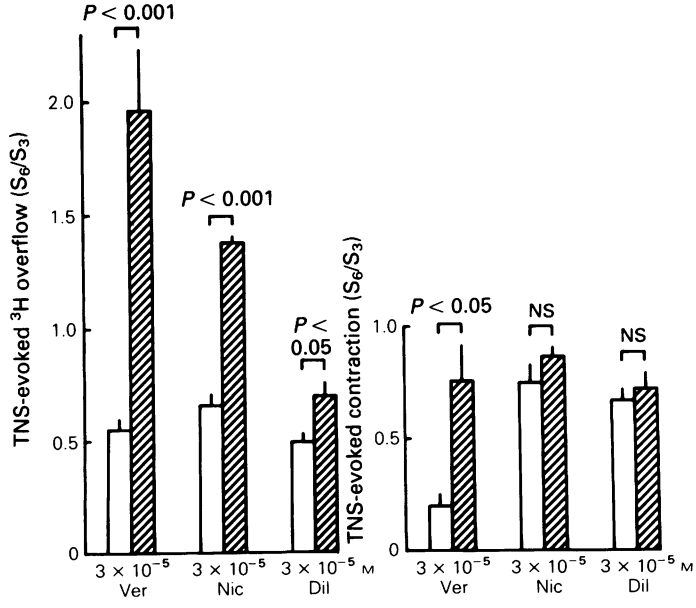

Figure 4 Effects of 4-aminopyridine (hatched columns) on the inhibition of tritium overflow and contraction evoked by transmural nerve stimulation by Ca antagonists in veins preloaded with [^{3}H]-noradrenaline. 4-Aminopyridine and Ca antagonists were superfused from 10 min before S_6 to 10 min before S_7 and from 10 min before S_4 to 6 min after S_6, respectively. Values are expressed as the mean from 3 to 6 experiments with s.e.mean shown by vertical lines. The results without 4-aminopyridine (open columns) were taken from our previous paper (1984). NS = not significant. Other abbreviations are shown as in Figure 3.

4-AP. Figure 4 shows the effects of 4-AP on the inhibition of the TNS-evoked tritium overflow and contraction by 3×10^{-5} M verapamil, nicardipine or diltiazem. A further application of 3×10^{-4} M 4-AP to the veins superfused with 3×10^{-5} M verapamil or nicardipine increased the TNS-evoked tritium overflow approximately 2 to 3 fold, compared with the evoked overflow observed during the superfusion with verapamil and nicardipine (Takata & Kato, 1984). 4-AP (3×10^{-4} M) also caused a slight but significant reversal of the inhibition of the TNS-evoked tritium overflow by 3×10^{-5} M diltiazem alone, which was much less than those for verapamil and nicardipine. On the other hand, 4-AP significantly reversed the inhibition of the contraction by verapamil, while it had no significant effect on the inhibitory actions of nicardipine and diltiazem on the contraction.

Effects of tetracaine on both tritium overflow and contraction induced by TNS in the presence and absence of 4-aminopyridine

Diltiazem and its (−)-*cis* isomer possess similar local anaesthetic potencies (Nagao *et al.*, 1972). Therefore, the effects of tetracaine (4×10^{-6} M), a local anaesthetic, on both responses to TNS were also studied in the presence and absence of 4-AP. Tetracaine was used in a concentration which was as potent as 3×10^{-5} M diltiazem in inhibiting TNS-evoked tritium overflow. Tetracaine significantly inhibited both responses to TNS in the absence of 4-AP, an action which was not affected by pretreatment with 4-AP (upper panel of Figure 5). When further 4-AP was added to the strips which had been treated with tetracaine, 4-AP again exerted no significant effect on the inhibition of both

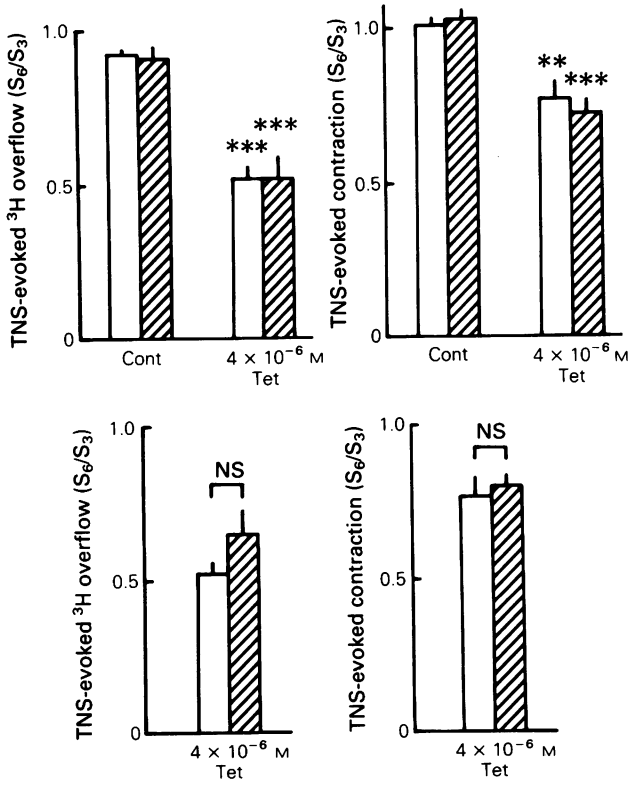

Figure 5 Effects of tetracaine (Tet) on both tritium overflow and contraction evoked by transmural nerve stimulation (TNS) in the presence (hatched columns) and absence (open columns) of 4-aminopyridine (3×10^{-4} M, 4-AP) in veins preloaded with [^{3}H]-noradrenaline. 4-AP was superfused from 10 min before S_3 to 6 min after S_7 (upper panel) or 10 min before S_6 to 10 min before S_7 (lower panel). Tet was superfused from 10 min before S_4 to 6 min after S_6 in each panel. Values are expressed as the mean of 4 to 6 experiments; vertical lines show s.e.mean. NS = not significant. Asterisks indicate significant difference from the corresponding control values: $^{**}P < 0.01$; $^{***}P < 0.001$.

responses to TNS by tetracaine (lower panel of Figure 5).

Effects of sodium salicylate on the TNS-evoked tritium overflow inhibiting actions of diltiazem and tetracaine in the presence of 4-aminopyridine

Tetracaine also causes a competitive antagonism of Ca^{2+}-induced contraction of the K^+-depolarized guinea-pig taenia caecum with a pA_2 of 5.3 (Spedding & Berg, 1985), this concentration being almost equal to that used in the present study. Such a Ca antagonist action of tetracaine is augmented by sodium salicylate (10 mM) (Spedding & Berg, 1985), while the effect of diltiazem is reduced (Spedding, 1984). We therefore determined, using sodium salicylate, whether the inhibitory effects of diltiazem on the TNS-evoked tritium overflow in the presence of 4-AP are related to a tetracaine-like Ca antagonistic action. As shown in Table 2, sodium salicylate (10 mM), when simultaneously applied with diltiazem (3×10^{-5} M) to the veins pretreated with 4-AP (3×10^{-4} M), failed to modify the inhibitory effects of diltiazem on the TNS-evoked tritium overflow. In contrast, the inhibition by tetracaine (4×10^{-6} M) was significantly enhanced by sodium salicylate.

Table 2 Effects of sodium salicylate on the inhibitory actions of diltiazem and tetracaine on the tritium overflow evoked by transmural nerve stimulation (TNS) in veins pretreated with 4-aminopyridine (3×10^{-4} M)

Drugs	*Concentration* (M)	*TNS-evoked ^3H overflow* (S_6/S_3)
Diltiazem	3×10^{-5}	0.44 ± 0.05
Diltiazem + sodium salicylate	3×10^{-5} + 10^{-2}	0.41 ± 0.02
Tetracaine	4×10^{-6}	0.52 ± 0.07
Tetracaine + sodium salicylate	4×10^{-6} + 10^{-2}	0.25 ± 0.03**

The veins were previously incubated with [^{3}H]-noradrenaline. 4-Aminopyridine was present from 10 min before S_3 to 6 min after S_7. Sodium salicylate and diltiazem or tetracaine were simultaneously superfused from 10 min before S_4 to 6 min S_6. Values are expressed as the mean ± s.e.mean from 5 experiments. Asterisks indicate significant difference from the corresponding drugs in the absence of sodium salicylate: $^{**}P < 0.01$.

Effects of 4-aminopyridine on the contraction induced by exogenous noradrenaline

To evaluate whether 4-AP enhances the TNS-induced contraction by acting on postsynaptic sites, we studied the effects of 4-AP (3×10^{-4} M) on the contraction produced by 10 nmol noradrenaline. In the control group of strips, the increases in tension to the second TNS and the second application of noradrenaline were 2.90 ± 0.67 and 2.97 ± 0.64 g ($n = 5$), respectively. The corresponding values in another group of strips before treatment with 4-AP were 2.92 ± 0.45 and 3.00 ± 0.31 g ($n = 5$), respectively. No significant difference between the contractions caused by the TNS and noradrenaline was observed in both groups. As shown in Table 3, 4-AP (3×10^{-4} M) caused no significant enhancement of the contraction induced by 10 nmol noradrenaline, suggesting that the augmentation of the TNS-evoked contraction by 4-AP may be due exclusively to an increase in neurotransmitter release.

Discussion

In the present study, 4-AP (3×10^{-4} M) augmented both tritium overflow and contraction evoked by TNS in the veins preloaded with [^{3}H]-noradrenaline. It has been reported that some vascular smooth muscles generate Ca spikes following perivascular nerve stimulation particularly in the presence of a drug that inhibits the K conductance of the membrane, such as 4-AP and tetraethylammonium, and that the spike is unaffected by treatment with either tetrodotoxin or Na^+-free solution, but is abolished by Ca antagonists (Itoh *et al.*, 1982). These authors also estimated that the influx of Ca during the spike is roughly 10^{-6} M and that this amount of Ca is enough to cause a contraction. The present results showing that tetrodotoxin completely inhibited the TNS-evoked tritium overflow and contraction in the presence of 4-AP may rule out the possibility that under the conditions used the canine saphenous veins generate spikes to produce the contraction. Furthermore, 4-AP failed to enhance

Table 3 Effects of 4-aminopyridine (4-AP) on the contraction induced by 10 nmol noradrenaline (NA)

Drugs	*Concentration* (M)	*NA-induced contraction* (NA_3/NA_2)
Control	—	1.03 ± 0.02
4-AP	3×10^{-4}	1.06 ± 0.02

Noradrenaline was injected 3 times every 30 min into the superfusion stream (NA_1-NA_3). 4-Aminopyridine was added to the superfusion medium 20 min before NA_3. Values are expressed as the mean ± s.e.mean from 5 experiments.

the contraction induced by noradrenaline exogenously applied at a concentration producing a similar contraction to that induced by TNS. These results suggest that the enhancement of the TNS-evoked contraction by 4-AP is exclusively attributable to an increased release of neurotransmitter, as previously described by Johns *et al.* (1976) using rabbit vas deferens.

We previously reported that high concentrations of verapamil and nicardipine significantly inhibited the TNS-evoked tritium overflow from the canine saphenous veins preloaded with [^{3}H]-noradrenaline (Takata & Kato, 1984). The present study shows that verapamil and nicardipine at the same concentration that we had previously used failed to inhibit the evoked tritium overflow from the veins pretreated with 4-AP. Thus, the inhibitory effects of the two drugs on the TNS-evoked tritium overflow were antagonized by 4-AP. Moreover, when 4-AP was applied to the verapamil- or nicardipine-treated veins, the inhibition of the evoked overflow by the two drugs was reversed by 4-AP. Since 4-AP increases Ca^{2+} influx during TNS into the nerve endings as a result of an inhibition of K conductance (Kirpekar *et al.*, 1977; Molgó *et al.*, 1977; Guerrero & Novakovic, 1980) and since 4-AP also facilitates Ca^{2+} influx through a direct action on the voltage-dependent Ca^{2+} channels (Lundh & Thesleff, 1977; Illes & Thesleff, 1978), the present results together with our previous study (Takata & Kato, 1984) suggest that both verapamil and nicardipine may decrease the release of neurotransmitter evoked by TNS by inhibiting Ca^{2+} influx into the nerve endings. This suggestion is consistent with the report that the inhibitory action of Cd^{2+}, a Ca antagonist devoid of Na^+ channel blocking properties (Kostyuk & Krishtal, 1977), on the electrically stimulated release of [^{3}H]-noradrenaline from the rat neocortical slices was strongly reduced when the release was enhanced by 4-AP (Schoffelmeer & Mulder, 1983). A similar result was also obtained by Zetler & Sowoidnich (1980), who reported that 4-AP antagonized the depressant effects of Mg^{2+}, a competitor with Ca^{2+} (Mordes & Wacker, 1978), on the twitch response of the field-stimulated guinea-pig vas deferens.

The inhibition of the TNS-induced contraction by verapamil was only partially antagonized by pretreatment with 4-AP. Thus, even under conditions where evoked tritium overflow was not suppressed, verapamil still caused a significant inhibition of the contraction produced by TNS, when compared with the control value obtained by TNS in the presence of 4-AP alone. This inhibition of the contraction by verapamil might be due to its interaction with α-adrenoceptors as suggested by Langer & Shepperson (1981) and by Karliner *et al.* (1982). High concentrations of verapamil have been reported to have a local anaesthetic action (Van der Kloot & Kita, 1975; Bondi, 1978). However, the inhibitory action of verapamil on the contraction may be unrelated to this effect, since local anaesthetic activity was observed at concentrations of 10^{-4} M or higher (Van der Kloot & Kita, 1975; Bondi, 1978). In this respect, tetracaine and lidocaine failed to inhibit the contractile response of canine saphenous veins to 40 mM KCl whereas the contraction was almost completely abolished by phentolamine (10^{-6} M) (Takata & Kato, 1985). Nicardipine also inhibited significantly the contraction in the presence of 4-AP, an effect almost equipotent to that in the absence of 4-AP. These findings suggest that the inhibition of the contraction by nicardipine in the absence of 4-AP may probably be due to an interference with Ca^{2+} availability in the postsynaptic sites rather than a decreased release of neurotransmitter. This is supported by the report that high concentrations of nicardipine inhibited the contractile response of the rabbit aorta to exogenous noradrenaline by potentiating intracellular calcium sequestration (Terai *et al.*, 1981).

Unlike verapamil and nicardipine, diltiazem at 10^{-5} M or more inhibited the TNS-evoked tritium overflow and the corresponding contraction in the veins pretreated with 4-AP, these effects being nearly equipotent to those in the absence of 4-AP. Moreover, 4-AP reversed the evoked overflow inhibiting action of verapamil much more effectively than that of diltiazem, although the two drugs suppressed, to a similar degree, the evoked tritium overflow in the absence of 4-AP (Figure 4). These observations make it questionable whether diltiazem decreases the TNS-evoked tritium overflow by inhibiting Ca^{2+} influx into the adrenergic nerve endings. Hence, we next investigated the effects of the (−)-*cis* isomer of diltiazem which is at least 100 times less potent than diltiazem in inhibiting the $CaCl_2$-induced constriction of the perfused rabbit ear arteries (Nagao *et al.*, 1982), on both responses to TNS in the presence and absence of 4-AP. This isomer at 10^{-5} M or more inhibited both tritium overflow and contraction evoked by TNS to the same extent as diltiazem. The inhibitions induced by (−)-*cis* isomer of diltiazem were also unaffected by pretreatment with 4-AP. Two possibilities may account for the observations concerning diltiazem. First, diltiazem and its (−)-*cis* isomer possess a local anaesthetic action, the ED_{50} values for diltiazem and its isomer being 6.2×10^{-3} and 6.4×10^{-3} M, respectively (Nagao *et al.*, 1972). The concentrations of the two drugs used in the present study were lower than the ED_{50} values. The slope of the dose-response curve for a local anaesthetic activity is known to be steep (Starke *et al.*, 1972). Furthermore, in the diltiazem-treated veins, 4-AP caused a slight but significant reversal of the inhibition of the evoked overflow by diltiazem, whereas it failed to reverse significantly that by tetracaine. Therefore, it seems unlikely that the in-

hibitory effects of diltiazem and its (−)-*cis* isomer on the TNS-evoked tritium overflow may be due to their local anaesthetic actions. The drugs which act on Na^+ channels, such as local anaesthetics, can also interact with Ca^{2+} channels (Hay & Wadsworth, 1982; Spedding & Berg, 1985). Tetracaine at the concentration used in the present study has such a Ca antagonistic action in the guinea-pig taenia caecum and this effect is augmented by sodium salicylate which increases the negative surface charge (Spedding & Berg, 1985); this was the case for the adrenergic nerve endings of canine saphenous veins. The present results where sodium salicylate failed to enhance the inhibition of the TNS-evoked tritium overflow by diltiazem also demonstrate that the effects of diltiazem are not attributable to a tetracaine-like Ca antagonistic action. Secondly, it is most likely that diltiazem inhibits the TNS-evoked tritium overflow through a mechanism independent on its ability to suppress Ca^{2+} influx. This mechanism remains unknown. However, it is speculated that diltiazem might interfere with the event which intervenes between Ca^{2+} influx during TNS and neurotransmitter release. In this connection, it is noted that relatively high concentrations (above 10^{-6} M) of diltiazem also act on intracellular sites (Saida & Van Breemen, 1983).

Diltiazem and its (−)-*cis* isomer at 10^{-5} M or more exerted a significant inhibition of the TNS-evoked contraction irrespective of the presence of 4-AP. Diltiazem at concentrations up to 10^{-4} M has no significant effect on the response of the canine saphenous veins to exogenous noradrenaline (Langer & Shepperson, 1981). The same concentrations of diltiazem also fail to suppress the contraction induced by 3×10^{-6} M noradrenaline in the rabbit ear arteries, although they inhibit the contraction evoked by perivascular nerve stimulation (Kajiwara & Casteels, 1983). These findings indicate that in contrast with verapamil and nicardipine, diltiazem and possibly its (−)-*cis* isomer may inhibit the TNS-evoked contraction only by decreasing neurotransmitter release.

The present study also indicates that 3×10^{-5} M verapamil significantly increased the spontaneous tritium overflow from the veins pretreated with 4-AP. The enhanced effects of verapamil were almost equipotent to those in the absence of 4-AP (Takata & Kato, 1984). On the other hand, significant increases in the spontaneous tritium overflow evoked by nicardipine, diltiazem and (−)-*cis* isomer of diltiazem in the absence of 4-AP (Takata & Kato, 1984) were no longer observed in the presence of 4-AP. Thus, the spontaneous overflow increasing actions of the three drugs were masked by the ability of 4-AP itself to increase the spontaneous tritium overflow which has been reported to be insensitive to external Ca^{2+} (Kirpekar *et al.*, 1977). Numerous investigators have found that several Ca antagonists increased the spontaneous tritium overflow from various tissues preloaded with [^{3}H]-noradrenaline (Kajiwara & Casteels, 1983; Karaki *et al.*, 1984; Takata & Kato, 1984; Zsotér *et al.*, 1984; Chaudhry & Vohra, 1985; Wolchinsky & Zsotér, 1985; Zsotér & Wolchinsky, 1986). The exact mechanism by which the Ca antagonists increase the spontaneous tritium overflow remains unclear. However, the increase in spontaneous tritium overflow induced by verapamil (Chaudhry & Vohra, 1985), diltiazem and nicardipine (Kajiwara & Casteels, 1983) consists mainly of tritiated 3,4-dihydroxyphenylglycol, a deaminated metabolite. Enhanced effects of verapamil on the spontaneous tritium overflow from the rat atria were also observed in the presence of cocaine and hydrocortisone (Chaudhry & Vohra, 1985). Therefore, it seems likely that verapamil and possibly the other antagonists act on noradrenaline storage vesicles to increase the leakage of the amine into the cytoplasm. This view is further supported by the present results that these antagonists did not change basal tension.

The present study together with our previous results (Takata & Kato, 1984) suggests that diltiazem but not verapamil and nicardipine may inhibit TNS-evoked neurotransmitter release through an action other than inhibition of Ca^{2+} influx into the adrenergic nerve endings, thereby allowing an inhibition of the corresponding contraction.

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Paf-acether-induced death in mice: involvement of arachidonate metabolites and β-adrenoceptors

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1 Intravenous Paf-acether (Paf, 15–80 $\mu g\,kg^{-1}$) killed conscious Swiss mice in a dose-dependent manner, without causing platelet aggregation in the lung microvasculature, or pulmonary oedema.

2 Propranolol (0.01–10 $mg\,kg^{-1}$, i.p.) potentiated the effects of an LD_{20} of Paf dose-dependently, while the β_1-adrenoceptor selective antagonist, metoprolol, was three orders of magnitude less potent in this respect. Salbutamol (1 $mg\,kg^{-1}$, i.p.) provided complete protection against an LD_{80} of Paf.

3 High doses of indomethacin, aspirin, benoxaprofen and FPL 55712 given i.p. failed to inhibit the effects of an LD_{80} of Paf, while BW 755C (50–100 $mg\,kg^{-1}$) exerted a dose-dependent protection and benzydamine (50 $mg\,kg^{-1}$) and nordihydroguaiaretic acid (200 $mg\,kg^{-1}$) were partially active. Dexamethasone (1–5 $mg\,kg^{-1}$, s.c.) exerted a dose-dependent protection, when administered at least 4 h before Paf.

4 In mice anaesthetized with urethane, Paf (1–30 $\mu g\,kg^{-1}$) produced hypotension which was not clearly dose-related. The effects of the highest dose were also tested on the resistance of the lungs to inflation and found to produce bronchoconstriction.

5 It may be concluded that pharmacological manipulation of β_2-adrenoceptors modulates Paf-induced death in mice, while arachidonate metabolites of the cyclo-oxygenase pathway and peptido-leukotrienes do not appear to be involved. However, lipoxygenase products, distinct from peptido-leukotrienes, may play a role in this phenomenon. It is suggested that bronchoconstriction, probably associated with cardiovascular effects, is a major determinant of the acute toxicity of Paf in mice.

Introduction

Paf (1-O-alkyl-2-acetyl - sn - glyceryl - 3 - phosphoryl-choline) is a novel mediator released from a variety of cell types (e.g. platelets, macrophages, polymorphonuclear cells) by immunological and non-immunological stimuli. In the last few years an increasingly significant role in pathophysiological events has been ascribed to this natural phospholipid, and its involvement in thrombosis, asthma, inflammation and gastric ulceration is being ascertained (Venuti, 1985; Rosam *et al.*, 1986).

Intravenous Paf produces lethal shock in the mouse (Myers *et al.*, 1983), a species whose platelets are resistant to the activating effects of the phospholipid (Namm *et al.*, 1982). This syndrome is completely prevented by cortisone acetate (Myers *et al.*, 1983) and, although no hypothesis was raised about the target organs of Paf toxicity in this model, it was supposed to be mediated by arachidonate metabolites of the lipoxygenase pathway. Since Paf-induced death in mice may represent a useful model of systemic anaphylaxis, we tried to reach a better understanding of its mechanism. In addition to investigating the role of lipoxygenase metabolites, we studied the effects of β-adrenoceptor antagonism, which causes a hyperreactive bronchomotor state (Bongrani *et al.*, 1983) and could modulate Paf-induced death, if bronchoconstriction participates in this phenomenon. In fact the latter is preceded by respiratory distress (Myers *et al.*, 1983), which could possibly be due to the well-known bronchoconstricor features of Paf, evident at high doses even in the rat (Dahlbäck *et al.*, 1984), another animal species whose platelets are unresponsive to Paf (Vargaftig *et al.*, 1981).

Methods

Male Swiss mice (Nossan, Corezzana, VA, Italy) weighing 25 to 35 g, fed *ad libitum*, were used. Groups of conscious animals were injected via a caudal vein with doses of synthetic Paf (C18, Bachem), which induced death in about 20% (LD_{20}) or 80% (LD_{80}) of

the animals, normally within 10 to 30 min. These doses were determined before each experimental session and were found to vary over several months between 15–25 and 40–80 $\mu g\,kg^{-1}$, respectively. Drugs were dissolved in saline, with the addition of 0.1 N sodium carbonate in the case of indomethacin, nordihydroguaiaretic acid and benoxaprofen, and administered i.p. unless stated otherwise. Dexamethasone was administered s.c. as a suspension in saline; in the 'repeated dose' experiment, dexamethasone was administered once a day, between 09 h 00 min and 10 h 00 min, for five consecutive days. Saline treated mice served as controls. The 24 h mortality rates of drug-treated groups were compared with those of matched control groups by the χ^2-test.

For histopathological studies ten mice were given Paf 50 $\mu g\,kg^{-1}$ i.v., a dose that caused 100% mortality within 30 min. Their lungs were removed immediately and fixed with 10% formalin. Sections of left lung lobes were processed to paraffin wax blocks, sectioned (5 μm) and stained with haematoxylin and eosin for light microscopical examination. Ten saline-treated mice, killed after 30 min by cervical dislocation, were used as controls.

In order to evaluate lung oedema, two additional groups of ten mice received Evans blue (10 $mg\,kg^{-1}$ i.v.) 20 min before Paf (50 $\mu g\,kg^{-1}$) or saline. Again, all Paf-treated mice died in less than 30 min, whereas controls were killed 30 min after administration of saline. Their lungs were removed immediately, rinsed in cold saline, blotted and weighed. Tissue Evans blue was determined according to Harada *et al.* (1971).

The effects of Paf (0.3–30 $\mu g\,kg^{-1}$) were tested on arterial blood pressure in older mice (body weight 45–50 g) and also (at the highest dose only) on the resistance of the lungs to inflation, by use of a modification of the Konzett & Rössler technique (1940). Briefly, the mice were anaesthetized with ethyl urethane 1.5 $g\,kg^{-1}$ i.p., a catheter was inserted into a carotid artery and connected to a Bentley Trantec 800 pressure transducer; blood pressure was recorded on a Basile 7070 Gemini Recorder. To measure lung function, mice were also given pancuronium bromide (2 $mg\,kg^{-1}$ i.v.) and were artificially ventilated through a tracheal cannula, connected to a Miniature Ideal Pump (Bioscience), calibrated at 0.5–0.6 ml and 100 r.p.m. The ventilation of the animal was regulated to equal a pressure of 6 cmH_2O and the volume of overflow air was diverted to a movable piston attached to a Basile 7006 isotonic transducer and recorded on a Basile 7070 Gemini recorder.

Drugs used were indomethacin and propranolol hydrochloride (Gianni), acetylsalicylic acid lysine salt (Maggioni), benzydamine hydrochloride (Angelini), nordihydroguaiaretic acid (Sigma), benoxaprofen (Lilly), salbutamol sulphate and dexamethasone (Glaxo), BW 755C (3-amino-1[*m*-(trifluoromethyl)-phenyl]-2-pyrazoline, Wellcome), FPL 55712 (sodium 7-[3(4-acetyl-3-hydroxy-2-propylphenoxy) -2-hydroxypropoxy] - 4 - oxo - 8 - propyl - 4H - 1 - benzopyran - 2 - carboxylate (Fisons), CV 3988 (rac-3-(N-*n*-octadecyl carbamoyloxy)-2-methoxypropyl-2-thiazolio ethylphosphate) (Takeda), metoprolol tartrate (Geigy), terbutaline sulphate (Farmitalia), pancuronium bromide (Organon) and ethyl urethane (C. Erba). All doses refer to the salts.

Results

Lethal doses of Paf induced prostration within 5 min of administration, followed by respiratory depression and gasping. Death occurred within 30 min of administration except in very few cases (less than 4%). Histopathological examination of the lungs failed to reveal platelet aggregates, pulmonary oedema or any other pathological signs, except for foci of leucocytes which were evident in occasional blood vessels in 4 out of 10 Paf-treated animals, but not in the controls. The weight of the lungs did not vary between treated and control groups and neither did the amount of Evans blue extracted, thus excluding a large fluid extravasation as a consequence of Paf treatment.

Table 1 shows that propranolol (0.01–10 $mg\,kg^{-1}$, i.v.) decreased survival following an LD_{20} of Paf dose-dependently, while metoprolol did not interfere in doses up to 1 $mg\,kg^{-1}$, but exerted a significant reduction at 10 $mg\,kg^{-1}$. As shown in Table 2, cyclo-oxygenase inhibitors (indomethacin and acetylsalicylic acid) gave no protection against Paf-induced death. Among dual lipoxygenase/cyclo-oxygenase inhibitors, benoxaprofen was inactive, while BW 755C exerted a dose-dependent protection, benzydamine was partially active at 50 $mg\,kg^{-1}$ (while causing convulsions at 100 $mg\,kg^{-1}$) and nordihydro-

Table 1 Potentiating effects of propranolol and metoprolol on Paf-induced death in mice

Treatment	*Dose* ($mg\,kg^{-1}$)	*Survivors* / *Tested*	*% survival*	*Significance* (χ^2)
Vehicle		97/120	81	
Propranolol	0.01	8/20	40	$P<0.01$
	0.1	4/20	20	$P<0.001$
	1	3/20	15	$P<0.001$
	10	0/10	0	$P<0.001$
Metoprolol	0.01	16/20	80	NS
	0.1	12/20	60	NS
	1	15/20	75	NS
	10	5/20	25	$P<0.05$

Test drugs were administered i.p. 30 min before a dose of Paf lethal in about 20% of the animals (15–25 $\mu g\,kg^{-1}$, i.v.).

Table 2 Effects of β-adrenoceptor agonists, cyclo-oxygenase and lipoxygenase inhibitors, a peptido-leukotriene antagonist and a Paf antagonist on Paf-induced death in mice

Treatment	*Dose* ($mg\,kg^{-1}$)	*Survivors* / *Tested*	*% survival*	*Significance* (χ^2)
Vehicle		45/290	16	
Salbutamol	1	19/20	95	$P<0.001$
Terbutaline	1	11/20	55	$P<0.05$
Indomethacin	5	2/10	20	NS
	50	4/20	20	NS
Acetylsalicylic acid	90	6/19	32	NS
Benzydamine	5	1/10	10	NS
	50	11/18	61	$P<0.05$
BW 755C	5	3/10	30	NS
	50	12/20	60	$P<0.01$
	100	9/10	90	$P<0.001$
Nordihydro-guaiaretic acid	50	10/29	34	NS
	100	3/10	30	NS
	200	7/10	70	$P<0.05$
Benoxaprofen	100	4/10	40	NS
	200	3/10	30	NS
FPL 55712	10	3/20	15	NS
	50	5/20	25	NS
CV 3988	1	13/20	65	$P<0.001$
	3	17/20	85	$P<0.001$
	10	10/10	100	$P<0.001$

Test drugs were administered i.p. 30 min (5 min in the case of FPL 55712) before a dose of Paf lethal in about 80% of the animals (40–80 $\mu g\,kg^{-1}$, i.v.).

Table 3 Protective effects of dexamethasone, administered in single and repeated doses, on Paf-induced death in mice

Treatment	*Pretreatment time* (h)	*Survivors* / *Tested*	*% survival*	*Significance* (χ^2)
		Single dose experiment		
Vehicle		8/45	18	
Dexamethasone	−0.5	11/30	37	NS
$5\,mg\,kg^{-1}$	−1	1/10	10	NS
	−2	10/20	50	NS
	−4	18/20	90	$P<0.001$
		Repeated dose experiment		
Vehicle		10/25	40	
Dexamethasone $5\,mg\,kg^{-1}$		19/20	95	$P<0.001$

In the single dose experiment, dexamethasone was administered subcutaneously at different times before a dose of Paf which was lethal in about 80% of the animals (40–55 $\mu g\,kg^{-1}$, i.v.). In the repeated dose experiment the test drug was administered by the same route for five days and, 30 min after the last administration, the animals were challenged with Paf.

guaiaretic acid produced a significant protection only at $200\,mg\,kg^{-1}$. FPL 55712 was tested over a range of doses ($1-50\,mg\,kg^{-1}$) and pretreatment times (1 and 5 min: only data obtained at the latter time are shown in Table 2), but no protection was observed in any case. The β_2-adrenoceptor agonists, salbutamol and terbutaline were very potent inhibitors of Paf-induced death. The selective Paf antagonist CV 3988 ($1-10\,mg\,kg^{-1}$) exerted a dose-dependent protection. The glucocorticoid dexamethasone was not significan-

Table 4 Reversal by propranolol of the protective effects of dexamethasone and salbutamol on Paf-induced death in mice

Treatment	*Dose* (mg kg^{-1})	*Route*	*Pretreatment time* (min)	*Survivors* / *Tested*	*% survival*	*Significance* (χ^2)
Vehicle + Vehicle	— —	i.p. i.p.	− 35 − 30	2/10	20	
Vehicle + Salbutamol	— 1	i.p. i.p.	− 35 − 30	9/10	90	$P<0.01$*
Propranolol + Salbutamol	10 1	i.p. i.p.	− 35 − 30	1/10	10	$P<0.01$§
Vehicle + Vehicle	— —	i.p. s.c.	− 30 − 240	1/20	5	
Vehicle + Dexamethasone	— 1	i.p. s.c.	− 30 − 240	15/20	75	$P<0.001$*
Propranolol + Dexamethasone	10 1	i.p. s.c.	− 30 − 240	7/20	35	$P<0.05$*§

The test drugs were administered at different times before a dose of Paf which was normally lethal in about 80% of the animals (40–70 μg kg^{-1}, i.v.).
* versus vehicle + vehicle; § versus vehicle + test drug.

tly active in single dose treatment (5 mg kg^{-1}, s.c.) when given 0.5–2 h before Paf, but exerted complete protection at 4 h (Table 3). After a repeated dose treatment, dexamethasone demonstrated a complete inhibitory effect even when Paf was injected only 30 min after the last dose (Table 3). As shown in Table 4, acute dexamethasone was active also at a lower dose. Its effects were reversed only partially by propranolol, at a dose that completely reversed the effects of salbutamol.

Paf did not affect mean arterial pressure in urethane-anaesthetized mice (basal values 45–60 mmHg) at a dose of 0.3 μg kg^{-1}, i.v. At 1 and 3 μg kg^{-1}, i.v., Paf transiently (5–20 min) reduced blood pressure by 23 ± 6% and 18 ± 6% respectively (mean values ± s.e.mean). A dose of 10 μg kg^{-1} was lethal in 2 out of 4 mice and a dose of 30 μg kg^{-1} in 3 out of 4, within 12–15 min of administration, while hypotension (20–36%) was observed in the surviving animals. Also when Paf was given in cumulative doses, similar

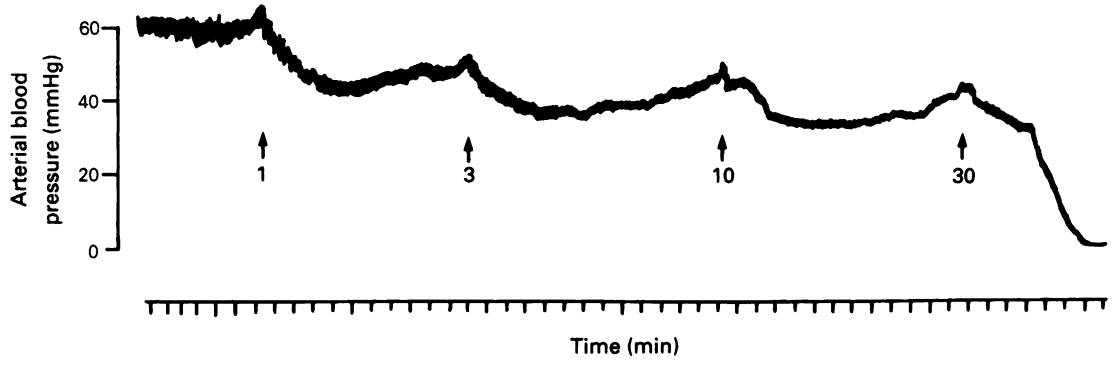

Figure 1 Effects of Paf 1–30 μg kg^{-1} i.v. (administered at the arrows) on arterial blood pressure of the anaesthetized mouse.

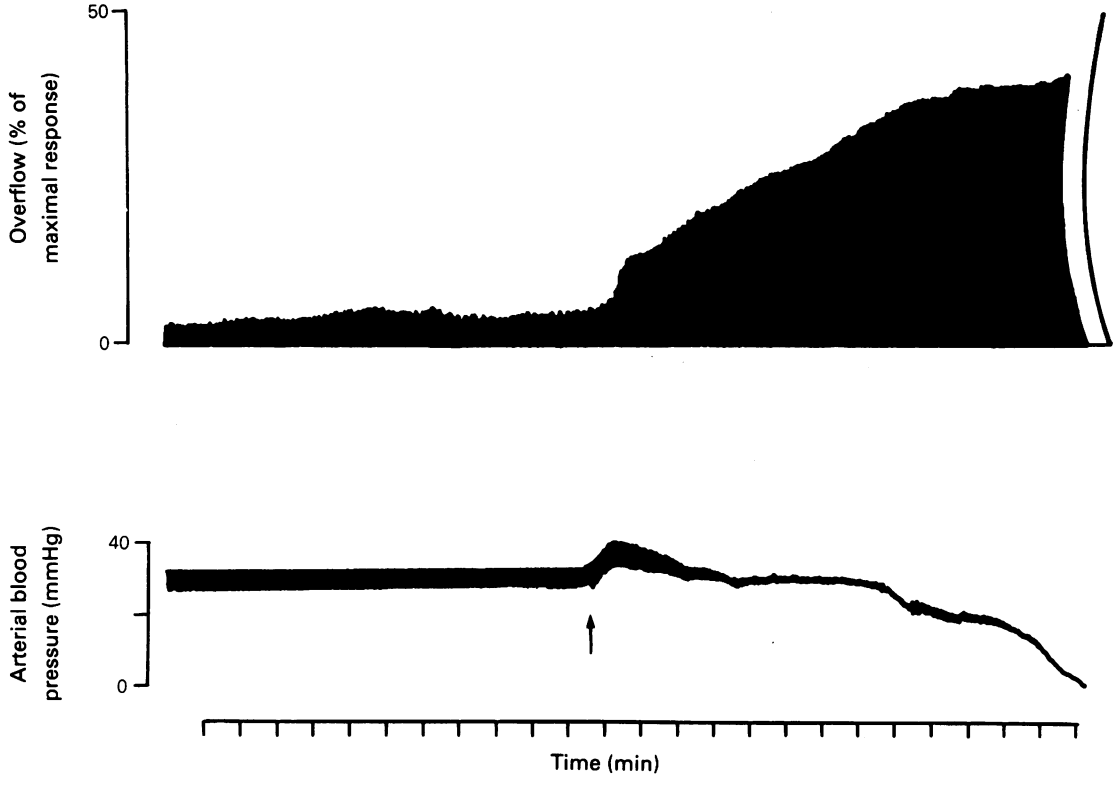

Figure 2 Effects of Paf 30 $\mu g\,kg^{-1}$ i.v. (administered at the arrow) on arterial blood pressure (b) and lung overflow (a) of the anaesthetized mouse, paralysed with pancuronium. At the end of the experiment maximal bronchoconstriction was achieved by clamping off the trachea, after a two fold decrease of the gain on the recorder.

effects were observed (Figure 1). When the animals were prepared according to the Konzett & Rössler (1940) technique, 3 out of 8 mice showed spontaneous increase of the lung overflow and were discarded. In the remaining 5 animals, Paf at 30 $\mu g\,kg^{-1}$, i.v. induced a bronchospasm which was 27 ± 5% of maximal bronchoconstriction (Figure 2), a value similar to those obtained with methacholine chloride 100–200 $\mu g\,kg^{-1}$ (data not shown).

Discussion

Paf may be a mediator of different types of shock, such as endotoxic shock (Terashita *et al.*, 1985) and anaphylaxis (Vargaftig *et al.*, 1986). Therefore, Paf-induced death in mice may be a convenient model for the *in vivo* screening of compounds potentially useful in the treatment of disorders such as these (Myers *et al.*, (1983). Anaphylaxis is an acute reaction manifested in a number of body organ systems, including the cardiovascular, respiratory, gastrointestinal and peripheral nervous systems as well as the skin. The most dramatic events are those of cardiovascular collapse and obstruction of the airways, which are the causes of death in fatal anaphylaxis (Schellenberg, 1985). Intravenous Paf does in fact produce an anaphylactic reaction in experimental animals, characterized by platelet-dependent bronchoconstriction and hypotension in guinea-pigs (Vargaftig *et al.*, 1980), rabbits (Lefer *et al.*, 1984) and baboons (McManus *et al.*, 1981), but the mechanism of Paf-induced death in mice, a species whose platelets are unresponsive to Paf (Namm *et al.*, 1982), is not clear.

Some of our results would seem to argue against the hypothesis that death is due mainly to a bronchopulmonary event: (a) morphological signs of bronchoconstriction were not seen (but such changes would be difficult to evaluate on histological sections, since the smooth muscle elements in mouse lungs are less prominent than in other species; A.J. Newman, personal communication); (b) platelet aggregates were not

seen in the sections examined; (c) morphological and functional signs of lung oedema were not detected. However, the foci of leukocytes, noted in 40% of Paf-treated mice, may indicate leukocyte activation, since Paf is capable of aggregating neutrophils in various animal species, releasing mediators and lysosomal enzymes, which can induce inflammation and bronchoconstriction (Lynch *et al.*, 1984). In fact bronchoconstriction was observed after the administration of a high dose of Paf to urethane-anaesthetized mice, although this result was obtained in rather unphysiological conditions. Paf ($1-30\,\mu g\,kg^{-1}$) also induced hypotension, followed by death within 15 min at the highest doses, but it was less potent than in the rat, where its hypotensive effects are evident from doses of $0.03-0.06\,\mu g\,kg^{-1}$, i.v. and are clearly dose-dependent (Caillard *et al.*, 1982; Lai *et al.*, 1983). Thus both bronchopulmonary and cardiovascular events probably contribute to Paf-induced death in mice.

However, some indirect evidence seems to indicate that an acute bronchoconstriction is a major cause of this phenomenon in conscious animals. Potentiation of Paf-induced death by propranolol (a β_1 and β_2-adrenoceptor antagonist; Weiner, 1985) but not metoprolol (a selective β_1-adrenoceptor antagonist; Weiner, 1985) and protection by β_2-adrenoceptor agonists are suggestive of bronchopulmonary effects of Paf, as it was shown that β_2-blockade potentiates the bronchospasms induced by numerous agonists, including Paf itself (McCulloch *et al.*, 1967; Bonnet *et al.*, 1983; Bongrani *et al.*, 1983), but do not influence their cardiovascular effects (Caillard *et al.*, 1982; Bongrani *et al.*, 1983).

The protective action of dexamethasone may also be explained by the activation of β_2-adrenoceptor mechanisms, since corticosteroids are known to increase the number and affinity of β-adrenoceptors in human neutrophils (Davies & Lefkowitz, 1981) and to abolish desensitization to β-agonists, possibly through the involvement of arachidonate metabolites (Abbracchio *et al.*, 1986). However, the fact that propranolol only partially reversed the protective effects of dexamethasone seems to indicate the involvement of some other explanation for these effects. Myers *et al.* (1983) hypothesized that the protective action of glucocorticoids in this model is dependent on the inhibition of the lipoxygenase pathway of arachidonate metabolism, through the well-known induction of factors inhibiting phospholipase A_2 and/or 5-lipoxygenase. While our study was in progress, Young *et al.* (1985) confirmed this hypothesis on the basis of the protection afforded by inhibitors of lipoxygenase, such as BW 755C and phenidone or the peptido-leukotrienes antagonist, FPL 55712. Our results are generally in good agreement with those described above, except that FPL 55712 was completely inactive in our hands, up to the highest dose of $50\,mg\,kg^{-1}$. The short duration of action of FPL 55712 cannot be invoked as an explanation for this result, since we found this compound inactive even when administered only 1 min before Paf. Thus it must be concluded that, at least under our experimental conditions, the peptido-leukotrienes do not play any significant role in Paf-induced death in mice. On the other hand the protection obtained with relatively high doses of BW 755C and another lipoxygenase inhibitor, benzydamine (Saeed *et al.*, 1979), may indicate that other products of lipoxygenase, such as leukotriene B_4 (LTB_4), a mediator of chemotaxis and bronchospasm released by leukocytes, are involved in this phenomenon. The weak activity of nordihydroguaiaretic acid does not contradict this hypothesis, since although this agent is a potent inhibitor of lipoxygenase *in vitro* (Salari *et al.*, 1984), it often showed little or no activity *in vivo* (Etienne *et al.*, 1985; Young *et al.*, 1985; McMillan *et al.*, 1986). Similarly the lack of activity of benoxaprofen is not surprising, this drug being 10 to 1000 fold weaker than BW 755C as a lipoxygenase inhibitor (Kingston, 1981). The lack of involvement in our model of LTC_4 and LTD_4, which are supposed to be major mediators of bronchoconstriction in man, does not necessarily contradict the suggested relevance of bronchopulmonary effects in Paf-induced death, since it has been demonstrated that the peptido-leukotrienes are not involved in the bronchospasm occurring in some other models of anaphylactic shock (Ufkes & Ottenhof, 1984).

In conclusion Paf-induced death in mice can be modulated by drugs which act at β_2-adrenoceptors, but not by cyclo-oxygenase inhibitors or peptido-leukotriene antagonists and may depend, at least in part, on acute bronchoconstriction.

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Effects of tachykinins on inositol phospholipid hydrolysis in slices of hamster urinary bladder

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1 Tachykinin-stimulated inositol phospholipid hydrolysis was examined in slices of hamster urinary bladder.

2 In the presence of lithium, to inhibit inositol monophosphatase activity, substance P, eledoisin and related tachykinins induced large, dose-dependent increases in [^{3}H]-inositol monophosphate accumulation.

3 The responses to substance P and eledoisin were not antagonized by the cholinoceptor antagonist, atropine.

4 The rank order of potency for various tachykinins was kassinin $\geq$ neurokinin A $\geq$ neurokinin B $\geq$ eledoisin $\geq$ physaelamin $\geq$ substance P $\geq$ substance P methyl ester.

5 The synthetic analogue [p-Glu^6, D-Pro^9] SP (6–11) was considerably more potent than its L-prolyl stereoisomer at stimulating inositol phospholipid hydrolysis.

6 These results suggest that in the hamster urinary bladder, tachykinin-induced inositol phospholipid breakdown is mediated via tachykinin receptors of the SP-E type, as opposed to the SP-P type.

Introduction

A variety of evidence now supports the existence of multiple receptors for tachykinin peptides in different tissues (see reviews by Watson, 1984a; Iversen, 1985; Table 1). At the SP-P receptor type, exemplified by the receptor present in guinea-pig ileum longitudinal smooth muscle, substance P is active in nanomolar concentrations and is approximately equipotent with eledoisin, kassinin and physaelamin (Lee *et al.*, 1982). In contrast, at the SP-E receptor, as observed in the rat vas deferens, whereas kassinin and eledoisin are active in nanomolar concentrations, physaelamin and substance P are several hundred times less active (Lee *et al.*, 1982). In addition the SP-P selective agonist, substance P methyl ester, although approximately equipotent with substance P and eledoisin on SP-P systems, is 100–1000 times less potent than substance

Table 1 Structures of naturally occurring and synthetic tachykinin peptides.

Tachykinin	*Amino acid sequence*
Substance P	Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2
Physaelamin	pGlu-Ala-Asp-Pro-Asn-Lys-Phe-Tyr-Gly-Leu-Met-NH_2
Eledoisin	pGlu-Pro-Ser-Lys-Asp-Ala-Phe-Ile-Gly-Leu-Met-NH_2
Kassinin	Asp-Val-Pro-Lys-Ser-Asp-Gln-Phe-Val-Gly-Leu-Met-NH_2
Neurokinin A	His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-NH_2
Neurokinin B	Asp-Met-His-Asp-Phe-Phe-Val-Gly-Leu-Met-NH_2
[pGlu^6, D-Pro^9] SP (6–11)	pGlu-Phe-Phe-D-Pro-Leu-Met-NH_2
[pGlu^6, L-Pro^9] SP (6–11)	pGlu-Phe-Phe-L-Pro-Leu-Met-NH_2

Conserved residues are underlined.
pGlu = pyroglutamic acid

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P on SP-E systems (Watson *et al.*, 1983).

In recent years, substance P and related tachykinins have been shown to stimulate inositol phospholipid hydrolysis in a variety of tissues, including rat salivary gland (Hanley *et al.*, 1980), guinea-pig ileum longitudinal muscle and rat hypothalamus (Watson & Downes, 1983). In these tissues substance P, eledoisin and substance P methyl ester possess similar nanomolar potencies at inducing this response, suggesting that inositol phospholipid breakdown is associated with SP-P receptor activation (Watson & Downes, 1983). However, in slices of rat ileum longitudinal muscle, eledoisin is considerably more potent than substance P at stimulating inositol phospholipid hydrolysis suggesting that in some tissues this response may be mediated by SP-E receptor (Watson, 1984b).

In order to investigate further the association of SP-E receptors with inositol phospholipid breakdown, we examined tachykinin-induced inositol monophosphate production in slices of hamster urinary bladder, a tissue whose contractile response exhibits a typical SP-E receptor profile (Watson *et al.*, 1983).

A preliminary account of this work has been presented to the British Pharmacological Society (Bristow *et al.*, 1986).

Methods

Incorporation of [^{3}H]-inositol into phospholipids

The effects of tachykinins on inositol phospholipid hydrolysis were examined by previously described methods (Berridge *et al.*, 1982; 1983). Golden Syrian hamsters (80–120 g, MB strain) were stunned, decapitated and their urinary bladders rapidly removed and placed in 10 ml freshly gassed (95% O_2 : 5% CO_2) Krebs solution containing (mM): NaCl 118, KCl 4.7, $CaCl_2$ 1.25, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2, and glucose 11 (pH 7.4). Following hemisection and washing, bladders were cross-chopped (350 μm × 350 μm × 350 μm) with a McIlwain tissue chopper. Slices were dispersed in 10 ml fresh Krebs solution by means of a Pasteur pipette and washed for 30 min at 37°C with 250 ml continuously gassed Krebs solution. After 30 min, the Krebs solution was removed and 25 μl aliquots (~0.5 mg protein) of the resultant tissue suspension were transferred into plastic tubes containing 2 μCi [^{3}H]-inositol, (Amersham International plc, specific activity 16.3 Ci mmol^{-1}), 10 mM LiCl, bovine serum albumin (1 mg ml^{-1}) and bacitracin (40 μg ml^{-1}) in 215 μl fresh Krebs solution. Lithium was added to inhibit inositol monophosphatase activity and hence prevent the hydrolysis of inositol monophosphate to free inositol (Hallcher & Sherman, 1980; Berridge *et al.*, 1982). Bovine serum albumin and bacitracin were included to inhibit metabolism of tachykinins by endogenous peptidases. Tubes were individually gassed (95% O_2 : 5% CO_2), capped and incubated for 30 min at 37°C in a shaking water bath to allow incorporation of [^{3}H]-inositol into tissue inositol phospholipids. Tachykinins (10 μl) were then added and the tubes gassed, capped and incubated at 37°C for a further 45 min. A maximally stimulating concentration of substance P (30 μM) was included in all experiments.

Separation of [^{3}H]-inositol monophosphate

Incubations were terminated by the addition of 1 ml chloroform/methanol (1 : 2; vol/vol) followed by 300 μl chloroform and 300 μl deionised water to separate the aqueous and organic phases. Aliquots (750 μl) of the upper aqueous phase were diluted with 2 ml deionised water and [^{3}H]-inositol monophosphate separated from other labelled water-soluble metabolites by anion exchange chromatography as described by Berridge *et al.* (1982). Samples were added to glass columns containing 1 ml of Dowex AG 1-X8 formate resin (1 vol Dowex : 1 vol deionised water suspension). Following elution of each column with 10 ml deionised water to remove unchanged [^{3}H]-inositol, followed by 15 ml 25 mM ammonium formate solution to elute [^{3}H]-glycerophosphoinositol, columns were eluted with 10 ml 150 mM ammonium formate containing 5 mM sodium tetraborate to remove [^{3}H]-inositol monophosphate. This latter fraction was collected in scintillation vials and counted in the gel phase for radioactivity following the addition of 10 ml Hydrofluor.

Analysis of results

Data were plotted as [^{3}H]-inositol monophosphate accumulation (d.p.m.) above basal versus log tachykinin concentration. Within an experiment, all data points were determined in triplicate. EC_{50} values (concentration of tachykinin inducing 50% of the maximum response as determined by 30 μM substance P) were obtained from dose-response curves involving at least four different tachykinin concentrations. Mean EC_{50} values were determined from 4–6 separate dose-response curves.

Peptides

All tachykinin related peptides were purchased from Bachem Biochemicals with the exception of substance P methyl ester which was obtained from Cambridge Research Biochemicals. [pGlu^6, D-Pro^9] SP (6–11) and [pGlu^6, L-Pro^9] SP (6–11) were synthesized and characterized in the Medicinal Chemistry Department of the MSDRL Neuroscience Research Centre.

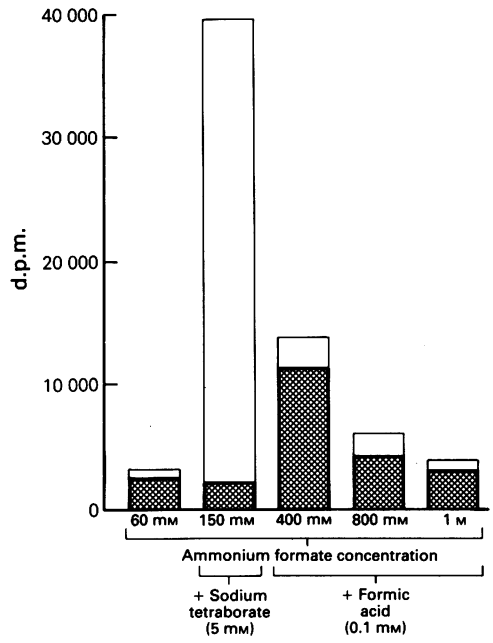

Figure 1 Separation of [^{3}H]-inositol labelled water soluble compounds from prelabelled slices of hamster urinary bladder exposed to a maximally stimulating concentration of eledoisin (10 μM) for 45 min (dashed lined) as compared with controls (continuous line). The method of Berridge *et al.* (1982) was used with minor modifications. Pooled, triplicate aqueous phase samples were added to Dowex anion exchange columns (see Methods), and phosphate esters removed by stepwise addition of formate solutions of increasing strength. Histograms represent the sum of the d.p.m. eluting with each formate solution. Glycerophosphoinositol and inositol-1, 2-cyclic phosphate were eluted with 15 ml 25 mM ammonium formate; myo-inositol monophosphate was eluted with 10 ml 150 mM ammonium formate containing 5 mM sodium tetraborate; inositol, bisphosphate was eluted with 10 ml 400 mM ammonium formate in 0.1 M formic acid. The more polar inositol phosphates were eluted with 10 ml 800 mM ammonium formate in 0.1 M formic acid followed by 10 ml 1 M ammonium formate in 0.1 M formic acid. [^{3}H]-inositol is not retained on the columns and was removed with 10 ml deionised water before the ammonium formate elution.

Results

Naturally occurring tachykinins

In the absence of added tachykinins, basal [^{3}H]-inositol monophosphate accumulation was 612 $\pm$ 103 d.p.m. per 25 μl slices (n = 11). Following a 45 min incubation in the presence of a maximally stimulating concentration of eledoisin (10 μM), [^{3}H]-inositol monophosphate production was increased 17 fold to 10410 $\pm$ 1029 d.p.m. per 25 μl slices (n = 5). This large increase in [^{3}H]-inositol monophosphate production reflects the ability of 10 mM lithium to block inositol monophosphatase activity. In view of the aqueous extraction procedures employed, significant increases in the levels of other labelled water soluble inositol monophosphates were not observed (Figure 1). A maximally stimulating concentration of substance P (30 μM) also stimulated [^{3}H]-inositol monophosphate production approximately 15 fold to 9399 $\pm$ 941 d.p.m. per 25 μl slices (n = 6).

Responses to both eledoisin and substance P were dose-dependent, and representative dose-response curves are illustrated in Figure 2. Similarly large dose-dependent increases in [^{3}H]-inositol monophosphate accumulation were also observed with the amphibian tachykinins, kassinin and physaelamin, and also the mammalian tachykinins, neurokinin A and neurokinin B. However, as detailed in Table 2, significant differences were observed in the relative potencies of these various tachykinins. Thus, with mean EC_{50} values of 0.007 μM and 0.045 μM respectively, kassinin and eledoisin were significantly more potent than physaelamin (EC_{50} value 3.97 μM) and substance P (EC_{50} value 5.09 μM). Neurokinin A and neurokinin B, with EC_{50} values of 0.013 μM and 0.036 μM respectively, were approximately equipotent with kassinin and eledoisin (Table 2).

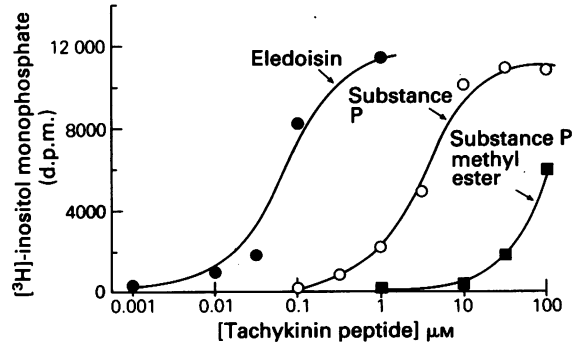

Figure 2 Dose-respone curves for the effects of eledoisin (●), substance P (○) and substance P methyl ester (■) on [^{3}H]-inositol monophosphate accumulation in slices of hamster urinary bladder. Results are expressed as d.p.m. [^{3}H]-inositol monophosphate per 25 μl slices over basal levels. Each dose-response curve was obtained in a separate experiment in which a maximally stimulating concentration of substance P (30 μM) induced a similar maximum response. Data points are the means for triplicate determinations.

Table 2 EC_{50} values for tachykinins and analogues for stimulation of [^{3}H]-inositol monophosphate production in slices of hamster urinary bladder

Tachykinin	*EC_{50} (μM) (− s.e.mean, + s.e.mean)*	
Kassinin	0.007	(0.005, 0.009)
Neurokinin A	0.013	(0.010, 0.018)
Neurokinin B	0.036	(0.030, 0.044)
Eledoisin	0.045	(0.033, 0.060)
Physaelamin	3.97	(3.16, 5.00)
Substance P	5.09	(3.95, 6.56)
Substance P methylester	106.6	(66.7, 170.6)
[pGlu^6, D-Pro^9] SP (6–11)	0.216	(0.164, 0.285)
[pGlu^6, L-Pro^9] SP (6–11)	28.97	(20.99, 40.00)

Mean EC_{50} values (concentration required to yield 50% maximum response as determined by 30 μM substance P) were calculated from 4–6 determinations each involving separate dose-response curves employing a minimum of four tachykinin concentrations. Results were calculated in the logarithms and are given as the antilog of the mean (− s.e.mean; + s.e.mean).

Synthetic tachykinin analogues

In contrast to the naturally occurring tachykinins, the synthetic SP-P receptor agonist, substance P methyl ester, failed to induce a maximum response at concentrations up to 1 mM (Figure 2). With an EC_{50} value of 106.6 μM, this synthetic tachykinin analogue was considerably less potent than the other tachykinins tested.

Two synthetic N-terminal fragments of substance P, [pGlu^6, D-Pro^9] SP (6–11) and [pGlu^6, L-Pro^9] SP (6–11) were also examined for their ability to stimulate [^{3}H]-inositol monophosphate production in slices of hamster urinary bladder. These analogues have been described as selective agonists for SP_1 and SP_2-receptors respectively (Piercey *et al.*, 1985), a tachykinin receptor classification based on the differential activity of N-terminal fragments of substance P in several biological systems (Piercey *et al.*, 1982). Interestingly, Lee *et al.* (1986) have recently described the differential selectivity of this pair of tachykinin analogues for SP-P and SP-E binding sites, with [pGlu^6, D-Pro^9] SP (6–11) being selective for SP-E sites whilst [pGlu^6, L-Pro^9] SP (6–11) is selective for SP-P sites. As illustrated in Figure 3, both analogues induced a dose-related increase in [^{3}H]-inositol monophosphate production, with [pGlu^6, D-Pro^9] SP (6–11) giving rise to a similar maximum response to that observed with a maximally stimulating concentration of substance P (30 μM). In comparison, [pGlu^6, L-Pro^9] SP (6–11) failed to induce a similar maximum response. Moreover, with a mean EC_{50} value of 40 μM (Table 2), [pGlu^6, L-Pro^9]-SP (6–11) was approximately 140 times less potent than [pGlu^6, D-Pro^9]-SP (6–11) (mean EC_{50} value 0.285 μM; see Table 2).

Effects of atropine

In addition to the various tachykinins, the cholinoceptor agonist carbachol (10 μM) also induced a large stimulation in [^{3}H]-inositol monophosphate accumulation (Figure 4). Given the observation that various tachykinins can stimulate [^{3}H]-acetylcholine release from previously labelled stores within the myenteric plexus of the guinea-pig ileum (Holzer &

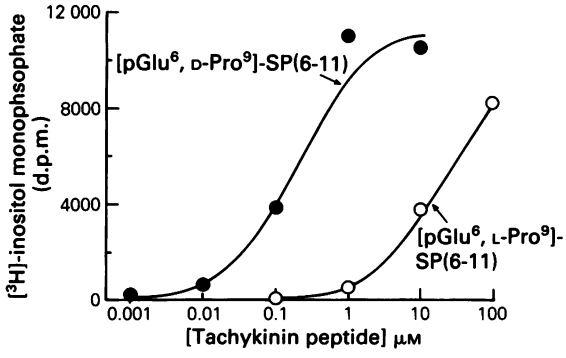

Figure 3 Dose-response curves for the effects of [pGlu^6, D-Pro^9] SP (6–11) (●) and [pGlu^6, L-Pro^9] SP (6–11) (○) on [^{3}H]-inositol monophosphate accumulation in slices of hamster urinary bladder. Results are expressed as d.p.m. [^{3}H]-inositol monophosphate per 25 μl slices over basal levels. Results are taken from a single representative experiment in which a maximally stimulating concentration of substance P (30 μM) induced a similar maximum response to that observed with 10 μM (pGlu^6, D-Pro^9] SP (6–11). Data points are the means for triplicate determinations.

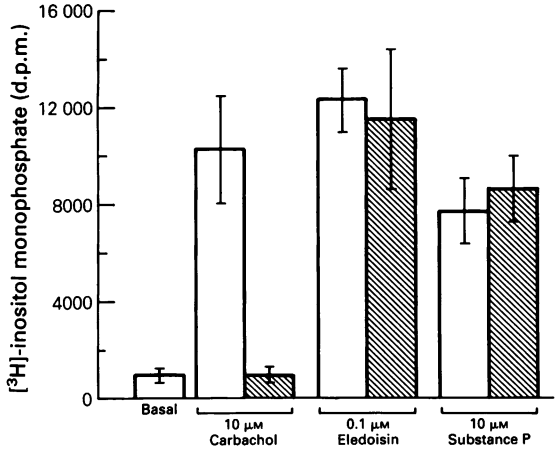

Figure 4 Effects of atropine (1 μM) on [^{3}H]-inositol monophosphate production in slices of hamster urinary bladder induced by carbachol (10 μM), substance P (10 μM) and eledoisin (0.1 μM). Results are expressed as d.p.m. [^{3}H]-inositol monophosphate per 25 μl slices over basal levels and represent the means of 4–8 separate experiments. Open column: agonist alone; hatched colums: agonist plus atropine (1 μM). In tubes receiving atropine, the antagonist was added 15 min before the addition of agonist.

Lembeck, 1980; Yau & Youther, 1982; Fosbraey *et al.*, 1984), the possibility exists that the increases in [^{3}H]-inositol monophosphate accumulation observed in response to tachykinins detailed in the present study may be mediated via acetylcholine released from parasympathetic neurones innervating the hamster urinary bladder. To test this hypothesis, the effects of atropine (1 μM), a muscarinic antagonist, were examined on both tachykinin- and carbachol-induced [^{3}H]-inositol monophosphate production. As shown in Figure 5, carbachol (10 μM), substance P (10 μM) and eledoisin (0.1 μM) all induced large responses in [^{3}H]-inositol monophosphate accumulation. However, whereas the response to carbachol was completely abolished in the presence of atropine 1 μM, the responses to both substance P and eledoisin were unaffected. It is concluded, therefore, that the effects of tachykinins on [^{3}H]-inositol monophosphate production in slices of hamster urinary bladder are mediated directly via an interaction with specific tachykinin receptors rather than indirectly via the release of acetylcholine.

Discussion

The results presented in this study indicate that various tachykinins, both naturally occurring and synthetic, can stimulate inositol phospholipid breakdown in slices of hamster urinary bladder. Three lines of evidence suggest that this response is mediated via a SP-E receptor type as opposed to a SP-P receptor type. First, there were marked differences in the relative potencies of tachykinins at stimulating inositol phospholipid breakdown in this tissue, a feature characteristic of SP-E receptors (Lee *et al.*, 1982). Thus, whilst kassinin and eledoisin stimulated inositol phospholipid hydrolysis with potencies in the nanomolar range, susbstance P and physaelamin were considerably less potent, possessing micromolar EC_{50} values. Kassinin, for example, was 700 times more potent than substance P at stimulating [^{3}H]-inositol monophosphate production. Secondly, substance P methyl ester, a selective SP-P agonist (Watson *et al.*, 1983) was significantly less potent than substance P at stimulating inositol phospholipid breakdown in this tissue. Thirdly, the synthetic tachykinin analogue [pGlu^6, D-Pro^9] SP (6–11) was considerably more potent than its L-prolyl stereoisomer at inducing inositol phospholipid breakdown. This result is particularly important in the light of a recent study by Lee *et al.* (1986) who reported that [pGlu^6, L-Pro^9] SP (6–11) is more potent than [pGlu^6, D-Pro^9] SP (6–11) at displacing [^{125}I]-Bolton Hunter labelled substance P binding to SP-P sites, whereas [pGlu^6, D-Pro^9]-SP (6–11) is more

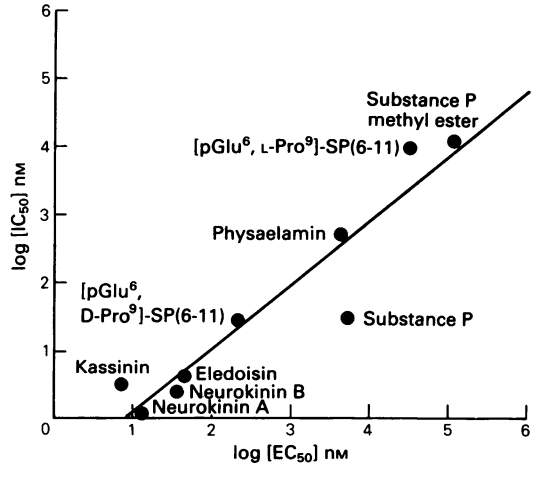

Figure 5 Correlation between EC_{50} values for tachykinin-induced inositol phospholipid hydrolysis in slices of hamster urinary bladder and IC_{50} values for tachykinin-induced displacement of [^{125}I]-Bolton Hunter labelled eledoisin binding in homogenates of hamster urinary bladder. The line was calculated by linear regression analysis; the correlation coefficient (r) was 0.941 (95% CI: 0.743, 0.988). Binding data were taken from Lee *et al.* (1986).

potent than [pGlu^6, L-Pro^9]-SP (6–11) at displacing the binding of [^{125}I]-Bolton Hunter labelled eledoisin to SP-E sites. Accordingly, SP-P and SP-E tissues exhibited very different D-Pro/L-Pro ratios (IC_{50} value of [pGlu^6, D-Pro^9] SP (6–11)/IC_{50} of [pGlu^6, L-Pro^9] SP (6–11). D-Pro/L-Pro ratios for various SP-P tissues were in the range 87–124, whereas in SP-E tissues, a ratio of 0.003 was calculated (Lee *et al.*, 1986). In the present study, the D-Pro/L-Pro ratio (EC_{50} value of [pGlu^6, D-Pro^9] SP (6–11)/EC_{50} of [pGlu^6, L-Pro^9] SP (6–11) for inositol phospholipid hydrolysis was approximately 0.007, thus providing further evidence that the tachykinin receptor mediating this response in the hamster urinary bladder is of the SP-E type. Finally, as illustrated in Figure 5, the EC_{50} values for various tachykinins obtained in the present study showed a good correlation ($r = 0.941$) with their IC_{50} values against [^{125}I]-Bolton Hunter labelled eledoisin binding to SP-E sites in homogenates of hamster urinary bladder as reported by Lee *et al.* (1986).

Recently, Laufer *et al.* (1985) have suggested that the neuronal substance P receptor mediating the release of acetylcholine from the guinea-pig myenteric plexus may represent a third tachykinin receptor type for which neurokinin B may be the preferred ligand. These authors proposed the term 'SP-N' to describe this tachykinin receptor type. In support of this hypothesis, Lee *et al.* (1986) observed that neurokinin B was at least 10 times more potent than other tachykinins at displacing the binding of [^{125}I]-Bolton Hunter labelled eledoisin to presumed SP-N sites in homogenates of rat cerebral cortex. This profile contrasts with that observed for [^{125}I]-Bolton Hunter labelled eledoisin binding to peripheral tissues, such as hamster urinary bladder and rat vas deferens, where neurokinin A, neurokinin B, kassinin and eledoisin are approximately equipotent, indicating the presence of an SP-E binding site. In the present study, the comparable potencies observed for neurokinin A, neurokinin B, kassinin and eledoisin argue against the existence of a 'SP-N' receptor type in the hamster urinary bladder. Whether SP-N receptors are coupled to inositol phospholipid breakdown remains to be seen.

In conclusion, however, the data presented in this paper, provide evidence in support of the original suggestion by Watson (1984) that both SP-P and SP-E receptors are coupled to inositol phospholipid hydrolysis.

Note added in proof:
At a satellite symposium on 'Substance P and Neurokinins' held in Montreal, Canada in July 1986 as part of the XXX International Congress of Physiological Sciences, a majority of participants agreed to rename SP-P, SP-E and SP-N tachykinin receptors NK-1, NK-2 and NK-3 receptors, respectively. Accordingly, the present paper describes the coupling of NK-2 tachykinin receptors to inositol phospholipid hydrolysis.

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Purinoceptors in the rat heart

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1 The effects of an intracoronary bolus of adenosine triphosphate (ATP), α,β-methylene ATP (APCPP), β,γ-methylene ATP (APPCP), adenosine diphosphate (ADP), adenosine monophosphate (AMP) and adenosine on coronary tone and ventricular myocardial contraction were investigated in the perfused rat heart.

2 Adenine nucleotides, given by bolus injection were negatively inotropic in amounts $> 3 \times 10^{-7}$ mol. The potency order was ATP > ADP > AMP. Adenosine ($< 1 \times 10^{-5}$ mol) had no effect on ventricular myocardial contraction.

3 Adenine nucleotides and adenosine (1×10^{-10}–1×10^{-7} mol) reduced coronary tone. The potency order was ATP > ADP > AMP = adenosine. The ATP analogue APPCP was less active than ATP at reducing coronary tone, and APCPP had no vasodilator effect. This suggests the presence of a P_2-purinoceptor, subclass P_{2Y}, which mediates vasodilatation.

4 ATP and ADP increased the concentration of prostacyclin (measured as 6-keto prostaglandin $F_{1\alpha}$) in the perfusate, but only after injection of $> 3 \times 10^{-7}$ mol, suggesting that the vasodilator responses to ATP and ADP were not mediated by prostacyclin. AMP and adenosine had no effect, even at 1×10^{-5} mol.

5 At a dose of 3×10^{-9} mol, approximately 40% of ATP and 70% of ADP was converted to AMP and adenosine whilst passing through the heart. The amounts of AMP and adenosine formed, however, were insufficient to account for the vasodilator effects of ATP and ADP.

6 Vasodilatation mediated by AMP and adenosine was inhibited by an infusion of 8-phenyltheophylline (8-PT; 2×10^{-5} M) indicating interaction with a P_1-purinoceptor. Vasodilatation induced by ATP (at doses at which AMP and adenosine had no action) was also depressed by 8-PT indicating either an action of ATP on P_1-purinoceptors, or an effect of 8-PT on P_{2Y} receptors.

7 Vasodilatation induced by AMP was unaltered during an infusion of α,β-methylene ADP (2×10^{-6} M, which inhibited breakdown of AMP to adenosine by $54.2 \pm 1.5\%$, $n = 4$). This suggests that AMP acted directly, and it did not require conversion to adenosine to induce vasodilatation.

8 The ATP analogues APCPP (1×10^{-9}–1×10^{-8} mol) and APPCP (1×10^{-8}–1×10^{-7} mol) increased coronary tone, as did high doses (1×10^{-5} mol) of ATP and ADP, indicating the presence of an additional P_2-purinoceptor, subclass P_{2X}, mediating vasoconstriction.

Introduction

The potent cardiovascular actions of extracellular adenine nucleotides and adenosine were first described by Drury & Szent-Gyorgyi (1929) and by Green & Stoner (1950). More recently these agents have been shown to alter myocardial performance (Moir & Downs, 1972; Hopkins, 1973; Collis & Pettinger, 1982; Burnstock & Meghji, 1983) and to reduce coronary resistance (Winbury *et al.*, 1953; Wolf & Berne, 1956; Moir & Downs, 1972; Paddle & Burnstock, 1974). Adenosine is now widely accepted as an agent involved in the regulation of coronary tone (see Berne, 1980 for a review). Adenine nucleotides may be present in the coronary circulation as a result of release from hypoxic myocardium (Paddle & Burnstock, 1974; Forrester & Williams, 1977), from endothelial cells (Pearson & Gordon, 1979) from damaged vessel walls (Born & Kratzer, 1984), and from aggregating platelets (Ingerman *et al.*, 1979), in sufficient amounts to cause local effects on the vasculature and the myocardium.

Adenine nucleotides are rapidly degraded to adenosine as they pass through the coronary circulation (Baer & Drummond, 1968; Hopkins, 1973; Paddle & Burnstock, 1974; Schwartzman *et al.*, 1981; Ronca Testoni & Borghini, 1982) and it is not clear to what extent their cardiac and coronary actions are due to

their intrinsic activity or to the activity of their immediate metabolites. Distinguishing between the effects of adenosine triphosphate (ATP) and adenosine offers the possibility of modulating the effects of released nucleotides by interfering with their metabolism.

This study set out to investigate the effects of adenine nucleotides on cardiac function and coronary tone in the perfused rat heart, to establish what types of receptors were involved, and to determine whether these effects were direct actions of the adenine nucleotides or due to their conversion to adenosine. Additionally, because ATP induces the production of prostaglandin (including prostacyclin) by various vascular beds and by cultured endothelial cells (Minkes *et al.*, 1973; Boeynaems & Galand, 1983; Pearson *et al.*, 1983; Hellewell & Pearson, 1984) we examined whether stimulation of prostaglandin production was involved in mediating the effects of nucleotides on rat coronary vessels.

Methods

Male Sprague-Dawley rats (200–300 g) were anaesthetized by intraperitoneal injection of a mixture of midazolam HCl (0.07 mg kg^{-1}), fentanyl citrate (0.17 mg kg^{-1}) and fluanisone (5.4 mg kg^{-1}). Heparin (500 u) was given intravenously. The hearts were excised, immersed in ice-cold buffer and dissected free of connective tissue. The aorta was cannulated and the coronary circulation perfused by the Langendorff method with a solution containing (in mM): NaCl 118, KCl 4.7, KH_2PO_4 1.9, $MgSO_4$ 1.9, $NaHCO_3$ 25.0, $CaCl_2$ 1.8, glucose 5.5, sodium pyruvate 5.5, gassed with O_2:CO_2 (95:5 by volume). Flow, controlled by a roller pump, was increased gradually until perfusion pressure (measured from a side arm of the aortic cannula) reached 60 mmHg (flow = 54.4 ± 1.6 ml min^{-1} g^{-1} dry tissue, mean ± s.e.mean of 65 experiments). Perfusate and tissue temperature were maintained at 37°C. Hearts were electrically stimulated (4 Hz, 12 ms with a supramaximal voltage) by a pair of platinum electrodes inserted into the right ventricle. Myocardial contraction was monitored by a water filled latex balloon within the left ventricle inflated such that left ventrical diastolic pressure did not exceed 10 mmHg. The preparations were allowed to equilibrate for 30 min before addition of drugs. No heart was perfused for longer than 120 min.

ATP, adenosine diphosphate (ADP), adenosine monophosphate (AMP), adenosine, α,β-methylene ATP (APCPP) or β,γ-methylene ATP (APPCP) were injected directly into the aortic cannula (50 μl, over 5 s). Changes in perfusion pressure and myocardial contractile force was monitored. Hearts in which ATP or adenosine (3×10^{-8} mol) reduced perfusion pressure by less than 3 mmHg were excluded from the study.

In some experiments the response to a bolus injection of adenine nucleotide was assessed in the presence of a background infusion of one of the following compounds: 8-phenyltheophylline (8-PT, 2×10^{-5} M), α,β-methylene ADP (APCP, 2×10^{-6} M), phentolamine (1×10^{-5} M), atropine (1×10^{-6} M) or methysergide (5×10^{-6} M).

The breakdown of adenine nucleotides in the coronary circulation was measured using 2-^{3}H-adenine nucleotides. 2-[^{3}H]-ATP, -ADP or -AMP (10 μ Ci) was injected into the aortic cannula and the effluent from the heart was collected for 50 s. The proportion of ^{3}H-nucleotides and nucleosides in this effluent was determined by counting on a t.l.c. linear analyser (Berthold LB 284) after t.l.c. separation using the method of Norman *et al.* (1974).

In other experiments the purine composition of the effluent was assessed by h.p.l.c. Adenine nucleotides were analysed using a 250 × 5 mm ODS Hypersil 5 μm column and a mobile phase of 5×10^{-2} M ammonium dihydrogen phosphate at a flow rate of 1 ml min^{-1} (Simmonds *et al.*, 1982). A linear 20 min gradient of 0–30% methanol was used for the analysis of adenosine. Absorbance was measured at 254 nm, and concentrations of nucleotides and nucleosides in samples were determined by quantifying peak areas relative to those produced by known standards.

In selected experiments prostacyclin production was assessed. Perfusate from the heart was collected in 3 s samples for 5 min after adenine nucleotide injection. The prostacyclin concentration in these samples was measured by radioimmunoassay of 6-keto-prostaglandin $F_{1\alpha}$ (6-keto $PGF_{1\alpha}$) as previously described (Ager *et al.*, 1982), using antiserum generously provided by Dr B.A. Peskar (Bochum, West Germany). The perfusate (0.1 or 0.3 ml) was added to the assay tubes without any solvent extraction. The detection limit of the assay was 3 pg per sample.

Drugs

Adenosine, adenosine monophosphate (AMP), adenosine diphosphate (ADP), adenosine triphosphate (ATP), α,β-methylene ADP (APCP), α,β-methylene ATP (APCPP), β,γ-methylene ATP (APPCP) and 8-phenyltheophylline (8-PT) were purchased from Sigma, methysergide was obtained from Sandoz. APPCP, APCPP and APCP, were free of contaminating adenine nucleotides and nucleosides (h.p.l.c. analysis).

All drugs except 8-PT were dissolved in distilled water. 8-PT was dissolved in 80% methanol in 2×10^{-1} M NaOH to give a stock solution of 2×10^{-2} M. The vehicle had no effect on perfusion pressure or myocardial contraction.

Results

Effects on ventricular myocardial function

Bolus injections (1×10^{-7} mol) of ATP, ADP or AMP, had no effect on myocardial contraction. Higher doses (3×10^{-7}–1×10^{-5} mol) had transient negative inotropic effects (Figure 1). The minimum dose needed to reduce myocardial contractile force was 3×10^{-7} mol for ATP, 1×10^{-6} mol for ADP and 2×10^{-6} mol for AMP. The highest dose of ATP, ADP or AMP used (1×10^{-5} mol) reduced myocardial contractile force by 96.2 ± 2.4% ($n = 5$), 84.4 ± 3.7 ($n = 5$) and 76.3 ± 4.4% ($n = 5$) respectively (all results are expressed as mean ± s.e.mean of n experiments). Adenosine (up to 1×10^{-5} mol) showed no negative inotropic activity (Figure 1).

Effects on coronary perfusion pressure

Adenine nucleotides and adenosine had effects on perfusion pressure at doses lower than those required to produce inotropic effects. Low doses (3×10^{-10}–1×10^{-7} mol) of ATP, ADP, AMP and adenosine transiently reduced perfusion pressure (Figure 2). This decrease peaked after 16 ± 1 s ($n = 30$) and returned to control values within 1 min (Figure 3). ATP and ADP were approximately equiactive and about ten times more potent than adenosine (Figure 2): maximum reduction in perfusion pressure occurred after injection of 1×10^{-8} mol ATP or ADP and 1×10^{-7} mol adenosine. With high doses of ATP and ADP (1×10^{-5} mol), which were negatively inotropic, an increase in perfusion pressure (6.5 ± 1.5 and 3.9 ± 1.2 mmHg respectively, $n = 5$) was seen, which peaked after 30 ± 2 s and returned to control values within 2 min (Figure 3).

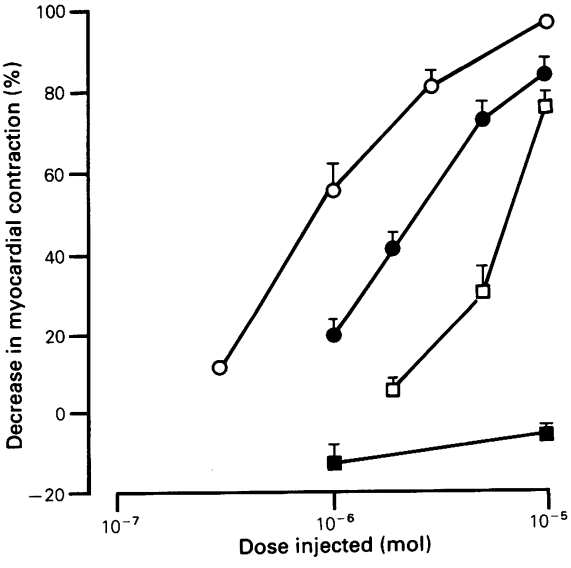

Figure 1 Effect of a 50 μl bolus of ATP (○), ADP (●), AMP (□) and adenosine (■), on ventricular myocardial contractile force. Vertical lines show s.e.mean (when larger than symbol) of 5 experiments.

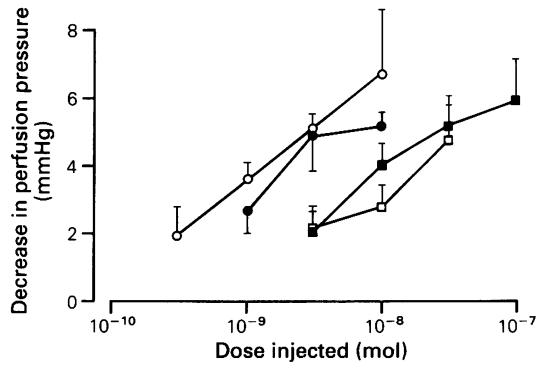

Figure 2 Effect of a 50 μl bolus of ATP (○), ADP (●), AMP (□) and adenosine (■) on perfusion pressure. Vertical lines show s.e.mean of 5 or more experiments.

Analogues of ATP also altered perfusion pressure (Figures 3 and 4); APPCP initially increased perfusion pressure in a dose-related manner (1×10^{-8}–1×10^{-7} mol) but this was rapidly followed by a reduction in perfusion pressure with a similar time course and over a similar dose range to adenosine. APCPP was about ten times more potent that APPCP at increasing perfusion pressure (Figure 4): APCPP (1×10^{-8} mol) increased perfusion pressure by 11.6 ± 3.9 mmHg ($n = 5$) whilst the same dose of APPCP increased it by only 3.6 ± 0.9 mmHg ($n = 7$). The response to APCPP peaked after 9 ± 1 s and perfusion pressure returned to control values within 2 min, there was no subsequent reduction in perfusion pressure (Figures 3 and 4). The response to APCPP was unaltered in the presence of a background infusion of atropine (1×10^{-6} M), methysergide (5×10^{-6} M) or phentolamine (1×10^{-5} M) $n = 3$, indicating that the response was not mediated by release of acetylcholine, 5-hydroxytryptamine, or noradrenaline.

When 2-^{3}H-adenine nucleotides were injected into the coronary circulation over 99% of the tritium recovered in the perfusate was collected within 39.5 ± 0.4 s ($n = 8$) – i.e. in a volume of 8.09 ± 0.21 ml ($n = 8$). Sequential collection of 1 s samples of effluent showed that a maximum of 10.8 ± 0.1% ($n = 8$) of the recovered tritium was collected in any sample. Thus

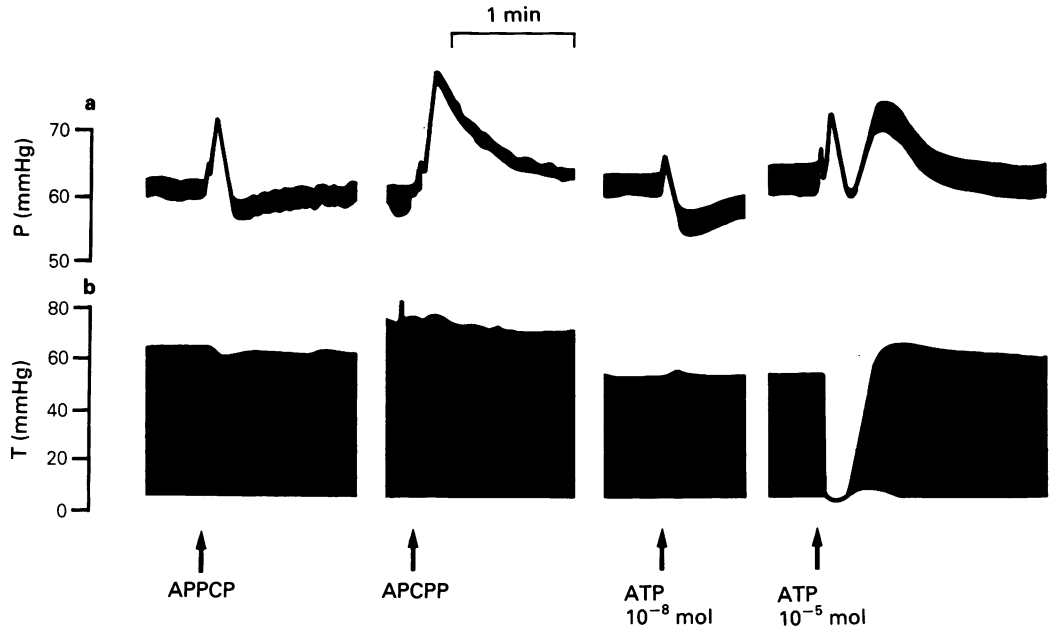

Figure 3 Experimental records of the effect of perfusion pressure (a) and myocardial contractile function (b) of: β,γ-methylene ATP (APPCP, 1×10^{-7} mol); α,β-methylene ATP (APCPP, 1×10^{-8} mol) and ATP, 1×10^{-8} mol and 1×10^{-5} mol. Note initial small increase in perfusion pressure due to the force of injection (at arrow) and the decrease in perfusion pressure caused by the negative inotropic action of the high dose of ATP (far right).

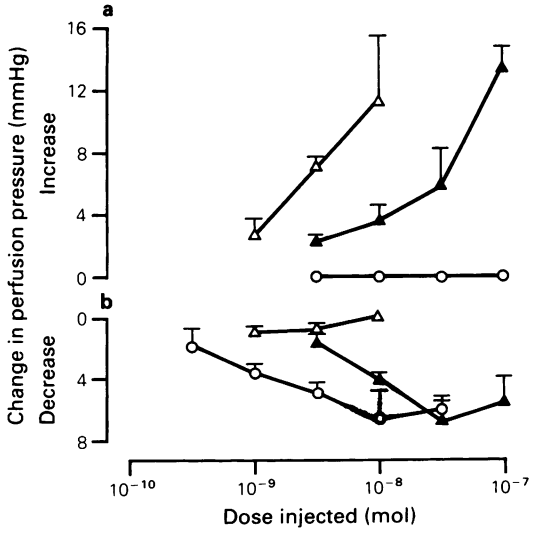

Figure 4 Comparison of the effect on perfusion pressure of ATP (○) with that of α,β-methylene ATP (△) and β, γ-methylene ATP (▲). (a) Initial increase in perfusion pressure, (b) the subsequent decrease in perfusion pressure. Vertical lines show s.e.mean of 5 or more experiments.

the mean concentration within the coronary circulation was calculated (in nmol ml^{-1}) as:

$$\frac{\text{dose injected (nmol)}}{\text{volume of distribution (ml)}} = \text{dose injected} \times 0.124,$$

and the peak concentration reached (in nmol ml^{-1}) as:

$$\frac{\text{dose injected (nmol)} \times \text{max. recovered in any fraction} \times 60}{\text{flow rate (ml min}^{-1})} = \text{dose injected} \times 0.536$$

Thin layer chromatography of the perfusate showed that 2-^{3}H-adenine nucleotides were metabolized as they passed through the coronary circulation (Table 1). When a dose of 3×10^{-9} mol of ATP or ADP was injected 37 ± 7% ($n = 6$) of the former and 69 ± 6% ($n = 5$) of the latter was converted to AMP or adenosine. The amounts of AMP and adenosine formed ($1.11 \pm 0.20 \times 10^{-9}$ mol and $2.08 \pm 0.18 \times 10^{-9}$ mol, respectively) were lower than those required to reduce perfusion pressure when these agents were injected directly into the coronary circulation, they were therefore insufficient to account for the decrease in perfusion pressure observed.

Assessment of the breakdown of APPCP and

Table 1 Metabolism of adenine nucleotides in the coronary circulation

		% 2-^{3}H-nucleotide on t.l.c. plate collected as					
	Dose injected	*ATP*	*ADP*	*AMP*	*Adenosine*	*Inosine*	n
ATP	3×10^{-9} mol	48 ± 7	11 ± 1	25 ± 4	12 ± 3	4 ± 1	6
	3×10^{-7} mol	54 ± 6	10 ± 1	21 ± 3	11 ± 3	3 ± 1	6
ADP	3×10^{-9} mol	—	19 ± 4	43 ± 3	26 ± 4	11 ± 5	5
	3×10^{-7} mol	—	22 ± 5	36 ± 7	29 ± 10	13 ± 4	5
AMP	3×10^{-8} mol	—	—	41 ± 8	54 ± 7	5 ± 3	4
	3 ± 10^{-7} mol	—	—	50 ± 12	50 ± 11	1 ± 1	3

Results show mean ± s.e.mean of *n* observations.

APCPP by h.p.l.c. showed that 78 ± 3% ($n = 4$) of a 3×10^{-8} mol dose of APPCP and 88 ± 5% ($n = 4$) of the same dose of APCPP remained as the parent compound after passage through the coronary bed.

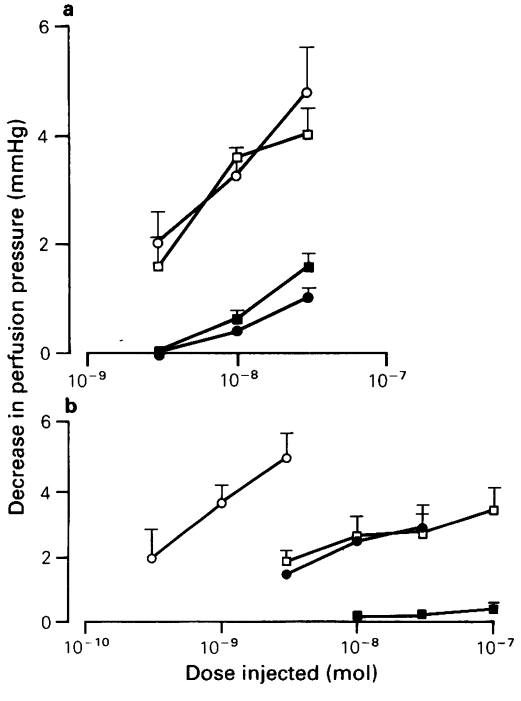

Figure 5 Effect of 8-phenyltheophylline (8-PT, 2×10^{-5} M) on the decrease in perfusion pressure in response to (a) a 50 μl bolus of AMP ((○) before, (●) after 8-PT) or adenosine ((□) before, (■) after 8-PT) and (b) a 50 μl bolus of ATP ((○) before, (●) after 8-PT) or β,γ-methylene ATP ((□) before, (■) after 8-PT). Vertical lines show s.e.mean of 5 experiments.

Inhibition of the formation or action of adenosine

8-Phenyltheophylline (8-PT) is known to inhibit competitively the interaction of adenosine with P_1-purinoceptors (Griffith *et al.*, 1981). In our experiments the vehicle in which 8-PT was dissolved had no effect on the vascular responses to adenine nucleotides or adenosine. A continuous infusion of 8-PT (2×10^{-5} M) abolished the response to 3×10^{-9} mol AMP and adenosine and severely depressed the responses induced by 1 and 3×10^{-8} mol (Figure 5a). The response to ATP (3×10^{-9}–3×10^{-8} mol) was also greatly reduced (Figure 5b), and the decrease in perfusion pressure caused by APPCP (1×10^{-8}–1×10^{-7} mol) was virtually abolished (Figure 5b).

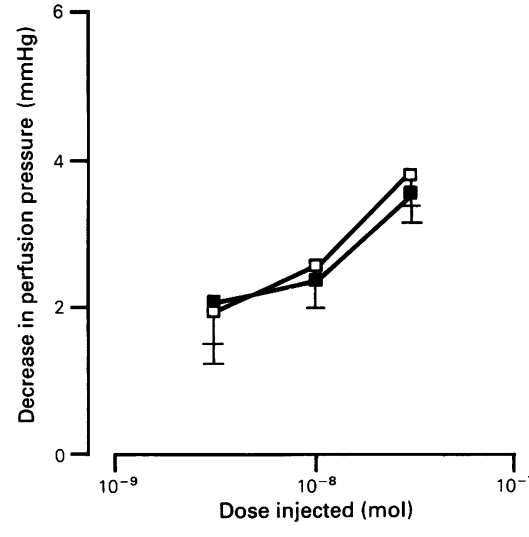

Figure 6 Effect of α,β-methylene ADP (APCP, 2×10^{-6} M) on the decrease in perfusion pressure in response to a 50 μl bolus of AMP ((□) before, (■) after APCP). Vertical lines show s.e.mean of 5 experiments.

APCP, a potent inhibitor of 5'-nucleotidase (Naito & Lowenstein, 1985), had no intrinsic vasoactivity in our experiments (up to 1×10^{-7} mol). A continuous infusion of 2×10^{-6} M had no significant effect on the reduction in perfusion pressure caused by AMP (Figure 6). H.p.l.c. analysis showed that in the absence of APCP $22.3 \pm 3.4\%$ ($n = 4$) of a 3×10^{-8} mol dose of AMP remained as the parent compound whilst $46.0 \pm 6.7\%$ was converted to adenosine. In the presence of APCP (2×10^{-6} M) the corresponding figures were $76.4 \pm 4.5\%$ AMP and $12.5 \pm 1.4\%$ adenosine ($n = 4$). Thus, the effect of AMP did not depend on its conversion to adenosine.

Prostaglandin release

Under basal conditions prostacyclin (measured as 6-keto $PGF_{1\alpha}$) was released into the perfusate from the heart (0.69 ± 0.04 ng min^{-1}; $n = 15$). ATP (3×10^{-7}–1×10^{-5} mol) induced a dose-related stimulation of prostacyclin release (Figure 7). The absolute amount of prostacyclin released into the effluent varied widely between hearts (the peak response to 3×10^{-6} mol ATP ranged from 0.72 ng min^{-1} to 22.33 ng min^{-1}), resulting in mean values with large standard errors.

Prostacyclin released in response to ATP peaked within 40 s of injection and returned to baseline values within 5 min. ADP stimulated prostacyclin release with a similar time course to ATP but was slightly less potent (mean maximal stimulation to 1×10^{-5} mol of ADP was 8.29 ± 2.90 ng min^{-1}, compared with 11.93 ± 3.23 ng min^{-1} in response to the same dose of ATP). AMP, adenosine (both up to 1×10^{-5} mol), APPCP and APCPP (both up to 1×10^{-7} mol) did not stimulate prostacyclin production.

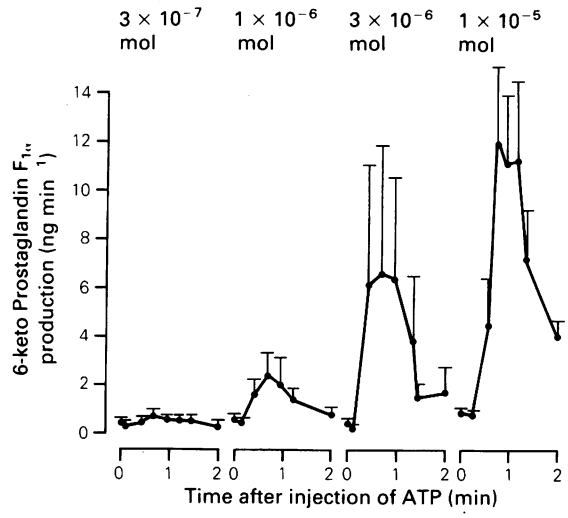

Figure 7 Stimulation of prostacyclin production (measured as 6-keto prostaglandin $F_{1\alpha}$) in response to ATP (3×10^{-7}–1×10^{-5} mol). Vertical lines show s.e.mean of 4 or more experiments.

Discussion

Adenine nucleotides are most likely to occur extracellularly within the coronary circulation as a result of platelet degranulation or release from damaged cells in the blood vessel wall. Because such release will be transient and localized, we studied the effects of bolus injections, rather than continuous infusion, on ventricular myocardial function and coronary tone.

Effects on myocardial function

Adenine nucleotides and adenosine have been demonstrated to be negatively inotropic in atrial myocardium (Drury & Szent-Gyorgyi, 1929; Collis & Pettinger, 1982; Burnstock & Meghji, 1983) and to slow sinoatrial conduction (James, 1965). The majority of studies on ventricular myocardial function show little or no effect of adenine nucleotides on ventricular conduction rate or force of contraction, although Burnstock & Meghji (1983) showed negative inotropism with high concentrations (3×10^{-4}–1×10^{-3} M) of ATP in the right ventricular strip of the rat, and a decrease in ventricular pacemaker rate has been found in guinea-pig isolated heart in the presence of adenosine (Szentmiklosi *et al.*, 1980; West *et al.*, 1982). In our experiments where chronotropic effects were prevented by electrical pacing and only ventricular function was measured, relatively high doses of adenine nucleotides ($> 3 \times 10^{-7}$ mol) were needed to produce negative inotropism. The intracoronary concentrations reached under these conditions; mean concentrations 3.7×10^{-5} M, peak concentration 1.6×10^{-4} M (estimated from the distribution profile of injected [^{3}H]-ATP), were similar to those used by Burnstock & Meghji (1983). The relative potencies of ATP, ADP and AMP, and the absence of a response to adenosine, indicate that the negative inotropic response is mediated by a P_2-purinoceptor (as defined by Burnstock, 1978).

Effects on perfusion pressure

In the perfused heart of the rat, where coronary flow is kept constant, any change in perfusion pressure, in the absence of inotropic effects on the myocardium reflects an alteration in coronary smooth muscle tone. In our experiments ATP, ADP, AMP and adenosine reduced coronary tone, APCPP increased it, and

APPCP and high doses of ATP and ADP had a biphasic effect.

Previous studies have shown that adenosine produces vasodilatation in many vascular beds, including the coronary circulation (see Berne, 1980, for a review) through stimulation of P_1-purinoceptors (as defined by Burnstock, 1978). Adenine nucleotides initiate endothelium-dependent relaxation of arterial smooth muscle (i.e. vasodilatation) in various vessels by interaction with endothelial P_2-purinoceptors (De Mey & Vanhoutte, 1981; Rapaport *et al.*, 1984; Kennedy *et al.*, 1985; White *et al.*, 1985). Stimulation of P_2-purinoceptors on smooth muscle cells in some vessels has been found to increase vascular tone (Verhaeghe, 1977; Su, 1981; Kennedy *et al.*, 1985; Kennedy & Burnstock, 1985a; White *et al.*, 1985).

Our experiments were designed to establish the type of purinoceptor (P_1 or P_2) responsible for the observed effects in the rat coronary circulation. The relative potencies of ATP and adenosine in altering coronary tone suggests that the receptor type primarily involved in the vasodilator response is P_2, although vasodilatation to adenosine may be mediated by interaction with a P_1-purinoceptor. Inhibiting 5'-nucleotidase with APCP had no effect on the response to AMP, suggesting that conversion to adenosine was not necessary for a vasodilator action and that AMP was either equipotent with adenosine at the P_1-purinoceptor or was a less potent agonist than ATP and ADP at the P_2-purinoceptor. The vasodilatation in response to adenosine was inhibited by 8-PT, a competitive antagonist at P_1-purinoceptors (Griffith *et al.*, 1981). However, 8-PT also inhibited the vasodilatation in response to ATP at doses where the amount of adenosine formed was below the response threshold. Thus, either ATP interacts with P_1-purinoceptors or, more probably, 8-PT is an antagonist at the vasodilator P_2-receptor.

Burnstock & Kennedy (1985) recently suggested that P_2-purinoceptors should be subdivided into two types on the basis of the relative potencies of ATP analogues and ATP. According to this classification, the decrease in coronary tone seen in our experiments is mediated by a P_{2Y}-purinoceptor (ATP more potent than APCPP and APPCP) as found in rabbit portal vein (Kennedy & Burnstock, 1985b), rat femoral artery (Kennedy *et al.*, 1985) and rat aorta (White *et al.*, 1985). The increase that we observed in coronary tone (with APCPP and APPCP more potent than ATP) is mediated by a P_{2X}-purinoceptor, as found in rat femoral artery (Kennedy *et al.*, 1985), rabbit ear artery (Kennedy & Burnstock, 1985a) and rat aorta (White *et al.*, 1985).

Interaction with P_2-purinoceptors on endothelial cells can induce increased prostacyclin production (Boeynaems & Galand, 1983; Pearson *et al.*, 1983) and prostacyclin is a potent vasodilator in many vascular beds including the coronary circulation of the rat (Schror *et al.*, 1980). In our experiments doses of ATP able to induce vasodilatation were one thousand times lower than those required to increase the prostacyclin concentration in the perfusate. Thus the vasodilatation produced by stimulation of P_{2Y}-receptors in this system was not mediated by prostacyclin.

In some smooth muscle preparations, increases in tone induced by ATP analogues have been shown to be mediated through release of acetylcholine (Moody & Burnstock, 1982), 5-hydroxytryptamine (Sakai, 1978) or noradrenaline (Su, 1981) from nerve endings. However, in our experiments atropine, methysergide and phentolamine had no effect on the response to APCPP, indicating that these neurotransmitters were not involved in purinoceptor-mediated vasoconstriction in the rat heart.

Conclusions

The rat coronary vasculature exhibits purinoceptors of at least two types: a vasodilator P_2-purinoceptor (P_{2Y}) stimulated by low concentrations of ATP and ADP, and a P_2-purinoceptor mediating vasoconstriction (P_{2X}), stimulated by higher concentrations of ATP and ADP and by slowly degraded ATP analogues. AMP and adenosine may act as less potent agonists at the P_2-purinoceptor or their effects may indicate the presence of an additional vasodilator P_1-purinoceptor. High concentrations of ATP and ADP also stimulate prostacyclin release and are negatively inotropic.

Significant extracellular concentrations of adenine nucleotides occurring locally within the coronary vasculature will normally increase coronary flow by stimulating vasodilator purinoceptors, thus limiting the extent of platelet aggregation and relieving hypoxia. If, as in the rat femoral artery (see Kennedy *et al.*, 1985), the vasoconstrictor P_{2X}-receptors are on smooth muscle cells whilst those mediating vasodilatation (P_{2Y}) are on endothelial cells then damage to the endothelial cells may result in a shifting of the ATP-mediated response from one of dilatation to one of constriction, especially if the metabolism of ATP and ADP is impaired because endothelial ectonucleotidase activity is compromised. Under such circumstances, increases in extracellular ATP and ADP could lead to further platelet aggregation, isolation of the hypoxic area by vasoconstriction and eventual myocardial infarction.

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Pharmacological characterization of 5-hydroxytryptamine-induced depolarization of the rat isolated vagus nerve

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1 A study has been made of the pharmacology of the 5-hydroxytryptamine (5-HT)-induced depolarization responses that can be recorded extracellularly from the rat isolated cervical vagus nerve.

2 Phenylbiguanide (PBG) and 2-methyl-5-hydroxytryptamine (2-methyl-5-HT) were found to mimic the effects of 5-HT on the vagus nerve. Their EC_{50} values were respectively 2.0 fold and 3.9 fold greater than that of 5-HT.

3 Metoclopramide behaved as a reversible competitive antagonist of depolarization induced by PBG and 2-methyl-5-HT, with pK_B values of 6.48 ± 0.04 and 6.64 ± 0.04, respectively. These agreed well with the pK_B value of 6.60 ± 0.04 obtained previously for metoclopramide against 5-HT on the rat vagus nerve. 5-HT, PBG and 2-methyl-5-HT had no demonstrable agonist effects at non-5-HT receptors on the rat vagus nerve.

4 Tropacaine and *m*-chlorophenylpiperazine were found to behave as reversible competitive antagonists of 5-HT-induced depolarization of the vagus nerve. The pK_B values were 6.29 ± 0.03 and 6.90 ± 0.03, respectively.

5 Quipazine, MDL 72222 and ICS 205-930 were also shown to be effective antagonists of 5-HT on the vagus nerve. However, although these compounds were highly potent, they all caused a marked concentration-dependent reduction in the amplitude of the maximum response to 5-HT. This behaviour was not consistent with a simple reversible competitive mechanism.

6 The results are discussed with reference to the current classification of mammalian peripheral neuronal 5-HT receptors.

Introduction

Recently, two novel, highly potent antagonists of the actions of 5-hydroxytryptamine (5-HT) on mammalian peripheral neurones have been described. These compounds, MDL 72222 (Fozard, 1984) and ICS 205-930 (Richardson *et al.*, 1985) are of special significance since they are the first 5-HT antagonists that have been demonstrated to both be effective on peripheral neurones and show clear specificity of action. Thus both MDL 72222 and ICS 205-930 are highly selective antagonists of the positive chronotropic effect of 5-HT on the rabbit isolated heart (Fozard, 1984; Richardson *et al.*, 1985), 5-HT-induced inhibition of compound action potentials in the rabbit isolated vagus nerve (Donatsch *et al.*, 1984b), and 5-HT-induced depolarization of the rabbit isolated nodose ganglion and superior cervical ganglion (SCG) (Azami *et al.*, 1985; Round & Wallis, 1986). In addition, both compounds are claimed to differentiate between sub-types of excitatory peripheral neuronal 5-HT receptors (Fozard, 1984; Richardson *et al.*, 1985). It would therefore seem desirable to use MDL 72222 and ICS 205-930 in any attempt to characterize the actions of 5-HT on other peripheral neurones.

To date, the only compound reported to behave as a reversible competitive antagonist of 5-HT-induced depolarization of the rat isolated vagus nerve is metoclopramide (Ireland *et al.*, 1982; Ireland *et al.*, 1983). In the present study, an attempt has been made to improve the pharmacological characterization of the receptors mediating this 5-HT-induced depolarization, by examining the inhibitory effects of a range of putative 5-HT antagonists – including MDL 72222 and ICS 205-930.

We also describe the effects of phenylbiguanide (PBG) and 2-methyl-5-hydroxytryptamine (2-methyl-5-HT) on the rat vagus nerve. PBG has previously been shown to mimic selectively the effects of 5-HT on mammalian peripheral neurones (see Fastier *et al.*,

1959; Gyermek, 1964; Drakontides & Gershon, 1968). 2-Methyl-5-HT was of interest since it has been shown to mimic the effects of 5-HT on the rabbit vagus nerve and heart (Richardson *et al.*, 1985); it was about half as active as 5-HT on both these tissues. In contrast, on the smooth muscle of the rat uterus, 2-methyl-5-HT was approximately 2000 times weaker than 5-HT (Richardson *et al.*, 1985). In the present study, both PBG and 2-methyl-5-HT were found to depolarize the rat vagus nerve. The effects of metoclopramide on these depolarization responses have been quantified, and compared with the results obtained previously with this antagonist against 5-HT-induced depolarization. The effects of some non-5-HT antagonists on PBG and 2-methyl-5-HT-induced depolarization have also been examined.

A preliminary account of some of the work in this paper has been presented to the British Pharmacological Society (Fortune *et al.*, 1983; Fortune & Ireland, 1984).

Methods

Preparation of tissues

Male hooded rats weighing 200–350 g were stunned by a blow to the head and killed by cardiac puncture. Segments of cervical vagus nerve, approximately 10 to 20 mm long and minus the nodose ganglion, were excised as rapidly as possible and placed in oxygenated Krebs-Henseleit medium (greater than 25 ml per tissue) at room temperature (approximately 21°C). The connective tissue sheath around each isolated vagus nerve was then carefully removed.

Extracellular recording

Within one hour of dissection, de-sheathed vagus nerves were transferred to two-compartment Perspex baths to permit extracellular recording of agonist-induced depolarizations. Each nerve was positioned so that approximately 50% lay in the first compartment, while the remainder projected through a greased slot (Dow-Corning high vacuum grease) into the second. The d.c. potential between the two compartments was recorded via silver-silver chloride electrodes connected to the tissue preparation through agar-saline/filter paper bridges and was displayed on a potentiometric chart recorder (Servogor 220 or SE 130). Each compartment of the bath was perfused continuously at a constant rate of approximately 1–2 ml per min with Krebs-Henseleit medium dripped directly onto the tissue. Drugs were applied at known concentrations via the superfusion stream into the first compartment only.

The temperature of each preparation was maintained at 27 ± 1°C by passing solutions through heat exchangers immediately before applying them to the tissue, and by placing the recording bath and electrodes in a temperature controlled chamber. This temperature was chosen since, in preliminary experiments, recorded base-lines were more stable at 27°C than at the more physiological 37°C (results not shown).

Measurement of the effects of agonists and antagonists

In the present study, concentration-response curves for agonist-induced depolarization were constructed non-cumulatively using serially increasing concentrations. Each application of agonist was continued until apparent equilibrium was reached. In practice, this usually meant contact times of 3 min or less. Preparations were always allowed to repolarize fully between each application of agonist; typically, this took 15–30 min.

The effect of the agonists PBG and 2-methyl-5-HT were quantified in separate experiments. These were performed on a minimum of four vagus nerve preparations, each obtained from a different rat. Every preparation was exposed alternately to 5-HT and to the chosen test agonist. Half the total number of vagus nerves used in each experiment was dosed in the sequence: test agonist–5-HT–test agonist; this was reversed in the remainder.

The EC_{50} and maximum (E_{max}) for each concentration-response curve for agonist-induced depolarization were estimated by direct fit of a logistic function:

$$y = E_{max}\frac{[A]^n}{[A]^n + EC_{50}{}^n}$$

where y is the observed response, [A] the concentration of agonist, and n a constant. The method used for fitting logistic curves to the experimental data was based on that of Parker & Waud (1971; see also Snedecor & Cochran, 1968). The computer programme used for curve fitting in the present study was written by Miss F.J. Illingworth, Department of Computer Science, Glaxo Group Research Ltd., Greenford, Middlesex.

5-HT-induced depolarization responses of rat vagus nerve are stable and reproducible when full repolarization is allowed between each application of the agonist (see Ireland, 1984). Therefore, a standard procedure was adopted for the measurement of antagonist effects. Firstly, a control 5-HT concentration-depolarization response curve was constructed on each tissue. Then the antagonist was applied and allowed to reach apparent equilibrum. This was taken to have occured when two successive applications of an approximate EC_{50} of agonist in the presence of the antagonist gave responses equal to within 10%. Fin-

ally, a second 5-HT concentration-depolarization response curve was constructed on each tissue. Only one concentration of antagonist was applied to each vagus nerve preparation. Lateral displacements of concentration-depolarization response curves were measured at the control half-maximal response level. The negative logarithm of the apparent dissociation constant for an antagonist (pK_B) was estimated by calculation of the mean (± s.e.) of the individual results: $pK_B = \log$ (dose-ratio -1) $-\log$ (antagonist concentration).

At least four different concentrations of each antagonist were used to calculate the pK_B value. Concentrations were chosen to cover as wide a range as possible, and to give dose-ratios of between approximately 3 and 300. The effects of each concentration of antagonist were measured in at least four individual vagus nerve preparations, each obtained from a different rat.

Drugs and solutions

The composition of the Krebs-Henseleit medium used in the present study was (in mmol l^{-1}): NaCl 118, $NaHCO_3$ 25, KH_2PO_4 1.18, KCl 4.7, $MgSO_4$ 1.18, $CaCl_2$ 2.5 and glucose 11.0; it was gassed with 95% O_2 and 5% CO_2. The medium was prepared in glass-distilled water, and reagents, which were all A.R. grade, were purchased from commercial sources.

The following drugs were used: 5-HT creatinine sulphate (Sigma), 2-methyl-5-HT creatinine sulphate (Glaxo), 1-phenylbiguanide (Aldrich), 1, 1-dimethyl-4-phenylpiperazinium iodide (DMPP) (Sigma), dopamine hydrochloride (Sigma), isoprenaline sulphate (Wellcome), γ-aminobutyric acid (GABA) (Sigma), bicuculline (Sigma), phentolamine mesylate (Ciba), metoclopramide hydrochloride (Beecham), quipazine maleate (Miles), 1-(*m*-chlorophenyl) piperazine dihydrochloride (MCPP) (Aldrich), exo-8-methyl-8-azabicyclo [3.2.1]-octan-3-ol benzoate hydrochloride (tropacaine) (Aldrich), methiothepin maleate (Roche), metergoline (Farmitalia), methysergide hydrogen maleate (Sandoz), ketanserin (Salford Fine Chemicals), haloperidol (Janssen), pipamperone hydrochloride (Janssen), spiperone (Janssen), domperidone (Janssen), xylamidine (Wellcome), cinanserin hydrochloride (Squibb), MDL 72222 (1αH, 3α, 5αH-tropan-3yl-3, 5-dichlorobenzoate) gift of Dr J.R. Fozard, Merrel-Dow, Strasbourg) and ICS 205-930 ((3α-tropanyl)-1H-indole-3-carboxylic acid ester) gift of Dr G. Engel, Sandoz, Basel). All drugs were dissolved in Krebs-Henseleit medium unless otherwise stated, to give a final concentration of 1×10^{-3}–1×10^{-2} M. Solutions were prepared immediately before use. Stock solutions of (−)-noradrenaline, 1×10^{-2} M, isoprenaline 1×10^{-3} M, and dopamine, 1×10^{-2} M, contained ascorbic acid, 1×10^{-3} M, to suppress oxidation. Haloperidol, spiparone, ketanserin, metergoline, bicuculline and domperidone were all insoluble in water. The first two compounds were dissolved to give 1×10^{-2} M solutions in 0.1 M (±)-tartaric acid, metergoline was dissolved to give a 1×10^{-2} M solution in 4×10^{-2} M ascorbic acid, and bicuculline was dissolved in 0.1 M hydrochloric acid. Domperidone was dispersed in Dioxalane (Cambrian Chemicals) plus 1.5 M hydrochloric acid. All these solutions could be diluted with normal Krebs-Henseleit medium, without causing visible precipitation. Appropriate solvent controls were found to be without effect on 5-HT-induced depolarization of the rat vagus nerve (results not shown).

Results

Phenylbiguanide and 2-methyl-5-hydroxytryptamine

PBG, 1×10^{-7}–3×10^{-5} M, and 2-methyl-5-HT, 3×10^{-7}–1×10^{-4} M, induced rapid, concentration-related depolarizations of the vagus nerve that closely resembled those produced by 5-HT, 1×10^{-7}–3×10^{-5} M. The amplitude of the maximum response induced by PBG was estimated to be 79.0 ± 1.4% of the 5-HT maximum ($n = 6$); the corresponding value for 2-methyl-5-HT was 68.3 ± 3.7% ($n = 4$) (Figure 1). Both PBG and 2-methyl-5-HT were slightly less potent than 5-HT. The means of the quotients: agonist EC_{50}/5-HT EC_{50} (where both EC_{50} values were determined in the same vagus nerve preparation) were 2.0 ± 0.2 ($n = 6$) and 3.9 ± 0.3 ($n = 4$), respectively.

Metoclopramide, 1×10^{-6}–1×10^{-4} M, caused parallel rightward displacements of the concentration-depolarization response curves for PBG and 2-methyl-5-HT on the vagus nerve. Plots of the log of the dose-ratio -1 against the log of the concentration of the antagonist (Arunlakshana & Schild, 1959), were straight lines with gradients of 0.92 ± 0.05 and 0.95 ± 0.06 respectively (Figure 2). Neither was significantly different from unity ($P > 0.05$, t test). The pK_B values, calculated by constraining the gradients to unity, were 6.48 ± 0.04 ($n = 20$) against PBG, and 6.64 ± 0.04 ($n = 23$) against 2-methyl-5-HT.

Haloperidol, 3×10^{-5} M, had negligible inhibitory activity against depolarizations of the vagus nerve induced by either PBG or 2-methyl-5-HT ($n = 4$, each agonist). The pA_2 values calculated according to Schild (1947) were both less than 4.5.

Control depolarizations of the vagus nerve induced by GABA (mean EC_{50} $2.8 \pm 0.3 \times 10^{-5}$ M, $n = 3$), DMPP (mean EC_{50} $3.5 \pm 0.3 \times 10^{-5}$ M, $n = 3$) and (−)-noradrenaline (mean EC_{50} $4.0 \pm 1.0 \times 10^{-6}$ M, $n = 3$) were essentially abolished by bicuculline, 1×10^{-5} M, hexamethonium, 3×10^{-4} M, and phen-

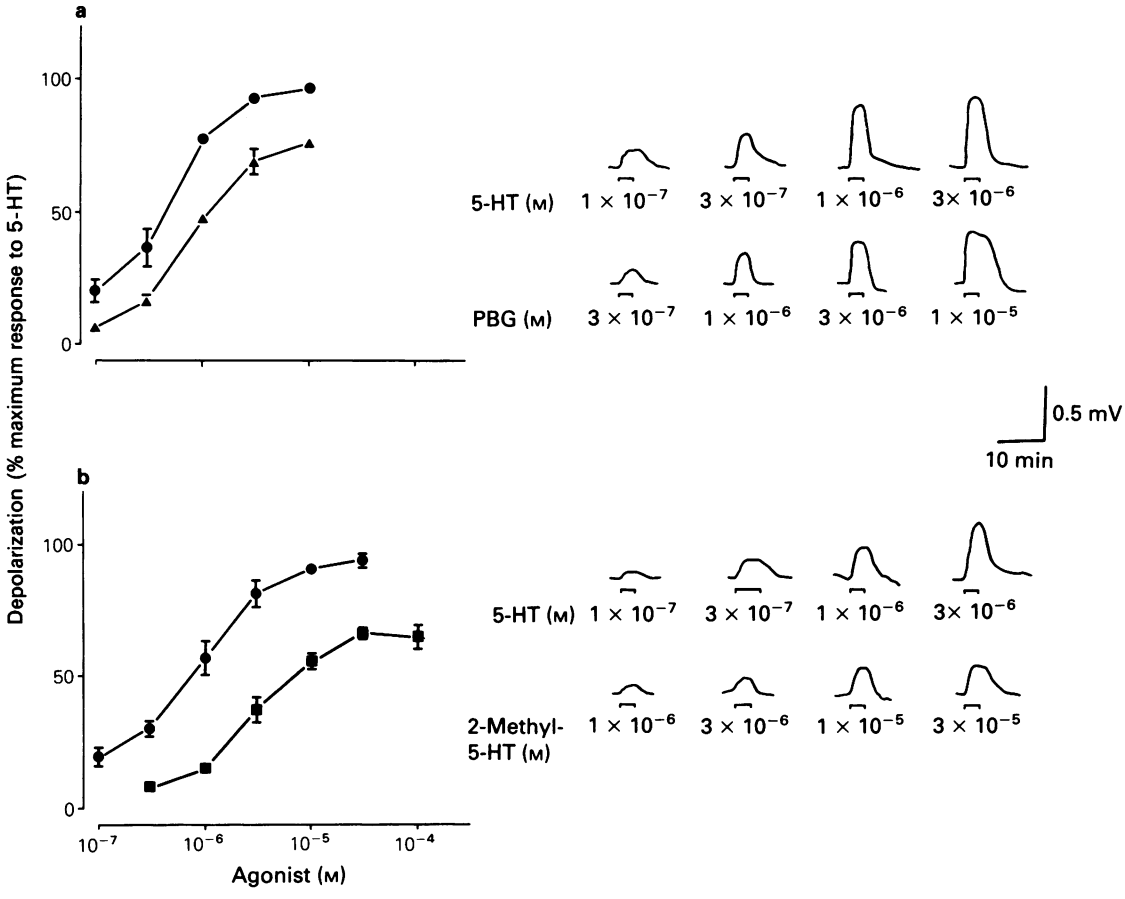


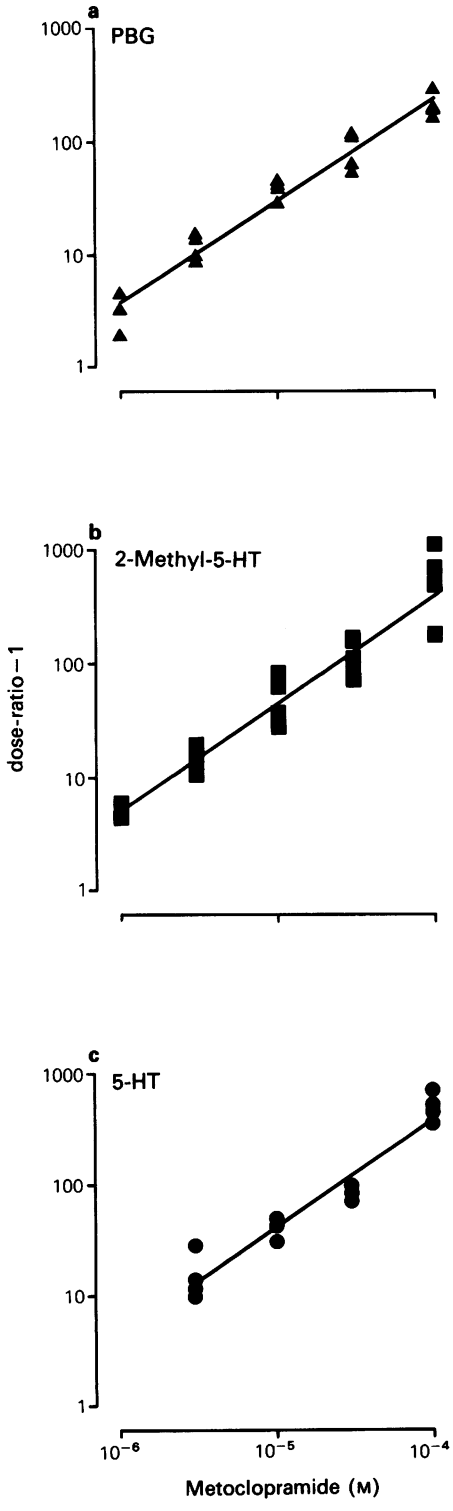
Figure 1 Comparison of the effects of 5-hydroxytryptamine (5-HT) and phenylbiguanide (PBG) (a) and 5-HT and 2-methyl-5-hydroxytryptamine (2-methyl-5-HT) (b) on the rat isolated vagus nerve. Each point on the concentration-depolarization curves is the mean of single determinations in 6 individual tissues (a) or 4 individual tissues (b) and vertical lines show s.e.mean. Symbols indicate responses to 5-HT (●), PBG (▲) or 2-methyl-5-HT (■). Also shown are discontinuous records of the effects of the agonists on two vagus nerve preparations: one was exposed to 5-HT and PBG (a), the other to 5-HT and 2-methyl-5-HT (b). Upwards deflection indicates depolarization; the solid bar under each response shows the approximate duration of the agonist application.

tolamine, 1×10^{-6} M, respectively. In contrast, these concentrations of the antagonists did not affect concentration-response curves for the depolarization induced by 5-HT, PBG or 2-methyl-5-HT constructed either in the absence or the presence of metoclopramide, 1×10^{-4} M.

Dopamine, 1×10^{-7}–1×10^{-5} M, and isoprenaline, 1×10^{-9}–1×10^{-7} M, produced very small depolarizations of the vagus nerve. The amplitudes of the responses induced by approximately maximally-effective concentrations of dopamine (1×10^{-5} M) and isoprenaline (1×10^{-7} M) were respectively 16.4 ± 4.5% ($n = 4$) and 19.2 = 2.4% ($n = 3$) of the maximum depolarization induced by 5-HT in the same tissue preparations. Muscarine, 1×10^{-9}–1×10^{-5} M, has previously been found not to depolarize the rat vagus nerve (Ireland *et al.*, 1982).

m-*Chlorophenylpiperazine and tropacaine*

m-Chlorophenylpiperazine (MCPP), 1×10^{-6}–3×10^{-5} M, and tropacaine, 1×10^{-6}–1×10^{-4} M, produced concentration-related parallel rightward displacements of the 5-HT concentration-depolarization response curve on the vagus nerve (Figure 3). Apparent equilibrium was attained within



one hour of starting the application of either compound. Plots of the antagonism data according to Arunlakshana & Schild (1959) had gradients of 0.92 ± 0.06 and 0.99 ± 0.04, respectively (Figure 3). Neither was significantly less than unity ($P > 0.05$, t test). The estimated pK_B values were 6.90 ± 03 ($n = 17$) and 6.29 ± 0.03 ($n = 21$), respectively.

Quipazine, MDL 72222 and ICS 205-930

Quipazine, $1 \times 10^{-8} - 1 \times 10^{-6}$ M, MDL 72222, $3 \times 10^{-8} - 1 \times 10^{-6}$ M and ICS 205-930, $1 \times 10^{-10} - 3 \times 10^{-9}$ M, all produced rightward displacements of the 5-HT concentration-response curve on the vagus nerve. These were accompanied by concentration-related reductions in the maximum response. This was particularly marked with ICS 205-930 (Figure 4). Quipazine, MDL 72222 and ICS 205-930 were devoid of effects on the extracellularly-recorded membrane potential. All three compounds took 2 to 3 h to reach apparent equilibrium, as judged by stabilization of the response to an approximate EC_{50} of 5-HT in the presence of the chosen antagonist.

Effects of other putative 5-hydroxytryptamine antagonists

A range of putative 5-HT antagonists known to block the so-called 5-HT autoreceptor, or displace radioligands from 5-HT binding sites, were tested against 5-HT-induced depolarizations of the rat vagus nerve. At 3×10^{-5} M, the compounds were generally found to have only marginal activity. Typical effects observed were small (less than 10 fold) rightward displacements of the concentration response curve for 5-HT-induced depolarization, accompanied by a reduction in the maximum response. pA_2 values were calculated for the compounds according to the method of Schild (1947); these are shown in Table 1.

Figure 2 Antagonism by metoclopramide of depolarization responses of the rat isolated vagus nerve induced by (a) phenylbiguanide (PBG), (b) 2-methyl-5-hydroxytryptamine (2-methyl-5-HT) and (c) 5-hydroxytryptamine (5-HT). Data for 5-HT are from Ireland *et al.* (1987). Each point represents the result obtained on a separate tissue. At least 4 tissue preparations, each obtained from a different rat, were used to measure the effect of each concentration of metoclopramide, on each agonist. Straight lines were fitted by linear regression.

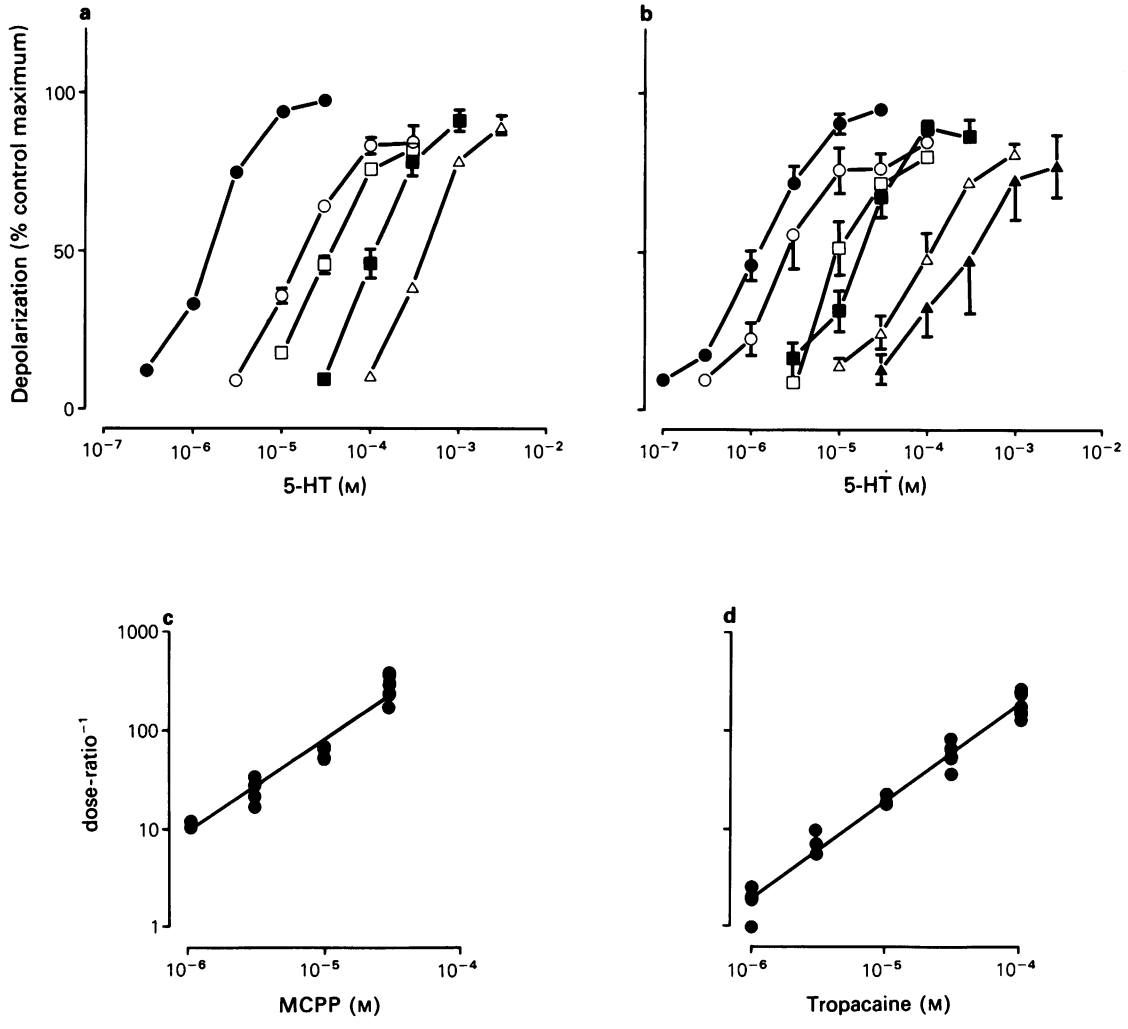


Figure 3 Antagonism by (a) *m*-chlorophenylpiperazine (MCPP) and (b) tropacaine of 5-hydroxytryptamine (5-HT)-induced depolarization of the rat isolated vagus nerve. In (a) and (b) points are means of single determinations from at least 4 separate tissues and vertical lines represent s.e.mean. Symbols indicate controls (●) or the presence of antagonist at 1×10^{-6} M (○), 3×10^{-6} M (□), 1×10^{-5} M (■), 3×10^{-5} M (△), 1×10^{-4} M (▲). (c and d) Data plotted according to Arunlakshana & Schild (1959). Each point represents the result from a separate tissue; straight lines were fitted by linear regression.

Discussion

On the rat isolated vagus nerve both PBG and 2-methyl-5-HT were found to produce depolarizations that closely resembled those induced by 5-HT. Metoclopramide was approximately equally effective as an inhibitor of the effects of all three agonists. Thus in the present study, metoclopramide behaved as a reversible competitive antagonist of depolarization induced by PBG and 2-methyl-5-HT, with pK_B values of 6.48 ± 0.04 and 6.64 ± 0.04, respectively. It has previously been shown to act similarly against depolarizations induced by 5-HT on this tissue; in this latter case, the pK_B value was 6.60 ± 0.04 (Ireland *et al.*, 1983). These results are consistent with the suggestion that 5-HT, PBG and 2-methyl-5-HT activate a common type of receptor on the rat vagus nerve.

Metoclopramide is not a specific 5-HT antagonist

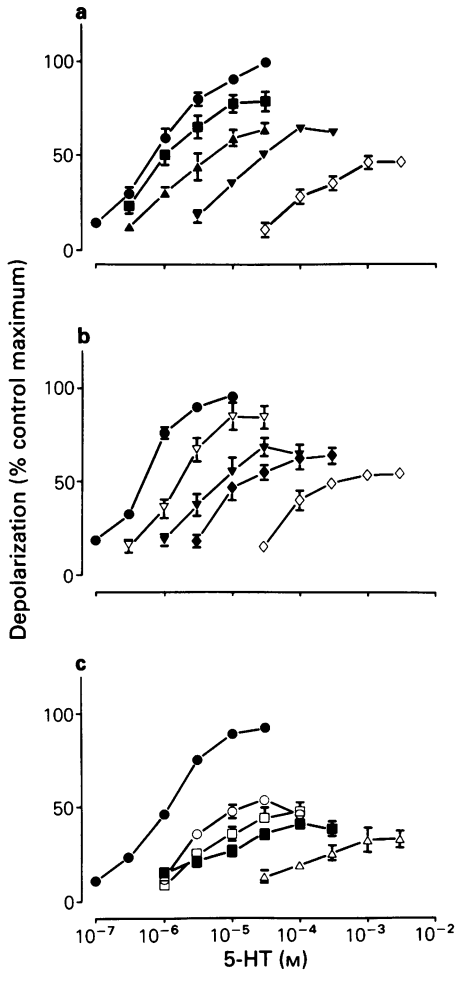


Figure 4 Antagonism by (a) quipazine, (b) MDL 72222 and (c) ICS 205-930 of 5-hydroxytryptamine (5-HT)-induced depolarization of the rat isolated vagus nerve. Points are means of single determinations from at least 4 separate tissue preparations and vertical lines represent s.e.mean. Symbols indicate controls (●), or the presence of antagonist at 1×10^{-10} M (○), 3×10^{-10} M (□), 1×10^{-9} M (■), 3×10^{-9} M (△), 1×10^{-8} M (▲), 3×10^{-8} M (▽), 1×10^{-7} M (▼), 3×10^{-7} M (◆), or 1×10^{-6} M (◇).

since it displaces [^{3}H]-spiperone and [^{3}H]-haloperidol binding in homogenates of rat corpus striatum, with pIC_{50} values of 6.95 and 5.8 respectively (Leysen *et al.*, 1978). However, it is unlikely that the antagonism by metoclopramide of depolarizations of the rat vagus nerve induced by PBG, 2-methyl-5-HT or 5-HT was due to an action at neuroleptic binding sites. Thus, haloperidol, which displaced [^{3}H]-spiperone and [^{3}H]-haloperidol binding with pIC_{50} values of 7.69 and 8.5 respectively (Leysen *et al.*, 1978), was essentially inactive against these depolarizations; the pA_2 values calculated according to Schild (1947) for antagonism of both PBG and 2-methyl-5-HT were less than 4.5, while that for 5-HT was 4.9. In addition, dopamine was a very weak agonist on the vagus nerve since the amplitude of the maximum depolarization induced by this agent was only $16.4 \pm 4.5\%$ of the maximum response to 5-HT ($n = 4$).

Evidence was also obtained to suggest that PBG, 2-methyl-5-HT and 5-HT had negligible activity at other non-5-HT receptors on the rat vagus nerve. Thus, responses by these agonists were unaffected by antagonists that blocked depolarizations induced by DMPP, GABA and (−)-noradrenaline. Further, these non-5-HT antagonists did not modify the apparent potency of metoclopramide as an antagonist of 5-HT, PBG or 2-methyl-5-HT on the vagus nerve.

PGB has previously been shown to mimic selectively some of the effects of 5-HT. For example, Fastier *et al.* (1959) have shown that both PBG and 5-HT cause reflex falls in heart rate and blood pressure in the cat, and induce pain following application to the bases of blisters in human subjects; unlike 5-HT, PBG did not contract rat blood vessels or stomach fundic strip *in vitro*. Further, Drakontides & Gershon (1968) showed cross-desensitization between PBG and 5-HT in the mouse isolated duodenum despite the fact that neither PBG nor 5-HT antagonized the effects of other spasmogens. They also found that unlike 5-HT, PBG had no direct effect on the smooth muscle of this tissue. Finally, Gyermek (1964) found that a series of biguanides, including PBG, mimicked the stimulating action of 5-HT on the cat inferior mesenteric ganglion *in situ*, while Wallis *et al.* (1982), showed that, like 5-HT, PBG depolarized the rabbit nodose ganglion *in vitro*.

2-Methyl-5-HT has previously been shown to mimic the effects of 5-HT in the rabbit isolated heart and vagus nerve, but does not appear to differentiate these two preparations from the rat vagus nerve. Thus, in the present study using the rat tissue, 2-methyl-5-HT was approximately 4 fold weaker than 5-HT in molar terms. Donatsch *et al.* (1984a) found that 2-methyl-5-HT was approximately 2 fold weaker than 5-HT on the rabbit neuronal preparations.

Tropacaine and MCPP were found to behave as reversible competitive antagonists of 5-HT-induced depolarizations of the rat vagus nerve, with pK_B values of 6.29 ± 0.03 and 6.90 ± 0.03, respectively. Like metoclopramide, tropacaine does not appear to discriminate between 5-HT-induced responses in the rat vagus nerve and rabbit heart (Table 2). Such comparative data are not available for MCPP. However, this latter compound is not a selective antagonist of the

Table 1 Effects of some putative 5-hydroxytryptamine (5-HT) antagonists on the rat vagus nerve, at 5-HT binding sites and at the 5-HT 'autoreceptor'

Compound	*Depolarization of rat vagus nerve* (pA_2)	*Effect of 5-HT inhibited Binding* (pIC_{50})		*Inhibition of 5-HT release* (pIC_{50})
		5-HT$_2$	*5-HT$_1$*	
Methysergide	4.0	8.50	7.21	6.00
Domperidone	4.5	6.58		
Ketanserin	4.5	8.88		
Pipamperone	4.5	8.58		
Cinanserin	4.8	8.18	5.88	6.00
Haloperidol	4.9	7.13		
Methiothepin	4.9		7.64	8.42
Spiperone	4.9	8.75		6.50
Metergoline	5.0	9.03	8.33	7.00
Xylamidine	5.4	8.30		
Metoclopramide	6.60*			<4.5‡
MCPP	6.90*		6.98	(Agonist)
MDL 72222	7.9	<5.0§	<5.0§	
Quipazine	8.5	6.1	6.49	6.17
ICS 205-930	11.0	<5.0†	<5.0†	

pA_2 values for blockade of 5-HT-induced depolarization of the vagus nerve were determined according to the method of Schild (1947), and are the means of single determinations on at least two individual tissue preparations. *Indicates a pK_B value. Unless stated otherwise, data for the displacement of [^{3}H]-ketanserin (5-HT$_2$) binding are from Leysen *et al.* (1981); data for the displacement of [^{3}H]-5-HT (5-HT$_1$) binding and blockade of the 5-HT 'autoreceptor' are from Martin & Sanders-Bush (1982). Symbols indicate data derived from the following sources: ‡Engel *et al.* (1983); §Fozard (1984); †Richardson *et al.* (1985). Abbreviation: MCPP = *m*-chlorophenylpiperazine.

effects of 5-HT on peripheral neurones since it has been shown to displace 5-HT$_1$-binding with a pIC_{50} of 6.98 (Martin & Sanders-Bush, 1982) and antagonize 5-HT-induced contractions of the rat isolated jugular vein with a pA_2 of 7.41 (Cohen & Fuller, 1983).

Quipazine, MDL 72222 and ICS 205-930 were all potent antagonists of 5-HT-induced depolarizations of the rat vagus nerve. However, since they caused concentration-dependent suppression of the maximum response to 5-HT, the mechanism of their antagonist actions appeared inconsistent with simple reversible competition. A full examination of the effects of quipazine against 5-HT on other mammalian neurones has not been published. MDL 72222 has been shown to cause non-parallel rightward displacements of concentration-response curves for 5-HT in the rabbit heart (Fozard, 1984), rabbit superior cervical ganglion (SCG) and rabbit nodose ganglion (Azami *et al.*, 1985; Fozard *et al.*, 1985). It is interesting to note that the latter authors observed that the nature of the antagonism produced by MDL 72222 changed from apparently competitive to insurmountable with increased time of exposure.

With ICS 205-930 there seems to be some disagreement as to the nature of the antagonism produced. In the present study on the rat vagus nerve, ICS 205-930, 1×10^{-10}–3×10^{-9} M, caused a pronounced reduction in the amplitude of the maximum depolarization induced by 5-HT. On the rabbit nodose ganglion and SCG, Round & Wallis (1986) noted similar effects with ICS 205-930, but only at concentrations of 1×10^{-9} M or greater. In contrast, Richardson *et al.* (1985) reported that ICS 205-930 behaved as a 'true competitive' antagonist when tested against the positive chronotropic effect of 5-HT in the rabbit heart, and against 5-HT-induced depression of the compound action potential in the rabbit vagus nerve. It may be of relevance that these latter effects were measured after equilibration periods of only 30 min on the heart, and 60 min on the vagus nerve, whilst the results given in the present study were obtained after at least 3 h exposure to ICS 205-930.

Given the nature of the 5-HT-antagonist effects of quipazine, MDL 72222 and ICS 205-930 on the rat vagus nerve, no attempt was made to calculate pK_B values. The approximate pA_2 values determined for these compounds according to the method of Schild (1947) (Table 2) do, however, give some guide to potency.

Quipazine, 1×10^{-6} M, has previously been shown

Table 2 Potencies of antagonists on 5-hydroxytryptamine (5-HT)-induced responses in isolated preparations of peripheral neurones

Antagonist	*Potency estimate*				
	Rat VN (pK_B)	*Rabbit Heart* (pA_2)	*Rabbit NG* (pA_2)	*Rabbit SCG* (pA_2)	*Rabbit VN* (pA_2)
Metoclopramide	6.60	7.2*, 7.1[¶]	—	—	7.3[¶]
Tropacaine	6.29	6.77†	—	—	—
MDL 72222	(7.9)	9.27‡, 8.9[¶]	7.81[§]	7.94[§]	7.9[¶]
ICS 205-930	(11.0)	10.6[¶]	9.75[§]	10.55[§]	10.2[¶]

Abbreviations: VN = vagus nerve; NG = nodose ganglion; SCG = superior cervical ganglion. MDL 72222 and ICS 205-930 did not behave as reversible competitive antagonists of 5-HT-induced depolarization of the rat VN; pA_2 values calculated according to the method of Schild (1947) are quoted. *Value from Fozard & Mobarok Ali (1978); †value from Fozard (1979); ‡value from Fozard (1984); [¶]data from Donatsch *et al.* (1984b); [§]data from Round & Wallis (1985).

to be an effective antagonist of 5-HT-induced depolarizations of the rabbit SCG and nodose ganglion, although pA_2 values were not calculated (Lansdown *et al.*, 1980; Wallis *et al.*, 1982). The potency of MDL 72222 as a 5-HT antagonist seems to depend on the tissue preparation used (Table 2). Thus, MDL 72222 appears to be approximately equipotent against 5-HT on the rat vagus nerve, rabbit vagus nerve, rabbit nodose ganglion and rabbit SCG, but is about 10 fold more potent against 5-HT in the rabbit heart. In contrast, the potency of ICS 205-930 appears to be largely independent of the tissue preparation on which it is tested (Table 2).

5-HT-induced depolarizations of the rat vagus nerve were not mimicked and were only weakly antagonized by compounds known to be potent in displacing radioligands from 5-HT_1, or 5-HT_2 binding sites, or in blocking the so-called 5-HT autoreceptor. There was no correlation between the potencies of these compounds on the vagus nerve and at the other sites in either absolute or rank-order terms (Table 1).

In conclusion, an attempt has been made to characterize 5-HT-induced depolarizations of the rat vagus nerve in terms of the effects of both agonists and antagonists. The results obtained with the two recently discovered, highly selective 5-HT antagonists MDL 72222 and ICS 205-930 were disappointing, since they did not seem to behave in a reversible competitive manner against 5-HT on this tissue preparation. This suggests caution in the use of MDL 72222 and ICS 205-930 as tools for the characterization of neuronal 5-HT receptors. Nevertheless, the observation that 5-HT-induced depolarizations of the rat vagus nerve were potently antagonized by these two compounds with approximate pA_2 values of 7.9 and 11.0 respectively, were essentially unaffected by ketanserin and methiothepin, and were mimicked by 2-methyl-5-HT with a potency similar to that of 5-HT itself, satisfy the recently proposed criteria for the involvement of a 5-HT_3-receptor (Bradley *et al.*, 1986) in their generation. However, we conclude that no good evidence is available to demonstrate convincingly any difference between the receptors that mediate 5-HT-induced depolarization of the rat vagus nerve, and those that mediate either 5-HT-induced depolarization of the rabbit nodose ganglion and SCG, or 5-HT-induced inhibition of action potential propagation in the rabbit vagus nerve.

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Nicotine cue in rats: effects of central administration of ganglion-blocking drugs

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1 In rats trained to discriminate nicotine from saline, a single intraventricular injection of a small dose of the quaternary ganglion-blocking drug chlorisondamine blocked the response to nicotine for four weeks.

2 Pentolinium was only weakly active and hexamethonium was inactive as a nicotine antagonist under the conditions used, even in doses that were just below those producing myoclonic jerks.

3 Chlorisondamine had no blocking effect in rats trained to discriminate the non-nicotinic drugs midazolam or morphine from saline.

4 Intraventricular injections of chlorisondamine have a specific and unusually persistent nicotine-blocking action, the mechanism of which requires further investigation.

Introduction

Most behavioural effects of nicotine are thought to be mediated primarily through central cholinoceptive sites resembling those in autonomic ganglia. For example, in rats trained to discriminate the effects of nicotine (s.c.) from saline, the ganglion-blocking drugs mecamylamine and pempidine act as antagonists when administered systemically (Morrison & Stephenson, 1969; Chance *et al.*, 1978). This antagonism is probably not competitive in nature, since the ganglion-blockers do not inhibit the binding of tritiated nicotine to putative receptor sites in brain and because the block cannot be overcome by increasing the dose of nicotine (Romano & Goldstein, 1980; Stolerman *et al.*, 1983).

Systemically administered quaternary ganglion-blocking drugs such as hexamethonium and chlorisondamine are not thought to penetrate well into the brain. These compounds are either ineffective or of very low potency in blocking the discriminative stimulus effects of nicotine (Morrison & Stephenson, 1969; Romano *et al.*, 1981; Stolerman *et al.*, 1983; Stolerman *et al.*, 1984). These observations have provided the main evidence that the discriminative effects of nicotine are central in origin. If this conclusion is valid, then direct intracerebral injections of quaternary ganglion-blockers should block the discrimination of nicotine. In the first experiment of this type, Hazell *et al.* (1978) found that hexamethonium administered intracerebroventricularly (i.c.v.) had no blocking effect on nicotine given (s.c.) 10 min later. Interpretation of these results was compromised by the use of only a single (10 μg) dose of hexamethonium, but the conclusion was confirmed by Stolerman *et al.* (1983), who showed that hexamethonium in doses as large as 400 μg (i.c.v.) had no blocking effect on responses to nicotine (s.c.). These results contrasted with observations that i.c.v. administration of quaternary ganglion-blockers potently blocked convulsions produced by nicotine in mice (Aceto *et al.*, 1969; Caulfield & Higgins, 1983).

In the light of the preceding observations, it was notable that the quaternary ganglion-blocker chlorisondamine (2–5 μg i.c.v.) prevented the increases in locomotor activity otherwise induced by nicotine in rats (Clarke & Kumar, 1983; Clarke, 1984). Furthermore, this block was extremely persistent and lasted for at least four weeks after an injection of chlorisondamine. The present experiments were carried out to determine whether chlorisondamine was also able to block the discriminative effects of nicotine for long periods of time, to test whether other ganglion-blockers such as hexamethonium and pentolinium had a similar action, and to examine the specificity of the block produced by chlorisondamine. Specificity was assessed by testing the effect of chlorisondamine in rats trained to discriminate a benzodiazepine (midazolam) or an opiate (morphine) from saline. Garcha *et al.* (1985a) have given a preliminary account of some of these experiments.

Animals

Male, Lister hooded rats (Olac, Bicester) were housed individually in rooms maintained at about 22°C with a regular light-dark cycle (light from 08 h 00 min to 20 h 00 min). Initially, the rats weighed 210–280 g; throughout the experiments they were fed restricted amounts of food so as to maintain their weights at about 80% of those under free-feeding conditions. Water was available in the living cages at all times.

Apparatus

Standard experimental chambers (Campden Instruments) were contained in sound-insulated, ventilated enclosures. The chambers were fitted with two response bars separated by a recess in which 45 mg pellets of food could be presented. White noise was present at all times to mask external sounds. The experiments were controlled by programmes written in ONLIBASIC, running on CUBE microcomputers (Control Universal, Cambridge).

Training procedure

The procedure for establishing drug discriminations has been described in detail by Pratt *et al.* (1983) and Stolerman *et al.* (1984) and only the main features are presented here. The rats were first trained to press bars for food reinforcers without receiving any injections. Then, in sessions after subcutaneous administration of a drug, the animals were reinforced for pressing one of the two bars: presses on the other bar were reinforced in sessions after saline injections. The final schedule of food reinforcement was tandem variable-interval 1 min fixed-ratio 10; under this schedule, food was presented following the tenth consecutive response on the correct bar after a randomly-determined interval (mean = 1 min). Responses during the intervals were not reinforced. Session length was either 15 min (for rats trained on morphine or nicotine) or 10 min (for rats trained on midazolam).

Most rats were trained with nicotine (s.c.); the dose was 0.4 mg kg^{-1} initially and it was reduced progressively to 0.1 mg kg^{-1}. In two experiments, midazolam (0.1 mg kg^{-1} s.c.) or morphine (3 mg kg^{-1} s.c.) was used for training. The dose of morphine was similar to that used by Shannon & Holtzman (1976). The dose of midazolam was chosen, after preliminary experiments, to produce discriminative effects close in magnitude to those produced by nicotine (0.1 mg kg^{-1}); the 0.4 mg kg^{-1} dose of midazolam used by Garcha *et al.* (1985b) acquired stimulus control over behaviour much more rapidly and reliably than did the very small dose of nicotine used for training.

Generalization test procedure

Testing the effects of putative long-term blocking drugs raises special problems in drug discrimination procedures that normally assume rats to be fully responsive to drugs in training sessions that take place between successive tests. Training to maintain stable baselines is not possible if the effects of the drug are attenuated by a long-acting antagonist. In the present work, it was necessary to carry out 8 tests (over four to six weeks) without retraining, after recovery from the surgery involved in injecting drug or saline (i.c.v.). These tests were performed with groups of 6–10 rats and consisted of 5 min extinction sessions during which no responses were reinforced. Tests took place twice weekly, on two successive days, with saline and the drug used for training given in counterbalanced order. In order to maximize baseline stability during the repeated tests, the doses of drugs were twice those used for training.

The main index used to assess discriminative effects was the number of responses on the bar appropriate for the training drug expressed as a percentage of the total number of responses on both bars. This quantitative index was calculated separately for each rat and means were then taken. Comparisons between different mean values were made, after arc-sine transformations, by mean of analyses of variance and *t* tests (Winer, 1971). A separate repeated measure analysis of variance was calculated on the data from the two extinction tests that took place each week; repeated measures were involved since both drug and saline tests were carried out in each group of rats. A supplementary (quantal) index was obtained by calculating the percentage of rats selecting the drug-appropriate bar; the selected bar was defined as the one on which the rat first totalled ten responses. Colpaert *et al.* (1977) have maintained that under certain conditions, valid results from drug discrimination experiments with antagonists may only be obtained with a quantal index. The total number of responses on both bars in a 5 min extinction test was used as an index of overall response rate.

Intraventricular injections

Rats were mounted in a stereotaxic frame (Stoelting) under halothane anaesthesia. Injection needles (outside diameter, 0.42 mm) were then lowered into the brain through burr holes, aimed at the left lateral cerebral ventricle. With the incisor bar 5 mm above the interaural line, co-ordinates were 6.0 mm anterior to the interaural line, 1.5 mm lateral and 4.0 mm ventral to the top surface of the dura (Pellegrino *et al.*, 1979). Intraventricular injections were given in volumes of 1 μl and were administered slowly over 1 min; the needle was left in place for a further min. The tonicity

of drug solutions was adjusted with sodium chloride. Injection sites were verified from 20 μm sections stained with Luxol fast blue and cresyl violet and examined under a light microscope.

Plan of investigation

In the first experiment, two sets of rats trained to discriminate nicotine were used to test the effects of chlorisondamine. Experimental rats received chlorisondamine (5 μg i.c.v. in 1 μl) and controls received the same volume of isotonic saline. In one set of rats, responses to saline and nicotine were tested 7 days after chlorisondamine and thereafter, at weekly intervals for four weeks. For the second set of rats, the first tests were 3 days after chlorisondamine, and thereafter at intervals of two weeks. In the second experiment, two sets of rats trained to discriminate nicotine were used to test the effects of pentolinium and hexamethonium. Experimental rats received hexamethonium or pentolinium and controls received saline. Hexamethonium was tested in doses of 50 μg and 200 μg and pentolinium in doses of 25 μg and 100 μg. Tests for all these rats began 7 days after injection and continued at weekly intervals. In the third experiment, two sets of rats trained to discriminate midazolam (0.1 mg kg^{-1}) or morphine (3 mg kg^{-1}) were used to test the effects of chlorisondamine. Experimental rats received chlorisondamine (5 μg) and control rats received saline; tests began after 7 days and took place at weekly intervals.

Drugs

Nicotine hydrogen tartrate (BDH, Poole, Dorset) was dissolved in isotonic saline and the pH was adjusted to 7 with a dilute solution of NaOH. The following drugs were dissolved in isotonic saline: chlorisondamine chloride (CIBA-Geigy, Basle), hexamethonium Br (Sigma, Poole, Dorset), midazolam (Hoffman-La Roche, Basle), morphine HCl (May & Baker, Dagenham, Essex) and pentolinium HBr (May & Baker). Nicotine, midazolam and morphine were injected subcutaneously in a volume of 1 ml kg^{-1} and all doses were calculated as those of the base. Injections were given 5, 15 and 30 min before each session for midazolam, nicotine and morphine, respectively.

Results

Experiment 1: chlorisondamine and discrimination of nicotine

Chlorisondamine (5 μg i.c.v.) completely blocked the discriminative effect of nicotine in tests 7 days after surgery. Figure 1a shows that control rats receiving

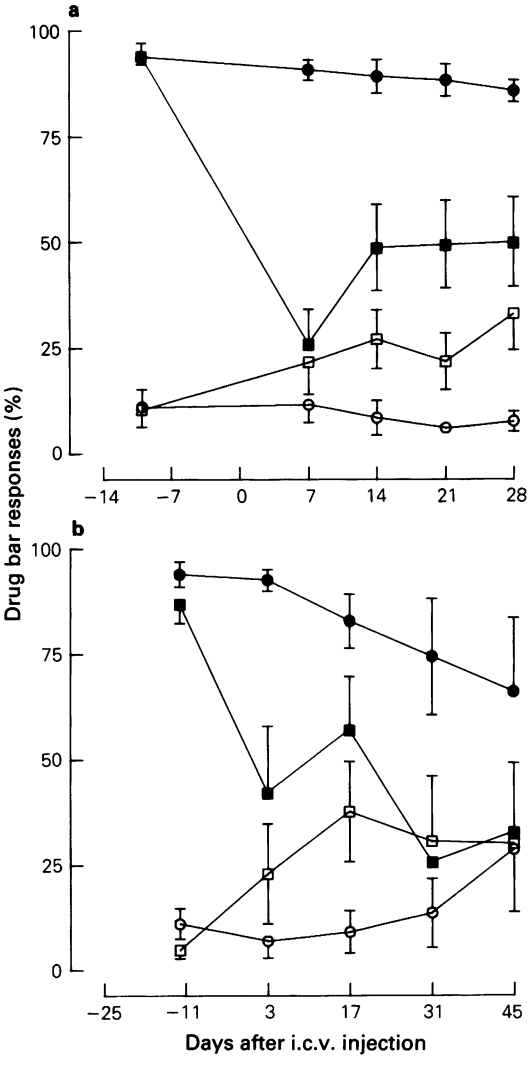

Figure 1 Discriminative effects of nicotine (0.2 mg kg^{-1} s.c.) in rats before and after an intracerebroventricular (i.c.v.) injection of chlorisondamine. (a) Shows results of extinction tests carried out at weekly intervals after chlorisondamine, whereas (b) shows tests at intervals of two weeks. Experimental rats ($n = 8-9$) received chlorisondamine (5 μg in 1 μl, i.c.v.) and were tested with both nicotine (■) and saline (□). Control rats ($n = 7-8$) received saline (1 μl i.c.v.) and were also tested with nicotine (●) or saline (○). In this and subsequent figures, results are shown as means, with vertical lines indicating s.e.means; overlapping s.e.means are omitted for clarity. Tests with nicotine and saline shown as taking place at, for example, 7 days were counterbalanced across days 7 and 8. Some rats did not respond sufficiently for discriminative effects to be assessed in all tests.

saline (i.c.v.) exhibited strong and consistent discrimination of nicotine from saline throughout the experiment. Initially, experimental rats did not differ from controls and showed strong discriminative responses to nicotine; 7 days after the i.c.v. injections, the effect of nicotine in experimental as compared with control rats was fully blocked (Figure 1a). The block was still present, albeit incomplete, in the tests 14, 21 and 28 days after chlorisondamine. Statistically, nicotine had significant effects on all test days (minimum $F = 28.0$, d.f. 1,15; $P < 0.001$), and the blocking action of chlorisondamine was verified by significant nicotine × chlorisondamine interactions ($F = 40.1$, 22.6, 13.2 and 12.0, for tests at 7, 14, 21 and 28 days, respectively; $P < 0.01$ in all cases). The interaction term in the analysis was insignificant for tests before the injection of chlorisondamine ($F < 1$, d.f. 1,16). Chlorisondamine itself slighly increased the percentage of responses on the nicotine-appropriate bar in tests at 14, 21 and 28 days (minimum $t = 2.25$, d.f. 16; $P < 0.05$).

The quantal index of response fully supported the results obtained with the quantitative index. For example, in the tests of response to nicotine at 7 days after i.c.v. injections, 0% of experimental rats selected the drug-appropriate bar, as compared with 100% of control rats. At 28 days, 50% of experimental rats selected the drug-appropriate bar, as compared with 100% of controls.

The effects of chlorisondamine were examined in a second set of rats with tests spaced over a wider range of times after the i.c.v. injections. Figure 1b shows that control rats receiving saline (i.c.v.) showed adequate discrimination of nicotine in tests at 3, 17 and 31 days (minimum $F = 10.2$, d.f. 1,7; $P < 0.05$, at 31 days). Nicotine was not reliably discriminated from saline in the tests at 45 days ($F = 4.3$, d.f. 1,7). Before treatment with chlorisondamine, experimental rats did not differ from controls and showed strong discriminative responses to nicotine; 3 days after the i.c.v. injections, the effect of nicotine in experimental as compared with control rats was attenuated (Figure 1b). The block was equivocal at 17 days but reappeared at 31 days. The action of chlorisondamine was verified by significant nicotine × clorisondamine interactions ($F = 14.3$, 43.3 and 9.9, for tests at 3, 17 and 31 days respectively; $P < 0.05$ in all cases). This interaction was insignificant for tests before the injection of chlorisondamine and at 45 days ($F < 1$ in both cases). Chlorisondamine itself increased the percentage of responses on the drug-appropriate bar in tests at 17 days only ($t = 2.31$, d.f. 11; $P < 0.05$). The pattern of results with the quantal index of response was similar and is not presented.

The repeated extinction tests carried out without any intervening training were associated with a steady decline in the total number of responses in all groups of rats. Figure 2 illustrates this effect for rats receiving weekly tests after injections of chlorisondamine. Nicotine slightly reduced the total number of responses (e.g. $F = 6.3$, d.f. 1,16; $P < 0.05$ during tests before surgery), whereas chlorisondamine alone had no effect (largest $F = 1.24$, d.f. 1,16). On all test days after surgery, chlorisondamine blocked the reductions in

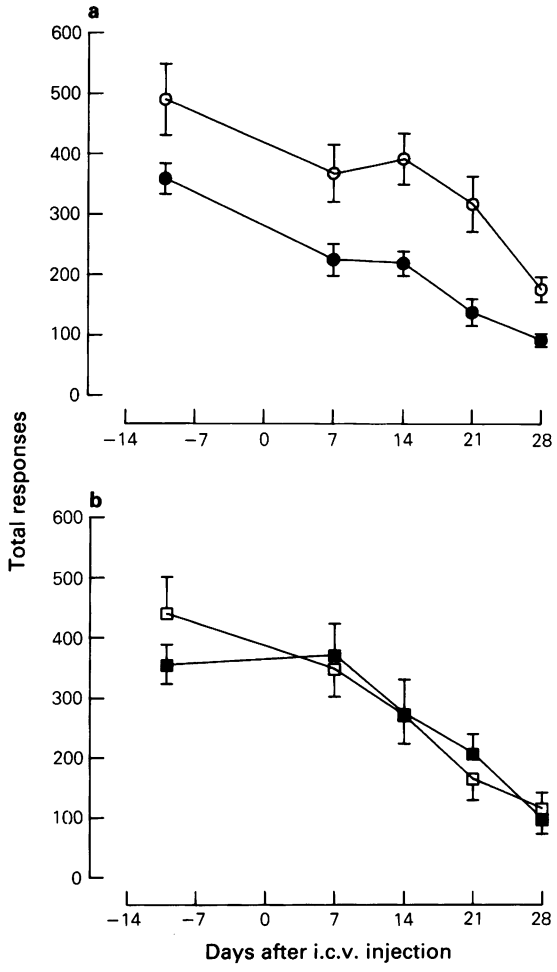

Figure 2 Total number of responses in rats receiving nicotine (0.2 mg kg^{-1} s.c.) or saline (s.c.) before and at weekly intervals after an intracerebroventricular (i.c.v.) injection of chlorisondamine. (a) Shows results of extinction tests in control rats ($n = 9$) receiving saline (1 μl i.c.v.) and tested with nicotine (●) or saline (○). (b) Shows tests in experimental rats ($n = 9$) receiving chlorisondamine (5 μg in 1 μl, i.c.v.) and tested with both nicotine (■) and saline (□).

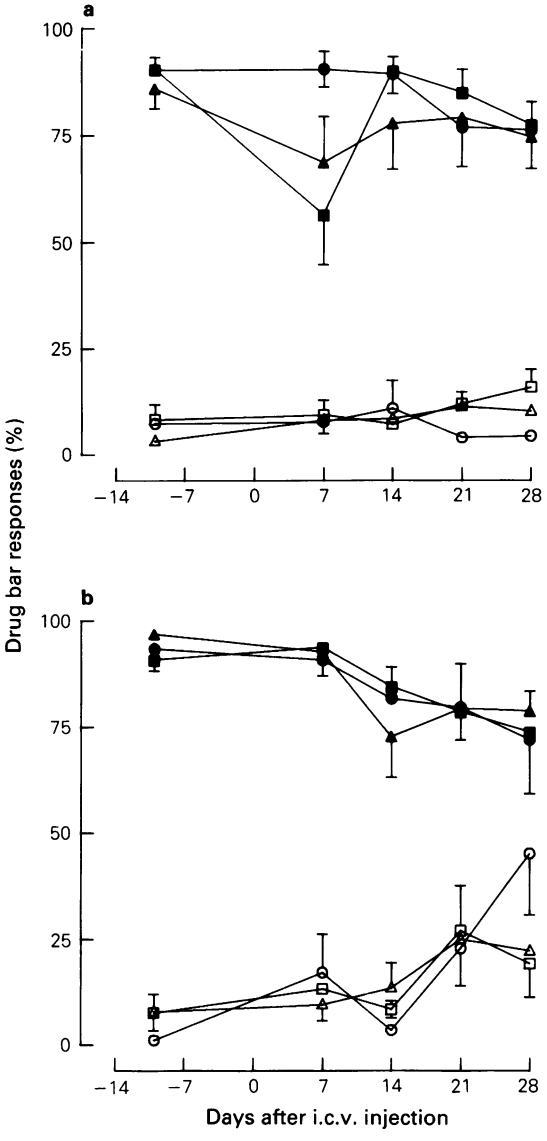

Figure 3 Discriminative effects of nicotine (0.2 mg kg^{-1} s.c.) in rats before and after intracerebroventricular (i.c.v.) injections of ganglion-blocking drugs ($n = 8$ throughout). (a) Shows results from tests with nicotine in rats receiving saline (●), pentolinium 25 μg (▲) or pentolinium 100 μg (■). Results from tests with saline in these rats are shown by corresponding open symbols (○, △, □). (b) Shows results from tests with nicotine in rats receiving saline (●), hexamethonium 50 μg (▲) or hexamethonium 200 μg (■). Results from tests with saline in these rats are shown by corresponding open symbols (○, △, □).

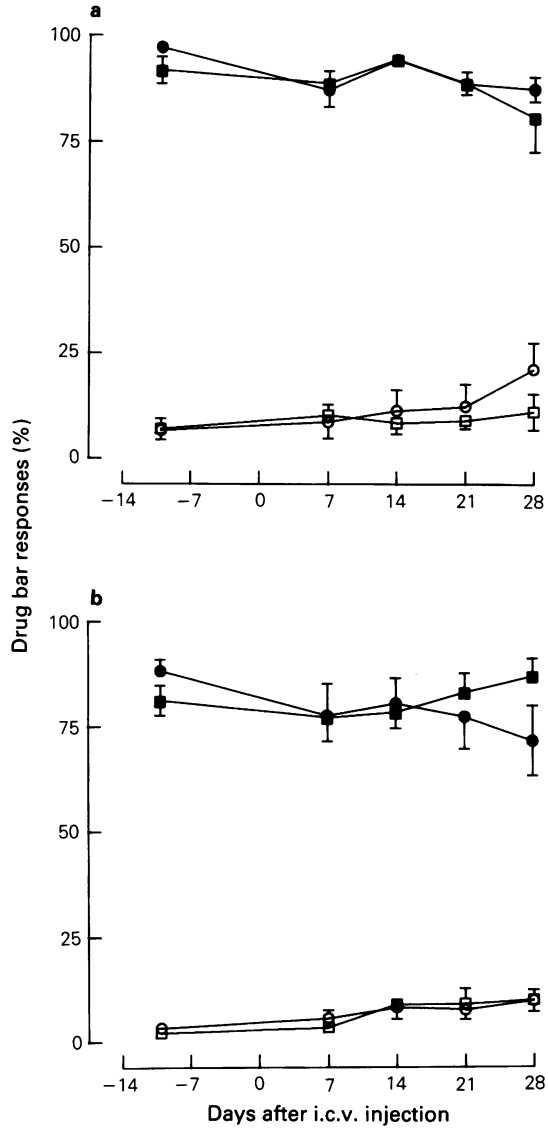

Figure 4 Discriminative effects of the non-nicotinic drugs midazolam and morphine in rats before and after an intracerebroventricular (i.c.v.) injection of chlorisondamine (5 μg). In (a) experimental rats ($n = 8$) received chlorisondamine and were tested with both midazolam, 0.2 mg kg^{-1} (■) and saline (□). Control rats ($n = 7$) received saline (i.c.v.) and were also tested with midazolam (●) or saline (○). In (b) experimental rats ($n = 10$) received chlorisondamine but were tested with morphine, 6 mg kg^{-1} (■) and saline (□). Control rats ($n = 10$) received saline (i.c.v.) and were tested with morphine (●) or saline (○).

total responses otherwise produced by nicotine, as verified by significant nicotine × chlorisondamine interactions ($F = 12.0, 6.4, 19.4$ and 4.1, for tests at 7, 14, 21 and 28 days respectively; d.f. 1,16; $P < 0.05$ in all cases). Similar trends over time were seen with other sets of rats tested in a similar manner, but these results are not presented since the drugs used had little effect on this index.

Experiment 2: pentolinium and hexamethonium on nicotine response

Pentolinium attenuated the discriminative effect of nicotine in tests 7 days after surgery. Figure 3a shows that control rats receiving saline (i.c.v.) showed strong, consistent discrimination of nicotine from saline throughout the experiment. Initially, experimental rats did not differ from controls, but 7 days after the i.c.v. injections, the effect of nicotine in experimental rats was weakened by pentolinium (Figure 3a). Block was absent in the tests 14, 21 and 28 days after pentolinium, and even in tests at 7 days after the largest dose of pentolinium, nicotine produced about 50% drug-appropriate responding on the basis of both the quantitative and the quantal indices. Pentolinium alone did not increase drug-appropriate responding.

Statistically, nicotine had significant effects on all test days (minimum $F = 100.8$, d.f. 1,21; $P < 0.001$). The blocking action of pentolinium in tests at 7 days was verified by a significant nicotine × pentolinium interaction ($F = 3.52$, d.f. 2,21; $P < 0.05$). In these tests, pentolinium, 100 μg, attenuated the response to nicotine ($t = 2.77$, d.f. 21; $P < 0.05$) but pentolinium, 25 μg, was without significant effect ($t = 1.91$, d.f. 21). Neither dose of pentolinium had any significant effect in tests at 14, 21 or 28 days ($F < 1$ in all cases).

Hexamethonium did not attenuate the discriminative effect of nicotine in tests 7, 14, 21 or 28 days after surgery. Figure 3b shows that control rats receiving saline (i.c.v.) demonstrated some discrimination of nicotine from saline throughout the experiment, although the effect seemed to weaken by 28 days. Experimental rats did not differ from controls at any stage, and hexamethonium neither blocked nicotine nor increased drug-appropriate responding. Statistically, nicotine had significant effects on all test days (minimum $F = 44.9$, d.f. 1,17; $P < 0.001$) but there was no significant nicotine × hexamethonium interaction (largest $F = 2.03$, d.f. 2,21).

Experiment 3: chlorisondamine on responses to midazolam or morphine

Chlorisondamine did not attenuate the discriminative effect of either midazolam or morphine in tests 7, 14, 21 or 28 days after surgery. Figure 4 shows that control rats receiving saline (i.c.v.) clearly discriminated these drugs from saline throughout the experiment. Experimental rats did not differ from controls at any stage, and chlorisondamine neither blocked these discriminative effects nor increased drug-appropriate responding. Statistically, midazolam and morphine had significant effects on all test days (smallest $F = 88.7$, d.f. 1,12; $P < 0.001$ for midazolam; smallest $F = 130.6$, d.f. 1,16; $P < 0.001$ for morphine). There was no significant interaction of chlorisondamine with either training drug (largest $F < 1$ for midazolam; largest $F = 2.40$, d.f. 1,16 for morphine).

Similar results were obtained with the quantal index of response, and these are not presented in detail. For example, in the tests with midazolam at 7 days after i.c.v. injections, 88% of experimental rats selected the drug-appropriate bar, as compared with 71% of control rats. In the corresponding tests with morphine, 100% of experimental rats selected the drug-appropriate bar, as compared with 71% of control rats.

Discussion

Direct comparisons of the effects of different quaternary ganglion-blocking drugs on the discriminative effects of nicotine have not previously been reported. In contrast with hexamethonium and pentolinium, chlorisondamine was a potent and fully effective antagonist. Larger doses of pentolinium of hexamethonium could not be tested since their administration in preliminary studies was associated with convulsive effects. In contrast, chlorisondamine did not produce convulsions either at the 5 μg dose used in the behavioural experiments, or even at 100 μg (in separate tests). It is not known why hexamethonium and pentolinium were largely inactive; either different distributions within the CNS or different degrees of receptor selectivity may be responsible. The latter explanation raises the possibility of differences between peripheral ganglionic receptors (which are blocked by all three compounds) and those in the CNS. One speculative interpretation could be that there are different subtypes of CNS nicotinic receptors, upon which the different antagonists have varying degrees of effect. Some ligand-binding studies have been interpreted as evidence for nicotinic receptor subtypes (e.g. Sloan *et al.*, 1984).

Drug discrimination techniques have not generally been used for testing the effects of substances thought to have long durations of action as antagonists. The present results show that information about long-acting antagonists may be obtained with such techniques. Usable baselines of discriminative control were maintained over long periods of time without intervening training. Discrimination of nicotine, midazolam and morphine persisted across a total of 8

extinction tests spaced through four weeks, despite marked reductions in the total numbers of responses. These data further support the independence of the measures of discriminative and response rate effects. The tandem schedule of reinforcement used in the present experiments ensured a low, unpredictable frequency of reinforcement that may have made it difficult for rats to discriminate between reinforced training sessions and extinction tests; direct comparisons with other schedules are needed to test this explanation.

The experiments with midazolam and morphine tested whether the attenuating effects of chlorisondamine on the responses to nicotine represented a pharmacologically specific block. The lack of effect of chlorisondamine on the discrimination of these two non-nicotinic drugs shows that a general disruption of discriminative behaviour cannot account for the results, and points to at least some pharmacological specificity. Tests of systemically administered mecamylamine on the discriminative effects of a variety of psychoactive drugs also suggest that ganglion-blockers may have pharmacologically specific effects (Poling *et al.*, 1979; Meltzer & Rosecrans, 1981; Overton, 1983; Stolerman *et al.*, 1983).

The small but reproducible increases in drug-appropriate responding produced by chlorisondamine alone may indicate either a weak nicotine-like effect, or weakened discriminative control by the saline training condition (Figure 1). The former explanation seems the more likely since similar effects were not seen in the experiments with rats trained to discriminate midazolam or morphine (Figure 4). However, very small changes in drug-appropriate responding may be attributable to response biases like those described by McMillan & Wenger (1984).

The powerful, prolonged blocking action of chlorisondamine seems to occur with all behavioural effects of nicotine examined to date. Both increases and decreases in locomotor activity of rats, produced by different doses and numbers of exposures to nicotine, are also fully blocked (Clarke & Kumar, 1983; Clarke, 1984). In experiment 1, chlorisondamine attenuated the response-rate decreasing effect of nicotine (Figure 2). In other experiments, chlorisondamine has been found specifically to block conditioned taste aversions produced by nicotine (Reavill *et al.*, 1986) and unconditioned decreases in fluid intake produced by nicotine (Reavill & Stolerman, unpublished results).

Through what central mechanism does chlorisondamine persistently block response to nicotine? One possibility is that this quaternary compound, after i.c.v. injection, cannot readily leave the brain. This explanation is not convincing because other quaternary antagonists do not have similarly prolonged durations of effect. Locke & Holtzman (1985) have shown that a quaternary analogue of naltrexone blocks the discriminative effects of morphine, but for only a few hours after injection (i.c.v.). Chlorisondamine probably does not act as a neurotoxin for brain nicotinic receptors since no lesions in brain regions rich in binding sites for tritiated nicotine could be detected by light microscopy (Reavill, unpublished observations). Another possibility is that chlorisondamine interacts irreversibly with some component of the proposed nicotinic receptor complex (Stolerman, 1986); this component is unlikely to be the binding site for nicotine itself since chlorisondamine does not inhibit the binding of tritiated nicotine (Romano & Goldstein, 1980; Jenner *et al.*, 1986). Additional studies on the mode of action of chlorisondamine may help to clarify the nature of brain nicotinic receptors.

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Effects of morphine on catecholamine release and arrhythmias evoked by myocardial ischaemia in rats

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1 The effects of morphine (10 mg kg^{-1} i.p.) on haemodynamics, arrhythmias and plasma and myocardial catecholamines (CA) were studied after coronary artery occlusion in anaesthetized rats. Myocardial intraneuronal CA were assessed histofluorimetrically and CA concentrations measured by high performance liquid chromatography.

2 Morphine increased blood pressure, presumably due to higher plasma noradrenaline (NA) concentrations found in morphine-treated rats.

3 Morphine increased the area of catecholamine-containing fluorescing neurones in the myocardium (as a percentage of total field area) 60 min after sham-operation (0.87 ± 0.07%) or occlusion (0.57 ± 0.05%) compared to untreated animals (0.67 ± 0.06 and 0.38 ± 0.03% respectively). Tissue NA content was not significantly affected by coronary occlusion and/or morphine within the first 60 min.

4 Morphine had no effect on ischaemia-induced arrhythmias.

5 Whether the higher intraneuronal NA content following morphine resulted from reduced central sympathetic outflow to the heart, presynaptic inhibition of NA release, or increased uptake due to higher plasma concentrations is unclear. Ischaemia-induced local NA release appears independent of these mechanisms, as it was unaffected by morphine.

Introduction

In the early period of myocardial infarction, the occurrence of ventricular arrhythmias (VES) and ventricular fibrillation (VF) is the main cause of early, prehospital mortality. About 60% of deaths occur during the first 2 h after the onset of symptoms (Kannel & Thomas, 1982).

In addition to biochemical, metabolic and mechanical factors (Opie *et al*, 1979; Covell *et al*, 1981), the effects of catecholamines (CA) on the ischaemic and non-ischaemic myocardium seem to play an important role in the genesis of early arrhythmias. In several studies, a positive correlation between adrenergic activity and the incidence of arrhythmias has been demonstrated (Lown & Verrier, 1976; Sharma & Corr, 1983). Two different mechanisms seem to be responsible for the increased concentration of CA at the myocytes: (1) Due to pain, fear and haemodynamic alterations, the activity of the sympathetic nervous system is increased by central reflexes (Kliks *et al.*, 1975). (2) A reflex-independent local release of noradrenaline (NA) in the ischaemic myocardium, probably due to hypoxia, acidosis and increased extracellular potassium concentration has been described by several investigators (Holmgren *et al.*, 1981; Abrahamsson *et al.*, 1982; Hirche *et al.*, 1985).

Morphine is frequently used as an analgesic in patients suffering from myocardial infarction because of its rapid and reliable onset of analgesia, sedation of the anxious patient and its almost negligible effects on myocardial contractility and haemodynamics (Jaffe & Martin, 1980). Opiates have been shown to inhibit NA release from sympathetic nerve terminals by central sympatholysis (Laubie *et al.*, 1977) and by interaction with inhibitory presynaptic opiate binding sites on adrenergic nerve terminals in a variety of organs (Henderson *et al.*, 1979; Stickney & Eickenburg, 1981).

It was the purpose of this study to investigate the effects of morphine (10 mg kg^{-1}, i.p.) on systemic and local CA release in the ischaemic myocardium and the incidence of early arrhythmias after coronary artery occlusion in the rat.

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Methods

Experiments were performed on male rats (Ham Wistar) weighing 250–350 g. Anaesthesia was induced with pentobarbitone sodium (6 mg $100\,g^{-1}$, i.p.). The animals were ventilated artificially with room air (1 ml $100\,g^{-1}$, 60–70 strokes per min) with a Rodent Respirator 681A (Harvard App., Mass., U.S.A.) and set up to allow recording of arterial blood pressure, from the left common carotid artery, (Statham P23DB, Hato Rey, Puerto Rico) and the electrocardiogram (ECG) using standard limb leads. These parameters were recorded continuously on a four-channel recorder (Gould Brush 2400).

Rectal temperature was maintained at approximately 38°C. After left thoracotomy in the fifth intercostal space, sectioning of the fourth and fifth ribs approximately 2 mm from the left margin of the sternum and resection of the pericardium, the heart was exteriorised by gentle pressure on the right chest wall. A ligature (6–0, 0.7 metric Perma-Hand Seide, Ethicon) was placed around the left coronary artery close to its origin and the heart was replaced immediately in the chest cavity.

Experimental design: rats were divided into four groups (Table 1). Immediately after placing the ligature, NaCl 0.9% 1 ml kg^{-1} (groups I and II) or morphine HCl 10 mg kg^{-1} (groups III and IV) was administered intraperitoneally. This dosage has been shown to produce significant analgesia in rats for 1 h after administration (Clarke & Wright, 1984). After 15 min, the left coronary artery was occluded in groups II and IV.

In 5 rats of each group, 15 min after sham-operation or occlusion (i.e. 30 min after morphine or NaCl administration) 3 ml of arterial blood was taken from the carotid artery for the measurement of plasma CA concentrations. These animals were then excluded from further experiments.

In the remaining animals the hearts were rapidly removed and shock-frozen, by means of a Wollenberger clamp cooled in liquid nitrogen, 60 min after sham-operation or occlusion. The hearts were stored at −80°C until analysis. Frozen tissue samples were taken from the left ventricular anterior wall, representing ischaemic myocardium in rats with coronary occlusion, and non-ischaemic myocardium in sham-operated rats. Fluorescence of intraneuronal catecholamines was induced by the method of De la Torre (1980) in cryosections which were 16 μm thick and cut 200 μm subepicardially. The area of fluorescing adrenergic nerve fibres was assessed morphometrically by means of a Leitz u.v. microscope coupled to a residual light amplifying caesicon video camera (PIC 762, Kranz, F.R.G.) and a picture analysing system (Artec Counter 982, Fisher Sci., F.R.G.). The area of fluorescing fibres was expressed as a percentage of the total field area in 100 microscope fields (total area 3 mm^2) per section. In the remaining tissue sample and in plasma, CA were determined by high performance liquid chromatography (h.p.l.c.) using electrochemical detection (h.p.l.c. catecholamine analysing system, Waters GmbH, FRG).

Dysrhythmic activity was assessed from ECG and blood pressure recordings. The number of ectopic beats per minute was counted. A run of 7 or more consecutive ectopic beats at a higher than normal rate was defined as ventricular tachycardia (VT). Periods of irregular, high frequency electrical activity combined with a fall in blood pressure to almost zero were defined as ventricular fibrillation (VF). VT and VF were expressed as seconds per minute.

Statistical evaluation of the data was performed using Student's t test or the Chi-squared test. The 5% level was accepted as significant. All values are expressed as mean ± standard error ($\bar{x} \pm$ s.e.mean).

Table 1 Number of animals (n) in each experimental group

	Morphine			
Group I	0	Sham-op	Plasma CA (5)	Myocardial CA (10)
Group II	0	Occlusion	Plasma CA (5)	Myocardial CA (12)
Group III	10 mg kg^{-1}	Sham-op	Plasma CA (5)	Myocardial CA (10)
Group IV	10 mg kg^{-1}	Occlusion	Plasma CA (5)	Myocardial CA (12)
	−15	0	15	60 min

CA = catecholamines

Results

Haemodynamics

Figures 1 and 2 show the changes in mean arterial blood pressure (MAP) and heart rate (HR) in animals used for the assessment of myocardial CA. Thoracotomy and artificial ventilation caused a significant fall in MAP of about 30% ($P < 0.01$) in all animals to values between 90 and 120 mmHg (see Figure 1). In the first 15 min following the administration of morphine or NaCl, there were only slight changes in MAP in animals receiving NaCl, whereas the morphine-treated rats showed a significant increase in MAP ($P < 0.001$). In group III (sham-operated), this increase continued until 30 min after morphine administration to reach a maximum of 160 mmHg, followed by a slow decrease until the end of the experimental period. Coronary artery occlusion (groups II and IV) caused a fall in MAP in the first minute to values of 76 ± 6 mmHg (group II) and 82 ± 5 mmHg (group IV; both $P < 0.01$). In group II, MAP remained significantly decreased compared to pre-occlusion values until 30 min after the onset of ischaemia. In the morphine-treated animals, the depression of blood pressure lasted only until 5 min after occlusion. After 15 min, MAP was significantly elevated above pre-occlusion values in the morphine-

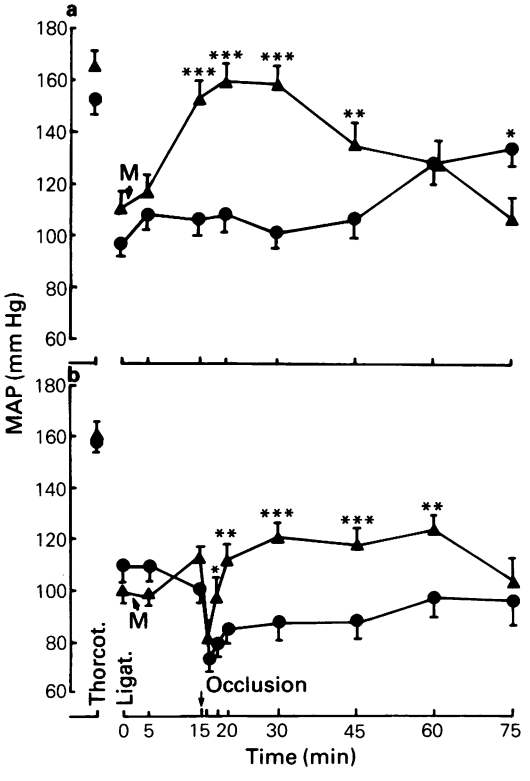

Figure 1 Mean arterial blood pressure (MAP) in sham-operated rats (a) and after coronary artery occlusion (b). Immediately after the ligature was placed around the left coronary artery, the animals received morphine hydrochloride 10 mg kg^{-1} (M, ▲), NaCl 0.9% 1 ml kg^{-1} i.p. (control ●). In (a) $n = 10$ for morphine-treated and control rats; in (b) $n = 12$ for morphine-treated and control rats. Points are mean values with vertical lines indicating s.e.mean. $*P < 0.05$; $**P < 0.02$, $***P < 0.001$ compared to untreated animals.

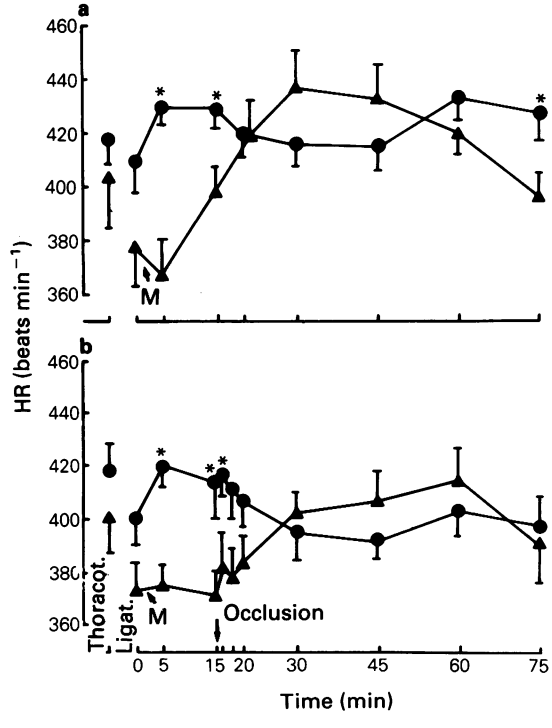

Figure 2 Heart rate (HR) in sham-operated rats (a) and after coronary artery occlusion (b). Immediately after the ligature was placed around the left coronary artery, the animals received morphine hydrochloride 10 mg kg^{-1} (M, ▲) or NaCl 0.9% 1 ml kg^{-1}, i.p. (control, ●). In (a) $n = 10$ for morphine-treated and control animals; in (b) $n = 12$ for morphine-treated and control animals. Points are mean values with vertical lines indicating s.e.mean. $*P < 0.05$ compared to untreated animals.

Table 2 Mean arterial blood pressure (MAP) and heart rate (HR) after sham-operation (groups I and III) or coronary artery occlusion (groups II and IV)

		Pre-Thorac.	*Pre-lig*	*Minutes after placing the ligature* 5	15 (Pre-occl.)	30
Group I	MAP	144 ± 9	116 ± 8[a]	109 ± 7	142 ± 14	129 ± 15
	HR	350 ± 23	360 ± 23	361 ± 34	368 ± 34	353 ± 37
Group II	MAP	162 ± 10	96 ± 7[aa]	83 ± 12	87 ± 11	76 ± 6
	HR	398 ± 18	360 ± 14[aa]	395 ± 17	368 ± 11	355 ± 9
Group III	MAP	148 ± 14	114 ± 12	104 ± 9	172 ± 5[bb]	180 ± 11[bb]
	HR	356 ± 21	346 ± 15	294 ± 12[b]	389 ± 16[b]	437 ± 18[bb]
Group IV	MAP	168 ± 11	96 ± 7[aa]	103 ± 15	130 ± 20	145 ± 5
	HR	400 ± 18	377 ± 8	349 ± 7[b]	365 ± 25	415 ± 19[c]

Immediately after placing the ligature, the rats received 0.9% NaCl 1 ml kg^{-1} (groups I and II) or morphine hydrochloride 10 mg kg^{-1}, i.p. (groups III and IV); 30 min later, 3 ml of arterial blood was taken for the determination of plasma catecholamine concentrations.
x ± s.e.mean, $n = 5$, [a]: $P < 0.05$, [aa]: $P < 0.01$ compared to pre-thoracotomy; [b]: $P < 0.05$, [bb]: $P < 0.01$ compared to pre-ligature; [c]: $P < 0.05$ compared to pre-occlusion.

treated rats. Morphine-treated rats also showed significantly higher MAP after both sham-op (group III) or coronary occlusion (group IV) compared to untreated animals (groups I and II respectively, Figure 1).

The changes in HR seen were less pronounced. In morphine-treated rats, HR was significantly lower for the first 15–18 min after administration compared to animals receiving NaCl (Figure 2).

Table 2 shows the haemodynamic changes in animals used for the determination of plasma CA. As can be seen, the haemodynamic changes were similar to those observed in animals used for the assessment of myocardial CA.

Arrhythmias

After coronary artery ligation, all animals developed arrhythmias in the first 30 min of ischaemia. The severity of these arrhythmias is shown in Table 3. Morphine had no significant effect on the arrhythmias developing as a result of coronary occlusion.

Catecholamines

Plasma CA concentrations were determined 30 min after NaCl or morphine administration (i.e. 15 min after sham-operation or occlusion). In a separate group of 5 rats, blood samples were taken immediately after thoracotomy to serve as control. These values are shown in Table 4. Thirty minutes after NaCl, significantly ($P < 0.01$) higher adrenaline (Ad) concentrations were found in sham-operated rats compared to control. In morphine-treated rats, plasma NA concentrations were almost three times higher than in untreated animals, and the increase in plasma Ad was not seen after morphine pretreatment.

Sixty minutes after coronary artery occlusion, the fluorescing area in the ischaemic myocardium was reduced by 49% compared to sham-operated hearts ($P < 0.001$, Figure 3). After morphine treatment, both ischaemic and non-ischaemic hearts showed a significantly higher fluorescing area than untreated hearts. However, the reduction in fluorescence of 35% at 60 min compared to morphine-treated, sham-operated hearts was almost the same as in untreated animals (Figure 3).

NA tissue concentration in the left ventricle was reduced by 14% after 60 min of ischaemia in untreated rats and by 13% after 60 min of ischaemia in morphine-treated rats compared to the appropriate sham-operated group. Morphine-treated rats showed slightly higher myocardial NA content after sham-operation or occlusion than untreated rats, however, none of these differences was statistically significant (see Figure 3).

Table 3 Ventricular arrhythmias following left coronary artery occlusion in untreated and morphine-treated rats

	Untreated	*Morphine-treated*
Number of rats	12	12
VES per 30 min	614 ± 166	618 ± 174
Number of rats with VT	11 (92%)	10 (83%)
Mean duration of VT (s)	27 ± 8	11 ± 2
Number of rats with VF	2 (17%)	5 (42%)
Mean duration of VF (s)	47 ± 2	11 ± 5

x ± s.e.mean, VES: ventricular extrasystoles; VT: ventricular tachycardia; VF: ventricular fibrillation.

Table 4 Plasma concentrations of noradrenaline (NA) and adrenaline (Ad) immediately after thoracotomy (Control) and 30 min after administration of 0.9% NaCl (1 ml kg^{-1}) or morphine hydrochloride (10 mg kg^{-1}) and sham-operation or coronary artery occlusion

		Sham-operated		*Occlusion*	
	Control	30 min + NaCl	30 min + morphine	30 min + NaCl	30 min + morphine
NA (pg ml^{-1})	138 ± 18	135 ± 51	363 ± 54*	92 ± 12	306 ± 93
Ad (pg ml^{-1})	214 ± 38	423 ± 42	225 ± 80	563 ± 128	81 ± 61*

x ± s.e.mean, $n = 5$, *: $P < 0.02$ compared to the values obtained after NaCl.

Discussion

Haemodynamics and arrhythmias

In untreated rats, coronary artery occlusion led to a reduction in MAP with no significant changes in HR. Similar haemodynamic effects have been described by other investigators (Clark *et al.*, 1980; Au *et al.*, 1983) and are presumably a consequence of reduced stroke volume due to alterations of ventricular function caused by ischaemia (Pfeffer *et al.*, 1979).

Ventricular ectopic activity began soon after occlusion and lasted approximately 30 min, with a maximum rate of 102 ± 48 VES min^{-1} after 8 min. Ventricular fibrillation or tachycardia were seen only during the first 15 min. These observations are also in agreement with those of other investigators using the same model (Clark *et al.*, 1980; Marshall *et al.*, 1981; Au *et al.*, 1983; Campbell & Parratt, 1983). Our finding of a total of 614 ± 166 VES during the first 30 min of ischaemia is lower than that reported by some other groups of workers. This may be due to differences in the strain of rats used (McCarty *et al.*, 1979), differences in the amount of stress to which the animals were exposed (the animals used in this study were bred in our Institute, and therefore not exposed to transport or cold stress), or periodic changes in the sensitivity of rats to arrhythmogenic factors (Abrahamsson & Almgren, 1981).

Catecholamines

An increased release of catecholamines in the ischaemic myocardium has been found in a number of species, such as the dog (Mathes & Gudbjarnason, 1971), pig (Hirche *et al.*, 1985; Holmgren *et al.*, 1985), and the rat (Abrahamsson *et al.*, 1982). However, the extent and time course of NA release and its underlying mechanisms are not yet completely clear.

We investigated myocardial catecholamines using two different methods. The morphometric assessment of fluorescing adrenergic nerve terminals in relation to

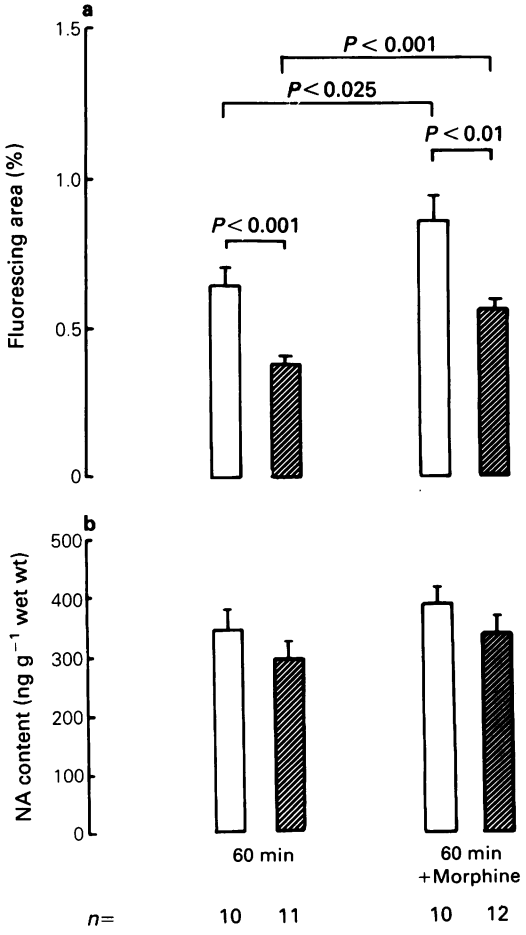

Figure 3 Fluorescing nerve fibres (as percentage of total field area) (a) and noradrenaline (NA) content (ng^{-1} g wet wt) (b) in samples of left ventricular myocardium 60 min after sham-operation (open columns) or coronary artery occlusion (hatched columns) in untreated and morphine-treated rats. Columns show mean values with vertical lines indicating s.e.mean.

total field area reflects the intra-neuronal NA content within certain limits. Jonsson (1971) showed in the rat iris that neurones which still contain 5–10% of their normal NA content no longer show a visible fluorescence, and that an increase above 40% of the maximum NA content is not accompanied by a further increase in fluorescence. This may explain why the perivascular sympathetic nerve plexuses preserve a visible fluorescence longer than terminal fibres in the ischaemic myocardium (Paessens & Borchard, 1980). For morphometric assessment we therefore excluded areas which contained such sympathetic plexuses. The determination of total tissue NA content measures both intra- and extra- neuronal NA, and this may explain the relatively poor correlation we found between values of fluorescing area and NA content ($r = 0.664$, $n = 28$, $P < 0.001$).

In agreement with Abrahamsson *et al.*, (1982) and Holmgren *et al.*, (1981), we found a significant reduction in fluorescing nerve fibres in the ischaemic myocardium after 60 min of occlusion compared to sham-operated animals. NA tissue content at this time was reduced by only 14%. The difference between the marked reduction in intra-neuronal CA and the only slight decrease in total NA content implies high extraneuronal CA concentrations. This may be a consequence of a decreased inactivation of released NA in the ischaemic myocardium. Reuptake, the most important mechanism of inactivation under normal conditions, is reduced by ischaemia in the rat heart (Schömig *et al.*, 1982). Washout of released NA, and the activity of the catabolic enzymes monoamine oxidase (MAO) and catechol-*O*-methyltransferase (COMT) would also be expected to be reduced under conditions of reduced blood flow and hypoxia.

Morphine effects

In clinical studies, reductions in blood pressure and vascular resistance after morphine administration have been described (Sethna *et al.*, 1982) but also hypertonic (Conahan *et al.*, 1973; Kistner *et al.*, 1979) or unchanged (Lee *et al.*, 1976) haemodynamics. Animal experiments have also shown differing haemodynamic effects, depending on route of administration, dose, and the additional effects of anaesthetics (Fennessy & Rattray, 1971; Holaday, 1983). In our experiments, morphine administration led to significant increases in MAP. This is presumably a result of the higher plasma NA concentrations found 30 min after morphine administration, when the haemodynamic effects were maximal. In preliminary studies, we found that a lower dose of morphine ($5\,mg\,kg^{-1}$, i.p.) had no marked effects on either plasma CA or haemodynamics. Fennessy & Rattray (1971) showed that the increase in MAP seen in the rat after morphine could not be prevented by adrenalectomy or hexamethonium, but was blocked by the α-adrenoceptor antagonist, phentolamine. They concluded therefore that, in the rat, morphine causes NA release from peripheral sympathetic nerves. Increased NA concentrations in plasma from rats treated with morphine have also been described by other investigators (Van Loon *et al.*, 1981; Conway *et al.*, 1983).

In the myocardium of morphine-treated rats 60 min after sham-operation or occlusion, a slightly higher NA content and a significantly higher area of fluorescing neurones was found than in untreated rats. This increase in fluorescing area suggests a higher intraneuronal NA content, and may be due to a number of possible mechanisms: (1) A morphine-induced 'central sympatholysis' (Laubie *et al.*, 1977) could reduce sympathetic outflow to the heart and so diminish NA release. This effect, however, does not seem to be present in all sympathetically-innervated organs, as the increased plasma NA concentration shows. (2) The higher intraneuronal NA content may be a consequence of an increased uptake of NA resulting from the higher plasma NA concentration. The heart has been shown to be capable of taking up about 80% of circulating CA during a single passage (Wurtmann *et al.*, 1963). (3) The increase in plasma NA and decrease in plasma Ad concentrations may reduce NA release via presynaptic α_2- or β-adrenoceptors. (4) Morphine may reduce NA release by presynaptic opiate receptor sites. The results reported for myocardial preparations are, however, controversial. In the rabbit heart, Starke (1977) found an increased NA release after morphine, whereas in guinea-pig isolated atria, a dose-dependent reduction in [^{3}H]-NA release has been shown for etorphine (Fuder, 1985). (5) Morphine may lead to an increased synthesis of CA. Increased CA concentrations as well as increased activity of tyrosine hydroxylase and dopamine-β-hydroxylase have been found in brain, adrenal medulla and plasma after morphine treatment (Anderson & Slotkin, 1976; Prasad *et al.*, 1976).

Which of these possible mechanisms plays the most important role in the increase in myocardial intraneuronal NA after morphine treatment found in the present study cannot, however, be determined from the available data.

The reduction in intraneuronal fluorescence in the ischaemic myocardium was not affected by morphine. This local release seems to be independent of efferent sympathetic impulses (Dart *et al.*, 1984). It appears to be mediated by a calcium-independent carrier, possibly identical to the uptake carrier, and ischaemia-induced increases in membrane permeability may also be involved (Schömig *et al.*, 1984). It has previously been shown that morphine can reduce nicotinic-induced, but not KCl-induced NA release from chromaffin cells (Kumakura *et al.*, 1980). In contrast to meptazinol, a partial agonist at opiate receptors

which has been shown to have antiarrhythmic properties (Fagbemi *et al.*, 1983), in our experiments morphine also had no effect on arrhythmias.

In conclusion, morphine administration increased plasma NA concentration and the NA content of adrenergic nerves in the rat heart. It is not possible on the basis of the present results to determine whether this was primarily a consequence of increased uptake of NA as a result of the increased plasma concentration, or whether morphine directly inhibited NA release, possibly via presynaptic opiate receptors. The local release of NA in the ischaemic myocardium was unaffected by morphine, as were the early ischaemia-induced arrhythmias, which may suggest a causal role for locally-released catecholamines in the genesis of these arrhythmias in the rat.

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(5Z)-carbacyclin discriminates between prostacyclin-receptors coupled to adenylate cyclase in vascular smooth muscle and platelets

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1 (5E)- and (5Z)-carbacyclin are prostacyclin (PGI_2) analogues endowed with antiaggregating and vasodilator properties, which stimulate adenylate cyclase activity in membranes from human platelets and cultured myocytes from rabbit mesenteric artery.

2 In platelets they display the same efficacy as prostaglandin E_1 (PGE_1), and hence PGI_2, both as activators of adenylate cyclase and as inhibitors of aggregation.

3 In contrast, in vascular smooth muscle cells (5Z)-carbacyclin fails to produce the same degree of stimulation of the enzyme as PGI_2, (5E)-carbacyclin and PGE_1, nor does it induce the maximal relaxation of the mesenteric artery as do the other prostaglandins.

4 (5Z)-carbacyclin is also able to antagonize the activation of adenylate cyclase and the relaxation elicited by PGE_1 or PGI_2 in the mesenteric artery, and therefore it displays partial agonist properties in these cells.

5 We conclude that the receptors for PGI_2 coupled to adenylate cyclase in platelets and vascular smooth muscle cells are different from each other, because (5Z)-carbacyclin can discriminate between them, being a partial agonist at myocyte but not at platelet level.

Introduction

Prostacyclin (PGI_2) is the most potent inhibitor of platelet aggregation (Moncada *et al.*, 1976) and, because of this property, it has many potential clinical applications. However, prostacyclin has some drawbacks which have so far limited a more widespread clinical use. In fact, not only is it chemically and metabolically unstable (Moncada *et al.*, 1976; Johnson *et al.*, 1976), but it also displays potent vasodepressor actions (Moncada *et al.*, 1976; 1978), when often only the anti-platelet effect is desired.

For these reasons, a great effort has been made toward the development of PGI_2 analogues more suitable for clinical use (Whittle & Moncada, 1984). The design of a PGI_2 analogue endowed with a high degree of selectivity for the platelet receptor will be successful only if this receptor differs from that on vasculature.

Because both inhibition of platelet aggregation and vasodilatation are supposed to be mediated by an increase in intracellular adenosine 3′:5′-cyclic monophosphate (cyclic AMP) levels (Gorman *et al.*, 1977; Tateson *et al.*, 1977; Miller *et al.*, 1979; Kukovetz *et al.*, 1979; Lombroso *et al.*, 1984; Oliva *et al.*, 1984a,b), through activation of adenylate cyclase (AC), we have addressed the problem of characterization of the platelet and vascular receptors for PGI_2 by investigating the activation of this enzyme in membranes from human platelets and from rabbit vascular myocytes. The evaluation in these systems of some prostacyclin analogues (PGE_1, 6ß-PGI_1, 6-keto-PGE_1), so far has not revealed any major difference between the platelet and the vascular receptors for PGI_2 coupled to adenylate cyclase (Lombroso *et al.*, 1984; Oliva *et al.*, 1984 a,b).

We describe here further studies performed with two epimers of carbacyclin (Morton *et al.*, 1979) (Figure 1) which share with PGI_2 the antiaggregating and vasodilator effects (Whittle *et al.*, 1980), and demonstrate that the 5Z epimer is able to discriminate between the platelet and vascular receptor.

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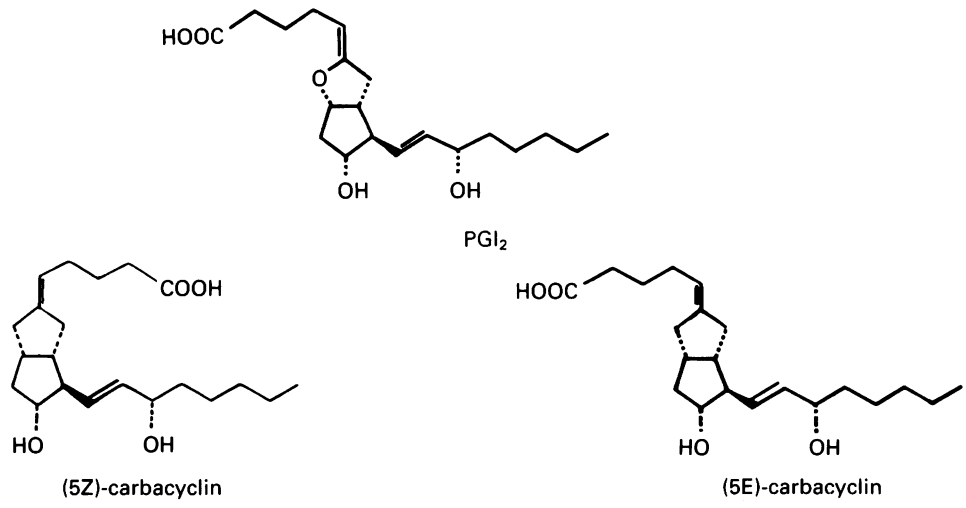

Figure 1 Structures of prostacyclin (PGI_2) and its analogues, the carbacyclins.

Methods

Materials

[8-^{14}C]-adenosine triphosphate ([8-^{14}C]-ATP) and [8-^{3}H]-cyclic AMP were from New England Nuclear, Boston, MA, U.S.A; ATP, cyclic AMP, guanosine triphosphate (GTP), creatine phosphate, creatine phosphokinase and sodium arachidonate were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Prostacyclin (PGI_2), PGE_1, (5E)-carbacyclin and (5Z)-carbacyclin were synthesized by the Upjohn Co., Kalamazoo, MI, U.S.A., and supplied by The Wellcome Research Laboratories, Beckenham, U.K. The solutions of PGI_2, which was stored in ethanol at $-20°C$, were freshly prepared immediately before use in 10 mM Tris-HCl buffer, pH 8. The other prostaglandins were dissolved in the same Tris buffer. In the experiments with myocyte membranes, where the high concentrations of (5Z)-carbacyclin gave solubility problems, it was more convenient to dissolve and dilute this prostaglandin, and therefore also the others, with 40% ethanol in Tris buffer (to yield a final ethanol concentration of 4% in the sample). The inclusion of ethanol in the adenylate cyclase assay did not modify the pattern of response to the prostaglandins. Eagle's minimum essential medium F11, foetal calf serum, trypsin-EDTA, penicillin (10,000 u ml^{-1}), streptomycin (10 mg ml^{-1}), tricine buffer (1 M) and non-essential amino acids (100 ×) were purchased from Grand Island Biological Co., Madison, WI, U.S.A.; disposable culture flasks and petri dishes were from Corning Glassworks, Amedfield, MA, U.S.A.

Cell cultures

Male white New Zealand rabbits (2–3 kg) were used. Cultures of smooth muscle cells from intima-medial layer of rabbit aorta and mesenteric arteries were prepared according to the method of Ross (1971), as previously described by Oliva *et al.* (1984b).

Preparation of membranes

Platelet concentrates (collected in citric acid/sodium citrate/sodium phosphate/dextrose) from 3–4 healthy male volunteers were pooled. A crude membrane preparation (pellet at 27,000 *g*) was prepared as described by Lombroso *et al.* (1984).

Smooth muscle cell monolayers from rabbit mesenteric artery (used between the 8th and 14th passage) were washed in 50 mM Tris-HCl buffer (pH 7.4), harvested by scraping, pooled and the membrane preparation (pellet at 15,000 *g*) was obtained as described by Oliva *et al.* (1984b).

Platelet aggregation studies

Blood was collected in 3.8% sodium citrate (9:1). Platelet-rich plasma (PRP) and platelet-poor plasma (PPP) were prepared as previously described (Tremoli *et al.*, 1979). Platelet count was adjusted to 300–400,000 μl^{-1} by adding PPP. Prostaglandin inhibition of platelet aggregation was measured in PRP samples stimulated by collagen 5 ng μl^{-1} using an ELVI Logos aggregometer by the turbidimetric technique of Born (1962).

Rabbit mesenteric artery studies

Spiral strips of rabbit mesenteric artery were prepared and set up using the laminar-flow technique of Ferreira & Costa (1976). The spirals were superfused with Krebs solution of the following composition ($g l^{-1}$): NaCl 6.9, KCl 0.35, KH_2PO_4 0.16, $MgSO_4.7H_2O$ 0.29, $CaCl_2$ 0.28, glucose 1 and $NaHCO_3$ 2.1. Flow rate was 0.2 ml min^{-1}, the buffer was kept at 37°C and gassed with 95% O_2 and 5% CO_2. The resting tone of the preparations was 1 g and contractions were recorded with isotonic transducers Mod. 7006 (Ugo Basile, Comerio, Italy) connected to a 2 channel Gemini recorder, Mod. 7070 (Ugo Basile). The tissues were equilibrated for 2 h and subsequently challenged with bolus injections of different agonists; the volume of the injected bolus was 0.1 ml.

Adenylate cyclase assay

The standard assay mixture (final volume: 100 µl) contained: 10 mM Tris-HCl buffer (pH 8); 0.10 mM [8-^{14}C]-ATP (50 dpm $pmol^{-1}$); 0.5 mM [8-^{3}H]-cyclic AMP (approximately 360 dpm $nmol^{-1}$); 2 mM $MgCl_2$; 2 mM creatine phosphate; 17 u ml^{-1} creatine phosphokinase; 10 µM GTP and the indicated prostaglandins. The incubation, started with the addition of the membrane preparation (0.04–0.09 and 0.06–0.10 mg protein per sample for platelet and mesenteric membranes respectively), was carried out at 30°C for 8 min. [8-^{3}H]-cyclic AMP was included in the assay mixture to permit correction for column loss and for the possible effect of phosphodiesterases (Katz *et al.*, 1978) which in any case was almost negligible.

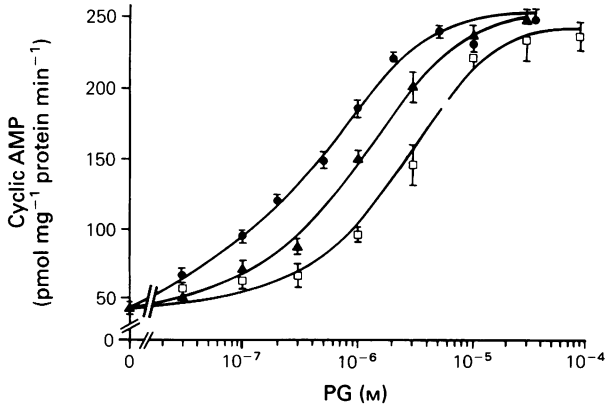

Figure 2 Dose-response curves for the activation of adenylate cyclase by different prostaglandins in membranes of human platelets: (●) (5E)-carbacyclin; (▲) prostaglandin E_1; (□) (5Z)-carbacyclin.

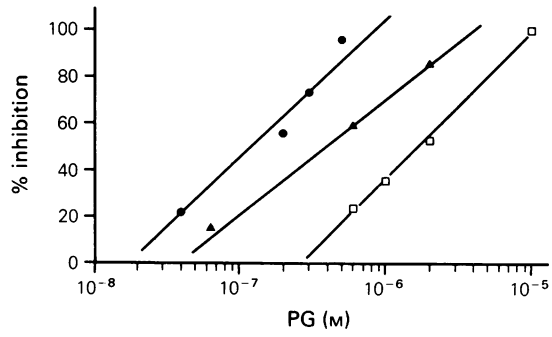

Figure 3 Dose-response curves for the inhibition of human platelet aggregation induced by collagen (5 ng μl^{-1}): (●) (5E)-carbacyclin; (▲) prostaglandin E_1; (□) (5Z)-carbacyclin.

[8-^{14}C, 8-^{3}H]-cyclic AMP was isolated and detected according to Salomon *et al.* (1974). Protein concentrations were determined according to Bradford (1976).

Expression of results

The results are expressed as mean ± s.d. of triplicate determinations in one experiment, performed at least three times with equivalent results.

Results

Human platelets

The effect of increasing concentrations of (5E)-carbacyclin and (5Z)-carbacyclin on AC activity was investigated in human platelet membranes and compared with that of PGE_1. Figure 2 shows that all the prostaglandins were able to stimulate AC in a dose-dependent fashion with approximately parallel curves, and that they were equieffective (maximal stimulation: 5 fold). The concentrations eliciting half-maximal stimulation (EC_{50}) were 0.307 ± 0.162 µM, 0.633 ± 0.351 µM and 2.83 ± 0.29 µM for (5E)-carbacyclin, PGE_1 and (5Z)-carbacyclin, respectively.

(5Z)-carbacyclin was tested for its ability to inhibit collagen-induced platelet aggregation (Figure 3) and was found to induce the same maximal response as PGE_1 and (5E)-carbacyclin (100% inhibition). The concentrations eliciting half-maximal inhibition (IC_{50}) were 105 ± 32 nM, 145 ± 153 nM, and 1.96 ± 0.99 µM, for (5E)-carbacyclin, PGE_1 and (5Z)-carbacyclin, respectively, in agreement with the results of Whittle *et al.* (1980).

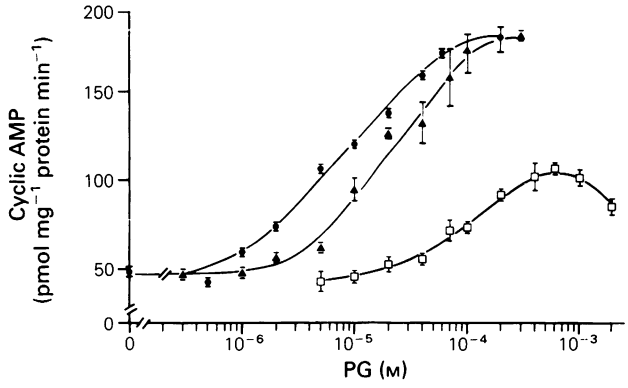

Figure 4 Dose-response curves for the activation of adenylate cyclase by different prostaglandins in membranes of myoctyes from rabbit mesenteric artery: (●) (5E)-carbacyclin; (▲) prostaglandin E_1; (□) (5Z)-carbacyclin.

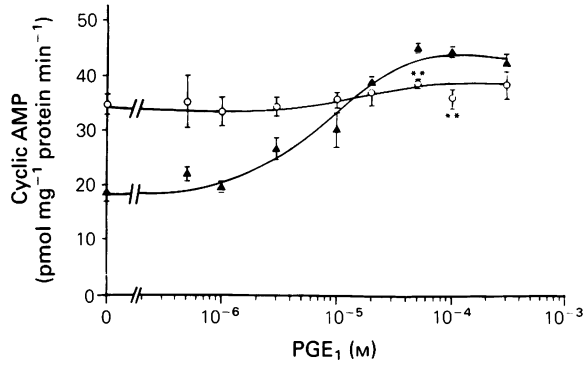

Figure 6 Dose-dependent effect of prostaglandin E_1 (PGE_1) on adenylate cyclase activity of myoctyes in the absence (▲) and the presence (O) of (5Z)-carbacyclin, 0.2 mM. $^{**}P < 0.001$ when compared with corresponding concentrations of PGE_1 alone.

Rabbit mesenteric arterial myocytes

In membranes of cultured smooth muscle cells from rabbit mesenteric artery (5E)-carbacyclin, PGE_1 and (5Z)-carbacyclin stimulated AC in a dose-dependent fashion (Figure 4). However, at variance with the results obtained in human platelet membranes, the maximal stimulation attained with the two carbacyclins was different. In fact, (5E)-carbacyclin activated AC to the same extent (3.5 fold) and approximately with the same potency ($EC_{50} = 5.93 \pm 2.10\,\mu M$) as PGE_1 ($EC_{50} = 14.9 \pm 8.6\,\mu M$).

In contrast, (5Z)-carbacyclin failed to reach the same response elicited by the other two prostaglandins, in that the maximal stimulation (attained at 0.4 mM) was 2 fold. (5Z)-carbacyclin had an EC_{50} of 0.104 ± 0.021 mM.

The lower efficacy of (5Z)-carbacyclin suggested that it might act as a partial agonist. In order to evaluate this hypothesis, the effect of increasing concentrations of (5Z)-carbacyclin on PGE_1-stimulated AC activity was investigated. As shown in Figure 5, (5Z)-carbacyclin displayed antagonistic properties in this system, since it was able to reduce the

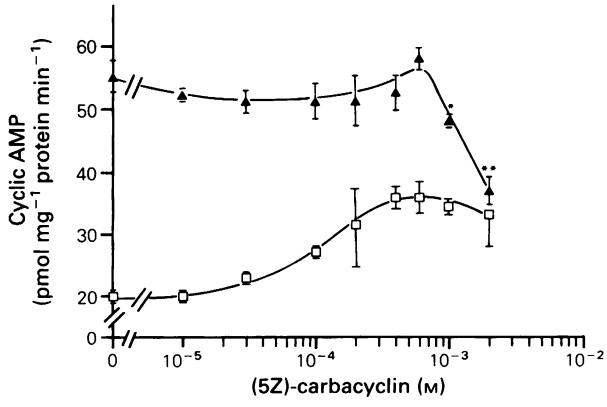

Figure 5 Dose-dependent effect of (5Z)-carbacyclin on adenylate cyclase activity of myocytes in the absence (□) and presence (▲) of prostaglandin E_1 0.3 mM. $^{*}P < 0.01$, $^{**}P < 0.001$ when compared with stimulation by prostaglandin E_1 alone.

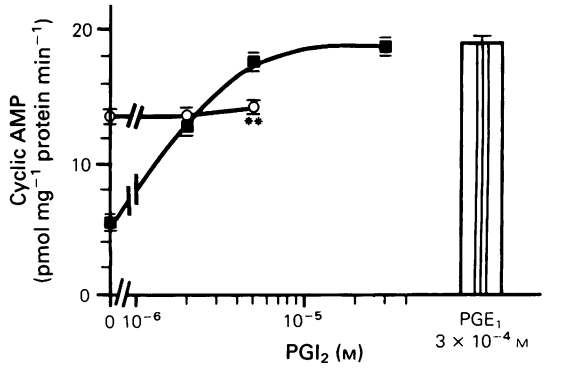

Figure 7 Dose-dependent effect of prostacyclin (PGI_2) on adenylate cyclase activity of myocytes in the absence (■) and presence (O) of (5Z)-carbacyclin 1 mM. $^{**}P < 0.001$ when compared with corresponding concentration of PGI_2 alone. The column represents the stimulation by a maximally effective concentration of prostaglandin E_1 (PGE_1).

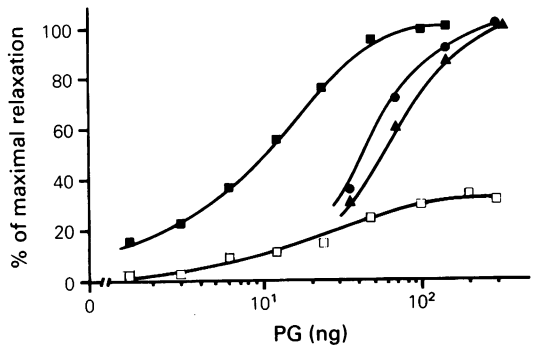

Figure 8 Dose-response curves for the relaxation of rabbit mesenteric artery: 100% relaxation was taken as the maximal effect attainable with prostacyclin (PGI_2); (●) (5E)-carbacyclin; (□) (5Z)-carbacyclin; (▲) prostaglandin E_1; (■) PGI_2.

stimulation of AC induced by 0.3 mM PGE_1. (5Z)-carbacyclin also antagonized the activation of AC elicited by 40 μM (5E)-carbacyclin (data not shown).

The agonist/antagonist properties of (5Z)-carbacyclin are further demonstrated by the experiment of Figure 6, where a dose-response curve for PGE_1 in the presence and absence of a fixed concentration of (5Z)-carbacyclin is shown. As expected from a partial agonist (Ariens *et al.*, 1964), this carbacyclin stimulates AC at low concentrations of the full agonist PGE_1, while it antagonizes the effects of higher concentrations of the latter.

The particular cell line used in the experiment shown in Figure 6 displayed a lower degree of stimulation than those used in other experiments (see, e.g. Figure 4), and therefore the difference between PGI_2 and (5Z)-carbacyclin efficacies appears smaller than in other experiments. However, as already discussed (Oliva *et al.*, 1984b), the pattern of AC stimulation was comparable to that of the other membrane preparations.

The experiment depicted in Figure 7 demonstrates that the partial agonist properties of (5Z)-carbacyclin are displayed not only versus PGE_1, but also versus PGI_2, as one would expect if the two latter prostaglandins act through the same receptor. In fact, a maximally activating concentration of (5Z)-carbacyclin (1 mM) elicited a lower enzyme stimulation than PGI_2 (which was equieffective with PGE_1). Furthermore, this same concentration of (5Z)-carbacyclin was not additive with an equieffective concentration of PGI_2 (2 μM), while it was able to inhibit the enzyme activation elicited by higher concentrations of PGI_2 (5 μM). These results are in line with the theory of partial agonist effects (Ariens *et al.*, 1964).

The effect of increasing amounts of the various prostaglandins in relaxing rabbit mesenteric artery was also investigated. Figure 8 shows that (5Z)-carbacyclin failed to reach the maximal relaxation of rabbit mesenteric artery attained with PGI_2, (5E)-carbacyclin and PGE_1. In fact, at 280 ng, (5Z)-carbacyclin elicited less than 40% of the maximal response.

Again, this result suggested that (5Z)-carbacyclin might be a partial agonist. This is confirmed by the data in Figure 9, which show that this carbacyclin antagonized the effects of PGI_2, decreasing the relaxation elicited by the latter, when they were coadministered. That the lower response was not due to a decreased sensitivity of the mesenteric artery is demonstrated by the unaltered response to the subsequent administration of PGI_2.

Discussion

(5E)- and (5Z)-carbacyclin are two chemically stable analogues of prostacyclin, the former being isosteric with the natural prostaglandin. Both carbacyclins

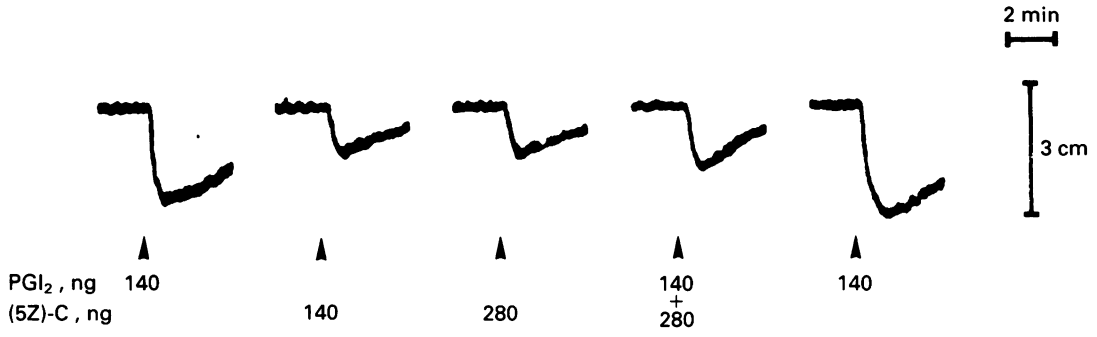

Figure 9 Relaxation of rabbit mesenteric artery by prostacyclin (PGI_2) and (5Z)-carbacyclin ((5Z)-C) alone, and their combined effect.

mimic the effect of PGI_2 and PGE_1, in that they are inhibitors of platelet aggregation *in vitro* and *ex vivo* and they lower arterial blood pressure (Whittle *et al.*, 1980). Since inhibition of platelet aggregation and vasodilatation are supposed to be mediated by increases in intracellular cyclic AMP levels, the study of adenylate cyclase stimulation by these prostaglandins might shed some light on the nature of the receptor involved in these phenomena. We have used PGE_1 as a reference compound in these studies, since we have demonstrated that it displays the same efficacy as prostacyclin, and, by means of binding (Lombroso *et al.*, 1984) or additivity studies (Nicosia *et al.*, 1987), that it acts through identical receptors.

In human platelet membranes, (5E)- and (5Z)-carbacyclins activate adenylate cyclase to the same extent as PGE_1, and therefore as PGI_2. The effect of (5E)-carbacyclin is in agreement with the data of Stein & Martin (1984), and was expected since this prostaglandin raises cyclic AMP levels (Ceserani *et al.*, 1980), and its anti-aggregating effect is potentiated by the phosphodiesterase inhibitor, theophylline (Whittle *et al.*, 1980; Morita *et al.*, 1980).

The stimulation of adenylate cyclase activity correlates well with the functional response of platelets, in that the rank order of potency for enzyme activation is paralleled by that found for inhibition of platelet aggregation (Whittle *et al.*, 1980). Furthermore, PGE_1 and the carbacyclins display parallel dose-response curves and attain the same maximal effect both in adenylate cyclase activation and in platelet aggregation studies.

The pattern is completely different in myocytes cultured from rabbit mesenteric artery. In this system, while (5E)-carbacyclin activates adenylate cyclase to the same extent as PGE_1, the (5Z) epimer has a markedly lower efficacy, the maximal stimulation of the enzyme being only 40–45% of that obtained with the other prostaglandins. It is noteworthy that both in smooth muscle and platelets the enzyme stimulation is in very good aggreement with the biological response; in fact, we demonstrate that (5Z)-carbacyclin is less efficient (40% of maximal) than the other prostaglandins in the relaxation of rabbit mesenteric artery *in vitro*.

The fact that (5Z)-carbacyclin is unable to produce the maximal effect elicited by PGI_2 or PGE_1 could be explained on the basis of two different hypotheses: either (5Z)-carbacyclin interacts with an independent receptor, different from the one shared by prostacyclin and other analogues, and possesses a lower intrinsic activity, or this carbacyclin behaves as a partial agonist at the prostacyclin receptor. That the latter is the more likely hypothesis is demonstrated by the experiments illustrated in Figures 5 to 7, which show that (5Z)-carbacyclin is able to decrease the stimulation of adenylate cyclase elicited by either PGE_1 or PGI_2. This carbacyclin seems therefore to be endowed with antagonistic properties at the PGE_1/PGI_2 receptor, as one would expect from a partial agonist (Ariens *et al.*, 1964).

While it is true that such antagonist properties appear only at relatively high concentrations, the relevance of our finding is supported by similar results which have been obtained recently with another PGI_2 analogue having the same configuration as (5Z)-carbacyclin at carbon 5: in fact, FCE 22176, i.e. (5Z)-13, 14-didehydro-20-methyl-carboprostacyclin, has been shown to be a competitive antagonist of PGI_2 on guinea-pig trachea and atrium (Fassina *et al.*, 1985). These results, taken together with our present findings, suggest that position 5 is a key one in determining the mode of interaction of PGI_2 with its receptors.

Whittle *et al.* (1980, 1984) had investigated the selectivity of a number of PGI_2 analogues, including (5Z)-carbacyclin, by means of the 'selectivity ratio', that is by calculating the ratio of the relative potency of the analogue to prostacyclin as a vasodepressor *in vivo* and a platelet inhibitor *in vitro*. The differences in this index obtained with a series of compounds seemed to indicate that some intrinsic differences exist between the platelet and vascular receptor for PGI_2. However, as the authors themselves pointed out (Whittle *et al.*, 1984), the 'selectivity ratio' must be interpreted with caution, mainly because it is inferred from the comparison of *in vivo* and *in vitro* data, which might be affected by differences in metabolism or pharmacodynamics among the various analogues.

Therefore, our results give evidence, obtained directly at the receptor-binding site level, of the possibility of discriminating between the platelet and vascular PGI_2 receptors, and suggest that the moiety comprising carbon 5 might play an important role in the design of an analogue with a high degree of selectivity between the cardiovascular and anti-platelet effects.

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1,4-Dithiothreitol-induced changes in histamine H_1-agonist efficacy and affinity in the longitudinal smooth muscle of guinea-pig ileum

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1 The effect of 1,4-dithiothreitol (DTT) on histamine H_1-receptor agonist affinity and efficacy has been investigated in longitudinal muscle strips of guinea-pig ileum.

2 Exposure of ileal smooth muscle to DTT significantly increased the maximal responses to the partial agonists SKF 71473 and DE-2PEA, indicative of an increase in agonist efficacy. This effect was paralleled by a small decrease in EC_{50} values. In contrast, DTT produced a parallel displacement of the concentration-response curve to the full agonist histamine in the same muscle strips.

3 Studies in which phenoxybenzamine and benzilylcholine mustard were used to reduce the maximum response to histamine suggested that DTT altered both agonist affinity and efficacy. The affinity constant for histamine, calculated by the method of Furchgott & Bursztyn (1967), increased by 2.7 fold in the presence of DTT. Furthermore, agonist efficacy also appeared to increase in the presence of DTT since the maximum response to histamine following phenoxybenzamine treatment increased on application of DTT.

4 [^{3}H]-mepyramine binding studies confirmed that DTT increased agonist affinity. DTT produced a significant parallel shift to the left of the displacement curves for histamine, 2-methylhistamine, 2-pyridylethylamine and 2-thiazolylethylamine.

5 The results of this study therefore suggest that DTT potentiates H_1-receptor-mediated contractile activity in guinea-pig ileum by increasing both agonist efficacy and affinity.

Introduction

The disulphide bond reducing agent 1,4-dithiothreitol (DTT) has been shown to potentiate histamine H_1-receptor-mediated contractile activity in both visceral (Donaldson & Hill, 1985a; 1986a) and vascular smooth muscle (Fleisch *et al.*, 1973; 1974; Carroll & Glover, 1977). This effect appears to be specific to the H_1-receptor since a similar potentiation of agonist activity is not observed with other smooth muscle spasmogens such as acetylcholine, 5-hydroxytryptamine and KCl (Fleisch *et al.*, 1973; Donaldson & Hill, 1985a; 1986a). The selective enhancement of H_1-receptor activity in rabbit colon and guinea-pig ileum does not, however, appear to be associated with an interference with histamine metabolism (Fleisch *et al.*, 1973; Donaldson & Hill, 1986a). These data suggest that the site of action of DTT is at the level of the H_1-receptor or at some point along the receptor-effector pathway proximal to the stage at which it is shared by responses elicited by other smooth muscle spasmogens.

In guinea-pig brain slices, DTT has been shown to potentiate both the H_1-receptor mediated accumulation of inositol phosphates and the H_1-mediated augmentation of adenosine-stimulated cyclic AMP accumulation (Donaldson & Hill, 1986b). Studies with partial agonists in slices of guinea-pig cerebellum and cerebral cortex suggest that this potentiating effect of DTT is associated, in part, with an increase in H_1-agonist efficacy (Donaldson & Hill, 1986b). Furthermore, ligand binding studies with [^{3}H]-mepyramine in homogenates of guinea-pig cerebral cortex and cerebellum indicate that DTT may also modify the binding affinity of agonists for the H_1-receptor in brain (Donaldson & Hill, 1986c).

The present series of experiments was undertaken to determine whether the potentiation of H_1-receptor mediated contractile activity in the longitudinal smooth muscle of guinea-pig ileum is related to

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changes in agonist affinity, efficacy or a combination of these two agonist determinants.

Methods

Organ bath studies

Hartley strain guinea-pigs of either sex (200–400 g) were killed by cervical dislocation and decapitation. Longitudinal smooth muscle strips of guinea-pig ileum were prepared essentially as described by Rang (1964). Muscle strips were suspended in 10 ml Krebs-Henseleit solution (mM): NaCl 118, KCl 4.7, $MgSO_4$ 1.2, $CaCl_2$ 2.5, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 5.5; pH 7.4 gassed with O_2/CO_2 (95:5) at 37°C in a conventional organ bath. The muscle strips were equilibrated for 60 min before being exposed to agonists, during which time the bathing fluid was changed at 15 min intervals to prevent the accumulation of metabolic products. Contractions to agonists were recorded isotonically. Agonists were in contact with the tissue for 15–25 s and applied at 2 min intervals. Concentration-response curves for histamine and a partial agonist were obtained essentially simultaneously by alternate dosing of the tissue with the two agonists. DTT (1 mM) was added to the reservoir solution and allowed to equilibrate with the tissue for 30 min before redetermination of agonist dose-response curves in the continued presence of DTT.

Experiments with irreversible antagonists

Benzilylcholine mustard was allowed to cyclise as a 0.1 mM solution in 10 mM Na-K phosphate buffer (7.6 mM Na_2HPO_4, 2.4 mM KH_2PO_4) pH 7.4, at room temperature for 60 min. The yield of the aziridinium ion under these conditions was expected to be greater than 90% (Burgen *et al.*, 1974). Phenoxybenzamine was cyclised in 0.9% saline for 60 min at room temperature at a concentration of 0.1 mM. Longitudinal smooth muscle strips of guinea-pig ileum were incubated in phenoxybenzamine (5 μM) for 30 min or benzilylcholine mustard (5 μM) for 40 min. Following treatment with the irreversible antagonist, muscle strips were washed with Krebs solution containing 1 mM sodium thiosulphate at 15 min intervals for 60 min before subsequent dose-response curves were determined. This agent reacts with any free aziridinium ions to form a biologically inert Bunte salt (Graham & Lewis, 1954).

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

Longitudinal smooth muscle strips from the ileum of two guinea-pigs were prepared in Krebs-Henseleit medium gassed with O_2/CO_2 (95:5), pH 7.4. Muscle strips were initially cut into small pieces before homogenization in 5 volumes of 50 mM Na-K phosphate buffer (37.8 mM Na_2HPO_4, 12.2 mM KH_2PO_4), pH 7.4, with a Polytron blender (setting 5, 3 × 20 s periods at 2 min intervals). The homogenate was then centrifuged at 18,000 *g* for 10 min at 4°C. The pellet was washed by resuspension in phosphate buffer and recentrifuged at 18,000 *g* for 10 min. The resulting pellet was finally resuspended in 5 ml phosphate buffer and kept on ice prior to measurement of [^{3}H]-mepyramine binding. The protein concentration of the final suspension was measured by the method of Lowry *et al.* (1951).

Aliquots of the freshly prepared membranes (40 μl, 0.3 to 0.5 mg protein) were added to 0.95 ml of 50 mM phosphate buffer, pH 7.4, and incubated at 37°C for 20 min in the presence or absence of DTT (1 mM). [^{3}H]-mepyramine and, where appropriate, competing ligand were then added in 10 μl of phosphate buffer and the incubation continued for a further 35 min. Incubations were terminated by the addition of 4 ml of ice-cold phosphate buffer containing 1 μM non-radioactive mepyramine (Daum *et al.*, 1982) and filtered immediately through Whatman GF/B glass fibre filters. The filters were washed twice with 4 ml of ice-cold buffer containing 1 μM mepyramine and tritium was determined by liquid scintillation counting. The level of non-specific binding was defined as that insensitive to inhibition by 2 μM promethazine.

Data analysis

Concentration-response curves from individual experiments, obtained in the presence and absence of DTT were either drawn by inspection or fitted to a Hill equation using the programme ALLFIT (DeLean *et al.*, 1978) as described previously (Donaldson & Hill, 1985b). The equation fitted was:

$$\% \text{ of maximum response} = \frac{E_{max} \times D^n}{D^n + (EC_{50})^n}$$

where D is the agonist concentration, n is the Hill coefficient, EC_{50} is the concentration of agonist giving half maximal stimulation and E_{max} is the maximal stimulation. Differences in the maximal stimulation (E_{max}) obtained with partial agonists, in the presence and absence of DTT, were analysed by means of a paired *t* test and the Wilcoxon signed rank test. Changes in EC_{50} value were tested for significance as described previously (Donaldson & Hill, 1986a). Affinity constants for histamine were determined in contractile studies according to Furchgott & Bursztyn (1967) following partial alkylation of the H_1-receptor population with phenoxybenzamine. Curves of the specific binding of [^{3}H]-mepyramine at different concentrations of the ^{3}H-ligand were fitted using

ALLFIT. The equation was:

$$\text{Specific binding of } [^3\text{H}]\text{-mepyramine} = \frac{B_{max} \times A^n}{A^n + (1/K_A)^n}$$

where B_{max} is the maximal specific binding site capacity (pmol g^{-1} protein), A is the concentration of [^{3}H]-mepyramine, K_A is the equilibrium affinity constant of [^{3}H]-mepyramine and n is the Hill coefficient. Curves of inhibition of [^{3}H]-mepyramine binding were also fitted to a Hill equation using ALLFIT. The actual equation fitted was:

$$\%\text{ inhibition of specific } [^3\text{H}]\text{-mepyramine binding} = \frac{100 \times I^n}{I^n + (IC_{50})^n}$$

where I is the inhibitor concentration, n is the Hill coefficient and IC_{50} is the concentration of non-radioactive inhibitor producing 50% inhibition of the specific binding of 1 nM [^{3}H]-mepyramine. Each point was weighted according to the reciprocal of the variance associated with it. ALLFIT was used to test for differences in the binding parameters (IC_{50} or n), obtained in the presence and absence of DTT, by inspecting the effect on the residual variance of forcing them to be equal (Delean *et al.*, 1978). Such a constraint (parameter sharing) normally increased the residual variance in addition to the number of degrees of freedom (number of points minus the number of estimated parameters). The appropriateness of a particular constraint was then evaluated by analysis of the residual variances using the following statistical test (Munson & Rodbard, 1980):

$$F = \frac{(R_2 - R_1)/(df_2 - df_1)}{R_1/df_1}$$

where R_1 and R_2 are the residual sums of squares and df_1 and df_2 are the degrees of freedom associated with the original fit and the analysis with shared parameters respectively. F represents the variance ratio with $(df_2 - df_1)$ and df_1 degrees of freedom.

The affinity constants for inhibitors of [^{3}H]-mepyramine binding were calculated from the IC_{50} values using the relationship: $K_A = ([M] \times K_M + 1)/IC_{50}$ where [M] is the concentration of [^{3}H]-mepyramine and K_M is its affinity constant, $1.4 \times 10^9\ M^{-1}$ (see results). In the special case of non-radioactive mepyramine this expression simplifies to $K_A = 1/(IC_{50}\text{-}[M])$, assuming that the substitution of one atom of tritium for hydrogen has no effect on the affinity constant.

Drugs

[^{3}H]-mepyramine (26 Ci $mmol^{-1}$) was purchased from the Radiochemical Centre, Amersham. Histamine dihydrochloride was obtained from BDH and mepyramine maleate and 1,4-dithiothreitol were obtained from Sigma. Gifts of 2-methylhistamine, 2-thiazolylethylamine (2-(2-aminoethyl)-thiazole), 2-pyridylethylamine (2-(2-aminoethyl)-pyridine), phenoxybenzamine, SKF 71473 (2-(2-aminoethyl)-imidazo- [1,2-α] pyridine) (all in hydrochloride form; Smith Kline & French), benzilylcholine mustard (Dr J.M. Young, University of Cambridge) and DE-2PEA (N,N-diethyl-2-(2-pyridyl)-ethylamine; Prof. D.A. Cook, University of Alberta, Canada) are gratefully acknowledged. Nuclear magnetic resonance (n.m.r.) analysis indicated that DE-2PEA was (2-pyridyl) rather than the (1-pyridyl) suggested originally (Kenakin & Cook, 1980).

Results

Contractile studies with partial agonists

DTT (1 mM, 30 min preincubation) produced a large parallel shift of the histamine concentration-response curve to lower agonist concentrations in longitudinal

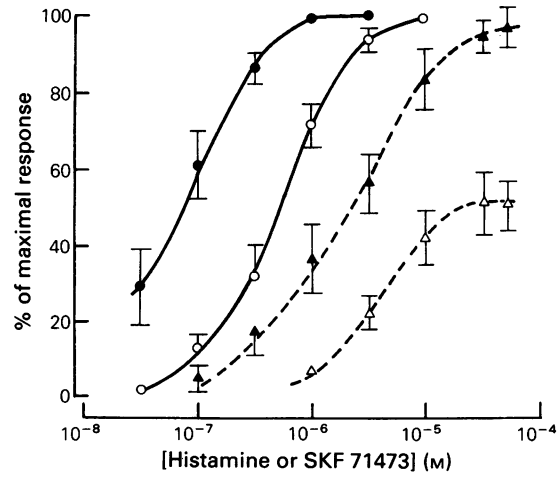
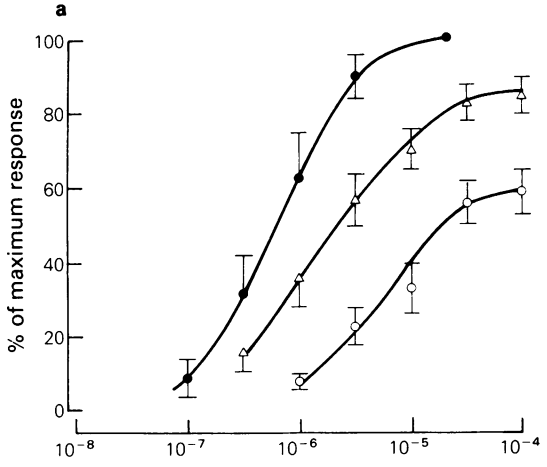


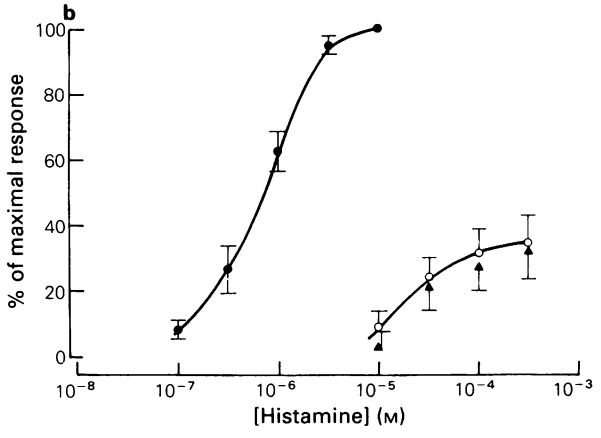
Figure 1 Influence of 1,4-dithiothreitol (DTT) on the contractile activity elicited by SKF 71473 and histamine in guinea-pig ileal smooth muscle. Concentration-response curves for both histamine (○, ●) and SKF 71473 (△, ▲) were obtained in each longitudinal muscle strip in the presence (filled symbols) and absence (open symbols) of 1 mM DTT. Data are expressed as a percentage of the maximal response obtained with histamine in the absence of DTT. Each point represents the mean of six experiments; vertical lines show s.e.means.

Table 1 Effect of 1,4-dithiothreitol (DTT, 1 mM) on the concentration-response parameters for SKF 71473 and N, N-diethyl-2-pyridylethylamine (DE-2PEA)

Agonist	*Maximum response* (%)†		EC_{50} [−DTT]/EC_{50} [+DTT]		
	Control	DTT (1 mM)	Agonist	Histamine	(*n*)
SKF 71473	58.3 ± 7.6	98.2 ± 1.2*	3.7 ± 0.9**	7.6 ± 0.8**	(6)
DE-2PEA	22.7 ± 6.4	71.3 ± 8.0*	5.7 ± 2.1**	10.1 ± 2.8**	(6)

Values represent mean ± s.e.mean. (†)The extent of the maximum response is expressed relative to the maximum response obtained with histamine (in the absence of DTT) in the same strip of ileal smooth muscle. *n* represents the number of paired determinations (agonist and histamine).
*$P < 0.01$ paired *t* test; $P < 0.05$, Wilcoxon signed rank test.
**$P < 0.05$ Wilcoxon signed rank test; EC_{50} in the presence of DTT compared to the EC_{50} of the control curve.





smooth muscle strips of guinea-pig ileum (Figure 1). This confirms our previous findings with DTT in this tissue (Donaldson & Hill, 1986a).

SKF 71473 (Durant *et al.*, 1973) acted as a partial H_1-receptor agonist in ileal smooth muscle producing a maximal response which was 58.3 ± 7.6% ($n = 6$) of the maximal response elicited by histamine in the same smooth muscle strips (Table 1; Figure 1). This analogue was also less potent than histamine in these experiments. The EC_{50} for SKF 71473 was 6.3 ± 1.7 μM while that for histamine was 0.5 ± 0.1 μM ($n = 6$ in each case). Incubation of muscle strips with DTT (1 mM) resulted in a significant increase in the maximal response to SKF 71473 from 58% to a value close to 100% of the maximal response to histamine (Table 1; Figure 1). Thus, DTT-treatment effectively increased the efficacy of SKF 71473 and converted it from a partial agonist into a full agonist. The increase in maximum was accompanied by a significant decrease in the EC_{50} value (Table 1).

N,N-diethyl-2-pyridylethylamine (DE-2PEA, Ken-

Figure 2 Effect of 1,4-dithiothreitol (DTT) on contractile responses to histamine in guinea-pig ileum following partial alkylation of the H_1-receptor population with phenoxybenzamine. (a) Concentration-response curves for histamine were determined in the following order: (●) control; (○) phenoxybenzamine (5 μM, 30 min); (△) DTT (1 mM). (b) Control experiments in which following determination of the concentration-response curve to histamine after phenoxybenzamine treatment (5 μM, 30 min; ○), the tissue was allowed to recover in normal Krebs-medium (i.e. without the addition of DTT) for a further 30 min before determination of a third dose-response curve for histamine (▲). (●) Control dose-response curve obtained prior to phenoxybenzamine treatment. Data are expressed as a percentage of the maximal response to histamine prior to phenoxybenzamine treatment. Each point represents the mean from seven (a) or four (b) experiments; vertical lines show s.e.mean.

Table 2 Effect of 1,4-dithiothreitol DTT (1 mM) on the dose-response parameters of the histamine-induced contractile response, following treatment of ileal muscle strips with phenoxybenzamine or benzilylcholine mustard

Alkylating agent	*Maximum response (%)*†		EC_{50} [−DTT]/EC_{50} [+DTT]	
	−DTT	+DTT		(*n*)
Phenoxybenzamine	59.9 ± 5.9	85.7 ± 4.4*	4.1 ± 0.7	(7)
Benzilylcholine mustard	44.5 ± 5.5	65.8 ± 5.6††	5.7 ± 1.3	(4)

Values represent mean ± s.e.mean. †The extent of the maximal contractile response is expressed relative to the maximum response produced by histamine before exposure to phenoxybenzamine (5 μM, 30 min) or benzilylcholine mustard (5 μM, 40 min). The number of experiments is showed in parenthesis.
*$P < 0.01$ (paired t test) or $P < 0.02$ (Wilcoxon signed rank test); ††$P < 0.01$ (paired t test).

akin & Cook, 1980) was also a partial H_1-receptor agonist in this tissue. In our hands DE-2PEA was much weaker than SKF 71473, producing a maximal response which represented 23% of the maximum response to histamine, and an EC_{50} value of 25 ± 8 μM (Table 1). In the presence of 1 mM DTT, there was a large increase in the size of the maximal response and a small decrease in the EC_{50} value (Table 1) similar to that found with SKF 71473.

Experiments with irreversible H_1-receptor antagonists

The ability of the irreversible H_1-antagonists phenoxybenzamine (Kenakin & Cook, 1976) and benzilylcholine mustard (Reasbeck & Young, 1975) to eliminate the spare receptor reserve and reduce the maximum response to full agonists provided an opportunity to investigate whether DTT potentiates histamine-induced contractile activity by increasing agonist affinity or efficacy.

Exposure of ileal strips to phenoxybenzamine (5 μM, 30 min) resulted in a dextral shift of the histamine dose-response curve to higher agonist concentrations and a reduction in the maximal response (Figure 2). Following incubation with DTT (1 mM) there was a significant increase in the maximal response (Table 2) and a significant decrease ($P < 0.02$) in the EC_{50} value (Table 2; Figure 2a). In four experiments the histamine dose-response curve was redetermined after a 30 min incubation period in the absence of DTT. No change in the maximal response or location parameter was observed under these conditions (Figure 2b). Similar results were obtained when benzilylcholine mustard (5 μM, 40 min) was used as the alkylating agent (Table 2).

The data obtained with phenoxybenzamine were used to calculate an affinity constant for histamine, in the absence of DTT, as described under methods (Table 3). In six experiments, muscle strips were treated with DTT (1 mM) before exposure to phenoxybenzamine (5 μM, 30 min). DTT potentiated the histamine response, producing a 10.2 ± 0.9 sinistral shift of the concentration-response curve. Subsequent treatment with phenoxybenzamine, in the continued presence of DTT, resulted in a dextral shift of the dose-response curve (70.5 ± 9.8 fold, $n = 6$) and a decrease in the maximal response to 49.3 ± 8.2% ($n = 6$) of the maximum obtained before treatment with the alkylating agent. Affinity constant determinations indicated that there was a significant increase in the affinity of histamine induced by DTT, when compared to the data obtained using the same alkylating agent in the absence of DTT (Table 3).

Effect of 1,4-dithiothreitol on [^{3}H]-mepyramine binding

DTT had no significant effect on the total or non-specific binding (determined in the presence of 2 μM

Table 3 Influence of 1,4-dithiothreitol (DTT) (1 mM) on the binding affinity of histamine for the H_1-receptor in guinea-pig ileum.

	Affinity constant (K_A, M^{-1}) *for histamine deduced from studies of:*	
	Contraction	*[^{3}H]-mepyramine binding*
−DTT	$1.8 \pm 0.2 \times 10^5$ (7)	$5.7 \pm 1.0 \times 10^4$ (4)
+DTT	*$4.9 \pm 1.1 \times 10^5$ (6)	†$2.5 \pm 0.4 \times 10^5$ (4)

Values represent mean ± s.e.mean. Affinity constants were determined either from contractile studies of muscle strips using phenoxybenzamine (5 μM, 30 min) or from inhibition of the binding of 1 nM [^{3}H]-mepyramine in homogenates of ileal smooth muscle as described under Methods. In binding experiments, affinity constants of histamine were measured in the presence and absence of DTT in each experiment. Determinations of these two affinities (+ or − DTT) from contractile studies were made in separate experiments. The number of experiments is given in parentheses.
*$P < 0.01$, unpaired t test and Mann Whitney U test.
†$P < 0.01$, paired t test.

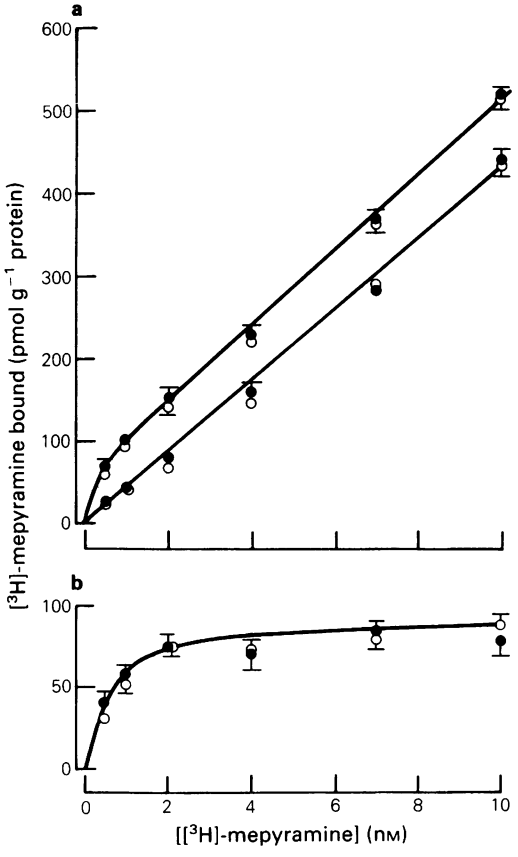


Figure 3 Effect of 1,4-dithiothreitol (DTT) on the binding of [^{3}H]-mepyramine to a membrane preparation of guinea-pig ileal smooth muscle. (a) The binding of [^{3}H]-mepyramine in the presence (○) and absence (●) of DTT (1 mM). The upper curve in (a) shows the total binding of [^{3}H]-mepyramine and the lower curve represents the binding obtained in the presence of 2 μM promethazine. Values represent mean of five replicate determinations in a single experiment with s.e.mean shown by vertical bars. Where no error bars are shown the s.e.mean was within the size of the symbol. (b) Promethazine-sensitive binding of [^{3}H]-mepyramine obtained in the presence (○) and absence (●) of DTT (1 mM). The curve drawn is the best-fit line to the control data obtained by non-linear regression using ALLFIT.

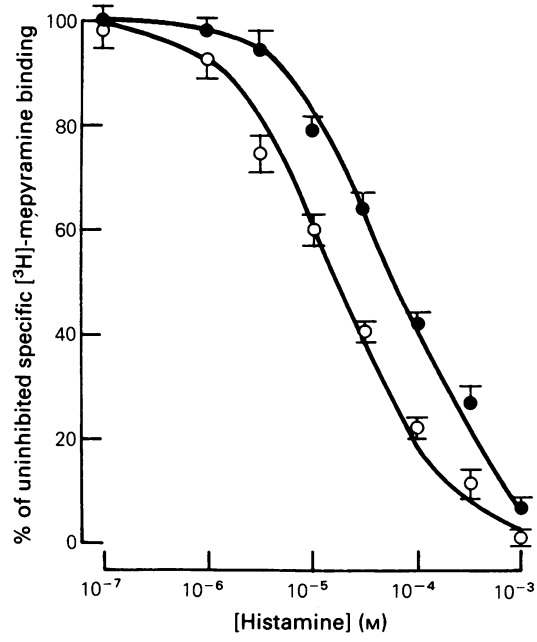


Figure 4 Effect of 1 mM 1,4-dithiothreitol (DTT) (○) on the inhibition of the specific binding of 1 nM [^{3}H]-mepyramine produced by histamine (●) in ileal smooth muscle membranes. Values represent the combined mean of five replicate determinations made in each of four separate experiments with s.e.mean shown by vertical bars. In each experiment non-specific binding was defined using 2 μM promethazine. Curves were fitted to the data points using the non-linear regression programme ALL-FIT, as described under Methods.

promethazine) of [^{3}H]-mepyramine to homogenates of guinea-pig ileal smooth muscle, over the concentration range 0.5 to 10 nM (Figure 3a). The mean affinity constants obtained for [^{3}H]-mepyramine in three such experiments were 1.45 ± 0.08 and $1.39 \pm 0.12 \times 10^9$ M^{-1}, in the absence and presence of DTT respectively. There was similarly no significant difference in the magnitude of the specific binding site capacity determined in the presence (102.3 ± 10.6 pmol g^{-1} protein, $n = 3$) and absence (100.3 ± 9.9 pmol g^{-1} protein, $n = 3$) of DTT (Figure 3b).

The lack of effect of DTT on the affinity of mepyramine in ileum was confirmed in studies of the inhibition of the specific binding of 1 nM [^{3}H]-mepyramine by non-radioactive mepyramine. The affinity constants (derived from the IC_{50} values) for mepyramine in the presence and absence of DTT (1 mM) were $6.3 \pm 0.8 \times 10^8$ M^{-1} and $5.6 \pm 0.7 \times 10^8$ M^{-1} respectively. The slope of the mepyramine competition curve was not affected by DTT and remained close to unity (1.15 ± 0.07 and 1.10 ± 0.06 in the absence and presence of DTT respectively). This is the value expected for a simple and reversible interaction with a single class of binding sites.

Table 4 Effect of 1, 4-dithiothreitol (DTT, 1 mM) on the inhibition of specific [^{3}H]-mepyramine binding by H_1-receptor agonists in guinea-pig ileal membranes

Agonist	*Hill coefficient* (n)		*IC_{50} (μM)*		*IC_{50} ratio*
	[−DTT]	[+DTT]	[−DTT] (A)	[+DTT] (B)	(A/B)
Histamine	0.87 ± 0.10	0.85 ± 0.09	60 ± 9	18 ± 3*	3.3 ± 0.7
2-Methylhistamine	1.13 ± 0.14	0.87 ± 0.10	172 ± 24	48 ± 6*	3.6 ± 0.7
2-Pyridylethylamine	1.07 ± 0.19	0.98 ± 0.13	228 ± 43	40 ± 6*	5.7 ± 1.4
2-Thiazolylethylamine	0.74 ± 0.08	0.70 ± 0.06	246 ± 33	35 ± 5*	6.9 ± 1.3

Values (mean ± s.e.mean) for the Hill coefficient (n) and IC_{50} value were obtained from the combined data obtained in four (histamine) or three (other agonists) experiments, using ALLFIT. In each experiment a direct comparison was made between control (−DTT) and DTT (1 mM)-treated membranes.
*$P < 0.005$ compared to control values (analysis of variance according to De Lean *et al.*, 1978).

Incubation of ileal smooth muscle membranes with DTT (1 mM) resulted in a small, but significant, parallel shift of the histamine displacement curve to lower agonist concentrations (Figure 4, Table 4). The apparent affinity constant deduced for histamine (after correction for competition with [^{3}H]-mepyramine assuming a simple equilibrium with a single class of binding sites) was significantly increased following incubation with DTT and the values obtained agreed reasonably well with those determined from contractile studies (Table 3). Treatment of ileal membranes with DTT produced a similar parallel leftward shift of the inhibition curves obtained for three other H_1-receptor agonists, namely 2-methylhistamine, 2-pyridylethylamine and 2-thiazolylethylamine (Table 4).

Discussion

The results presented here confirm our previous observation that treatment of ileal smooth muscle strips with DTT potentiates H_1-receptor-mediated contractile activity in this tissue (Donaldson & Hill, 1986a) and suggest that this effect is due to an increase in both agonist affinity and efficacy.

A direct comparison of the H_1-receptor agonist properties of SKF 71473 or DE-2PEA with histamine in the same muscle strips showed that both of these analogues produced submaximal tissue responses (relative to histamine), indicative of partial agonism. Similar findings have been made previously by Durant *et al.* (1973) for SKF 71473 and by Kenakin & Cook (1980) for DE-2PEA. In the presence of DTT the maximal responses elicited by these two partial agonists were significantly increased. The maximal stimulation elicited by DE-2PEA was increased from 23% to 71%, while that produced by SKF 71473 was changed from 58% to 98% of the maximal response to histamine in the same muscle strips. These data suggest that the efficiency of the receptor-effector coupling mechanism is increased in the presence of DTT. For SKF 71473 this change is sufficiently large effectively to transform it into a full agonist. Similar effects of DTT on partial agonist activity have been observed in slices of guinea-pig cerebral cortex and cerebellum (Donaldson & Hill, 1986b).

A change in agonist efficacy was also observed with the full agonist histamine in experiments in which the concentration of functional H_1-receptors was reduced with the irreversible antagonists phenoxybenzamine (Kenakin & Cook, 1975) and benzilylcholine (Reasbeck & Young, 1973). By reducing the receptor concentration, it is possible to reduce the efficiency of the receptor-effector system to such an extent that a maximal tissue contractile response is no longer attainable. Under these conditions the maximal response to histamine was reduced to 60% and 45% of that obtained before application of the alkylating agent, with 5 μM phenoxybenzamine (30 min) and 5 μM benzilylcholine mustard (40 min) respectively. Following the addition of DTT to the incubation medium the maximum size of these responses was significantly increased. This change in the extent of the maximum response could be due to a dissociation of the complex between the receptor and the irreversible antagonist (perhaps due to the unstable nature of the covalent bond) in the period following the scavenging of the free aziridinium ions with thiosulphate. However, the fact that no recovery from the effects of phenoxybenzamine was observed in control experiments in the absence of DTT makes this explanation unlikely. Furthermore, the similarity between the effects of DTT on the response to partial agonists (Table 1) and histamine (following treatment with an irreversible antagonist; Table 2) is striking.

Analysis of the effects of phenoxybenzamine on the response to histamine before and after prior treatment with DTT indicates that the effect of DTT on agonist efficacy may be accompanied by an increase in binding

affinity. Thus, the calculated affinity constant for histamine increased by a factor of 2.7 from 1.8×10^5 M^{-1} to 4.9×10^5 M^{-1} in the presence of DTT. However, there are difficulties in interpreting these values since the underlying assumption that the antagonist acts directly and irreversibly with only the receptor recognition site (Furchgott & Bursztyn, 1967) may not be entirely justified in the case of phenoxybenzamine. Kenakin & Cook (1975) have suggested that phenoxybenzamine may act allosterically to inhibit contractile activity on a site outside of the histamine receptor itself, in addition to producing alkylation of the receptor macromolecule. Studies in mouse neuroblastoma cells have indicated that this allosteric site might be a calcium channel (El-Fakahany & Richelson, 1981). This finding is supported by evidence obtained in rabbit aortic strips which shows that phenoxybenzamine can interfere with ^{45}Ca influx into smooth muscle cells (Shibata & Carrier, 1967). This complex interaction of phenoxybenzamine with the H_1-receptor clearly complicates the estimation of agonist affinity constants, particularly since the possibility remains that the apparent effect of DTT on agonist affinity could be contributed to by an interaction between phenoxybenzamine and DTT at the level of the effector system (i.e. the calcium channel). However, even with these caveats, the similarity of the effect of DTT on the apparent affinity of histamine for the H_1-receptor deduced from contractile studies and from inhibition of [^{3}H]-mepyramine binding is notable.

Treatment of membrane preparations of ileal smooth muscle with DTT altered agonist, but not antagonist, binding to a high affinity [^{3}H]-mepyramine binding sites in guinea-pig ileum. DTT did not modify the total or non-specific binding of [^{3}H]-mepyramine over the concentration range employed (0.5 to 10.0 nM ^{3}H-ligand). DTT was similarly without effect on the IC_{50} values or slope parameters determined from competition experiments involving non-radioactive mepyramine. These binding data confirm our previous observation in functional studies that DTT does not affect the affinity of mepyramine for the H_1-receptor (Donaldson & Hill, 1986a). DTT did, however, produce a significant reduction in the IC_{50} values obtained for histamine and the H_1-receptor agonists, 2-methylhistamine, 2-pyridylethylamine and 2-thiazolylethylamine. However, in contrast to previous binding data obtained in guinea-pig cerebellum and cerebral cortex (Donaldson & Hill, 1986c) the effect of DTT on agonist IC_{50} values was not accompanied by a change in slope parameter (Hill coefficient). With the exception of the data obtained with 2-thiazolylethylamine, the slopes obtained for H_1-receptor agonists were not significantly different from unity; the value expected for a simple equilibrium with a single class of binding sites. Thus, at least for histamine, 2-methylhistamine and 2-pyridylethylamine the data obtained with DTT are consistent with a simple change in agonist affinity similar to that observed in bovine aortic membranes (Carmen-Krzan, 1984). The values obtained for the affinity constant of histamine from binding studies are somewhat lower than those deduced from contractile studies, although as noted above there are problems in the calculation of these constants from functional studies. However, previous studies have shown that IC_{50} values for histamine obtained from binding studies in 50 mM Na-K phosphate buffer (used in this study) are in general higher than those measured in more physiological (Krebs-phosphate) buffers (Hill & Young, 1981).

The results of this study suggest that DTT potentiates H_1-receptor-mediated contractile activity in guinea-pig ileum by increasing both agonist affinity and efficacy. It remains to be established whether these two effects are a consequence of common or different mechanisms. For example, one possibility is that DTT may increase the coupling between the receptor and a transducer molecule (e.g. a GTP binding protein) leading to both an increase in affinity for agonists and an increase in agonist efficacy. Alternatively, the increase in efficacy may be unrelated to the change in agonist affinity and arise from an effect of DTT on a more distal site in the chain of events linking receptor occupancy to the final contractile response. The selectivity of action of DTT on contractile responses to histamine, but not other smooth muscle spasmogens (Donaldson & Hill, 1986a), does however suggest that any separate effect of DTT on agonist efficacy is at an early stage in the stimulus-contraction process.

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Contractile effects of substance P and neurokinin A on the rat stomach *in vivo* and *in vitro*

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1 Substance P and neurokinin A (substance K) were infused into the coeliac artery of anaesthetized rats at doses of 0.06–20 nmol min^{-1}. Both tachykinins caused contractions of the stomach, the threshold dose of neurokinin A being 10 times lower than of substance P. The dose-response curve for substance P was flatter than that for neurokinin A.

2 On circular muscle strips from the rat gastric corpus *in vitro*, the dose-response curves for both tachykinins were parallel, neurokinin A being 10 times more potent than substance P. The contractions in response to 10 μM neurokinin A and to 30 μM substance P were 58 and 54%, respectively, of the maximal contraction to bethanechol (1 mM).

3 The effect of substance P was reduced by atropine both *in vivo* and *in vitro*. *In vitro*, the contractions to substance P were also reduced by tetrodotoxin but left unaffected by methysergide. The action of neurokinin A was not affected by these drugs.

4 It is concluded that neurokinin A contracts rat stomach by a direct action on the circular smooth muscle, whereas the action of substance P is mediated, at least in part, by cholinergic interneurones.

Introduction

Substance P (SP) has previously been shown to contract the stomach of various animals in a variety of preparations both *in vivo* and *in vitro* (Bertaccini & Coruzzi, 1977; Milenov *et al.*, 1978a,b; Edin *et al.*, 1980; Milenov & Golenhofen, 1983). In the rat the stomach is innervated by SP-immunoreactive nerves (Schultzberg *et al.*, 1980) which are of both extrinsic (sensory) and intrinsic origin (Minagawa *et al.*, 1984; Sharkey *et al.*, 1984; Ekblad *et al.*, 1985). Neurokinin A-like immunoreactivity has a distribution pattern similar to SP (Theodorsson-Norheim *et al.*, 1984), and binding sites for both SP and neurokinin A (substance K, NKA) are present in high numbers throughout the gastric smooth muscle layers, particularly in the circular muscle (Burcher *et al.*, 1986).

So far, two different preprotachykinin mRNAs encoding NKA have been identified in mammals (Nawa *et al.*, 1984b; Krause *et al.*, 1987), both of which also encode SP. Thus it is highly probable that, wherever NKA occurs, it coexists with SP. Consequently, SP and NKA might act on the same postsynaptic structures or, at least, influence each other in this action. SP and NKA have been previously found to affect potently gastric emptying in the rat; emptying seemed to depend on whether contraction of the stomach or pylorus prevailed (Holzer, 1985). The precise nature of the effects of SP and NKA on gastric contractility is, however, not evident from these data. We therefore investigated the motor effects of the two tachykinins on gastric circular muscle and some of their pharmacological characteristics in more detail. The effects were tested both *in vitro* and *in vivo* in view of the finding that, in the dog gut, the effects of SP differ when examined on isolated preparations or in the living animal (Fox *et al.*, 1983).

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Methods

Animals

Sprague–Dawley rats (Institut für Versuchstierkunde, Himberg, Austria) of either sex weighing 250–300 g (females) and 300–400 g (males) were used. Before the experiments, the rats were fasted over night.

In vivo *experiments*

The rats were anaesthetized with urethane (1.25 g kg^{-1} i.p.). The trachea was cannulated, and blood pressure was monitored from a carotid artery. The oesophagus was ligated in the neck. The abdominal aorta was exposed through a midline laparotomy and a PE-20 cannula was introduced into the aorta in a retrograde direction until the tip of the cannula lay at the branching of the coeliac artery. This cannula was connected to an infusion pump and Tyrode solution was continuously infused at a rate of 60 $\mu l\,min^{-1}$. When the peptides were to be tested, the infusion was switched to the appropriate solution for 5 min. At least 15 min were allowed between two consecutive test infusions. By means of an infusion of Evans blue it was proved that such an intra-aortic infusion reached the upper gastrointestinal tract from the corpus of the stomach to the first 4–5 cm of the duodenum but spared the gastric fundus.

Changes in intragastric pressure were recorded through a catheter introduced into the stomach via the duodenum and fixed by a ligation around the pylorus. By means of a Y-shaped adapter, this catheter was connected to a reservoir of Tyrode solution and to a Statham pressure transducer. Fifteen min after the end of the operation, 3 ml Tyrode solution (2 ml for rats below 300 g body weight) was rapidly infused into the stomach.

For guanethidine pretreatment the rats received 10 mg kg^{-1} of the compound subcutaneously in the evening, and 10 mg kg^{-1} in the morning before the experiment.

In vitro *experiments*

The rats were killed by a blow on the head. The stomach was rapidly taken out and circular strips were cut from the gastric corpus. The mucosa was dissected off, and the muscular strips, 2 mm wide and 10–12 mm long, were suspended in a 3 ml organ bath containing Krebs solution at 37°C gassed with 5% CO_2 and 95% O_2 (composition in mM: NaCl 118.0, KCl 4.7, $MgSO_4$ 1.18, $CaCl_2$ 2.5, KH_2PO_4 1.18, $NaHCO_3$ 25.0, glucose 11.1). Isometric contractions were measured by a force displacement transducer (Hugo Sachs Elektronik, Freiburg, FRG). After an equilibration period of 45 min, the tissue was stimulated 2–3 times with a maximally effective dose of bethanechol (1 mM). Then, conventional dose-response curves were constructed. The tissue was washed as soon as the peak contraction had developed, which took up to 45 s for bethanechol, up to 3 min for SP, and up to 4 min for NKA.

When tetrodotoxin (TTX, 1 μM) was tested, it was added to the organ bath 30 s before each addition of agonist. Atropine (0.6 μM) or methysergide (0.1 μM) was added to the Krebs solution reservoir. Since a decrease in tissue sensitivity with time was observed, the series of agonists were added twice, TTX or one of the antagonists being added after the 2nd series of agonist. For control experiments, two identical series of agonists were tested on one strip. For evaluation of these experiments, the change in contractile force between the first and second addition of a particular dose of agonist on the same strip was calculated (as the difference between the first and second contraction). If

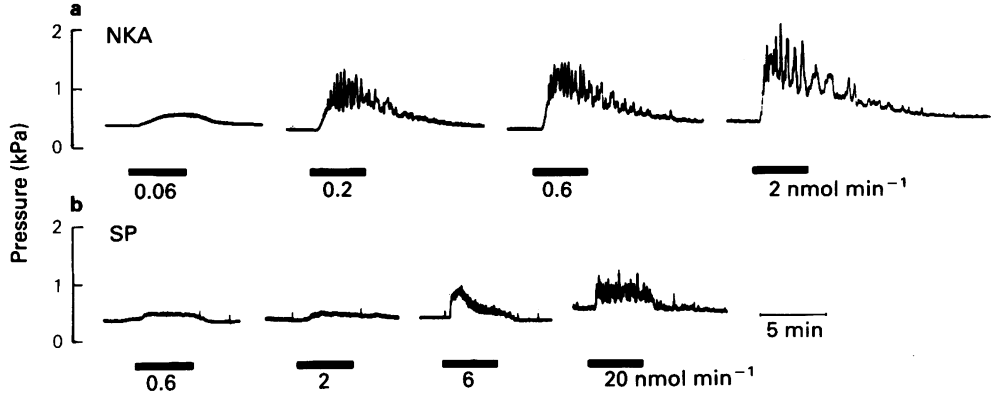

Figure 1 Typical tracing of the rat stomach *in vivo* contracting in response to infusions of (a) neurokinin A (NKA) or (b) substance P (SP) into the coeliac artery as indicated by the bars.

one of the antagonists added was to reduce the response to the agonist, the difference would be expected to be greater than in control strips without antagonist.

Substances

SP and NKA were obtained from Cambridge Research Biochemicals (Cambridge, UK), bethanechol-Cl from Schuchardt (München, FRG), atropine sulphate from Merck (Darmstadt, FRG), guanethidine from CIBA-Geigy (Basle, Switzerland), dimethylphenylpiperazinium-iodide (DMPP) and TTX from Sigma (Deisenhofen, FRG), 5-hydroxytryptamine creatinine sulphate from Fluka (Buchs, Switzerland), and methysergide maleate from Sandoz (Basle, Switzerland). Stock solutions (1 mM) of the peptides were made with 0.01 M acetic acid, of the other substances with 0.15 M NaCl. Dilutions for *in vivo* use were made with Tyrode solution, for *in vitro* use with isotonic saline.

Results

In vivo *experiments*

After filling the stomach with a volume of 2 or 3 ml Tyrode solution, intragastric pressure equilibrated at 0.1–0.3 kPa. No spontaneous phasic contractions occurred with this tone. Infusion of 0.6–20 nmol min^{-1} SP or 0.06–2 nmol min^{-1} NKA into the coeliac artery for 5 min led to dose-dependent tonic contractions of the stomach; the higher doses used also caused phasic contractions (Figures 1 and 2). The threshold dose of SP was somewhat more than 10 times higher than that of NKA. The dose-response curve for SP appeared flatter than that for NKA and, in the dose range used, the strongest effects produced by SP were smaller than those produced by NKA.

The two higher doses of SP (6 or 20 nmol min^{-1}) led to short decreases in blood pressure by 0.6–2.6 kPa, whereas hardly any hypotension was observed during an infusion of NKA.

Pretreatment of the rats with guanethidine (20 mg kg^{-1} s.c.) shifted the dose-response curves for both tachykinins to the right without changing their relative positions (Figure 2).

Atropine (1 mg kg^{-1} i.v.) reduced basal intragastric pressure if it was higher than 0.4 kPa at the time of injection. Atropine did not influence the effect of a test dose of NKA (0.6 nmol min^{-1}) (Figure 3). In contrast, the tonic contractions in response to 6 nmol min^{-1} SP were significantly reduced by atropine, and phasic contractions did not occur in 4 of 6 preparations. As expected, the contractions due to bethanechol (60 nmol min^{-1}) were completely abolished by atropine.

In vitro *experiments*

Circular muscle strips of the gastric corpus responded to a maximally effective dose of bethanechol (1 mM) with a mean force development of 58 ± 6 mN (n = 5). NKA and SP were less efficacious: 30 μM SP induced a maximal effect only 54 ± 4% (n = 6), and 10 μM NKA

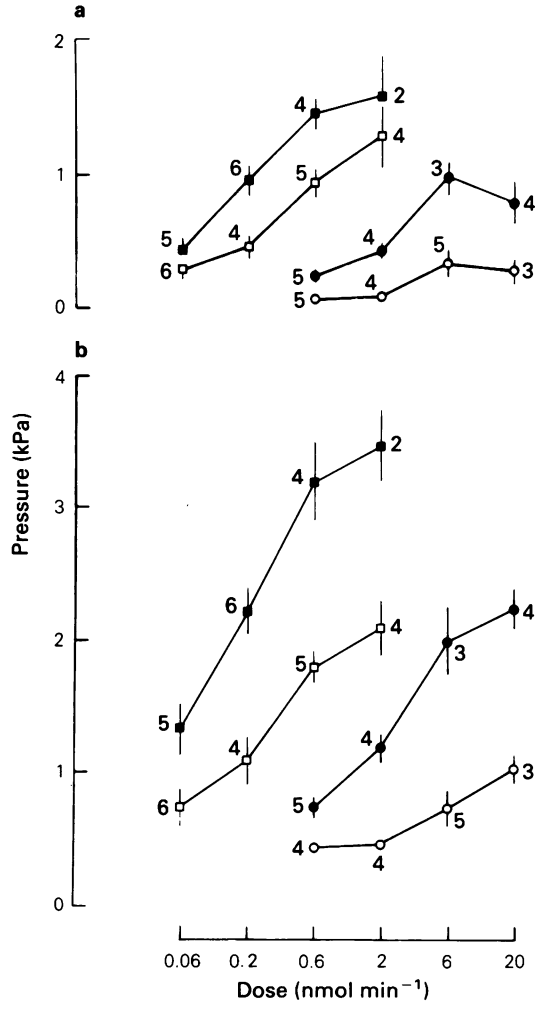

Figure 2 Dose-response curves for the contractile effects of substance P (SP, ○, ●) and neurokinin A (NKA, □, ■) on the rat stomach *in vivo* during infusion into the coeliac artery. (a) Increase in baseline pressure (tonic contraction). (b) Maximum pressure at the peak of the phasic contractions (○, □) Untreated rats; (●, ■) guanethidine-pretreated rats. Each point represents the mean and vertical lines show s.e.mean. n indicated by the number beside each point.

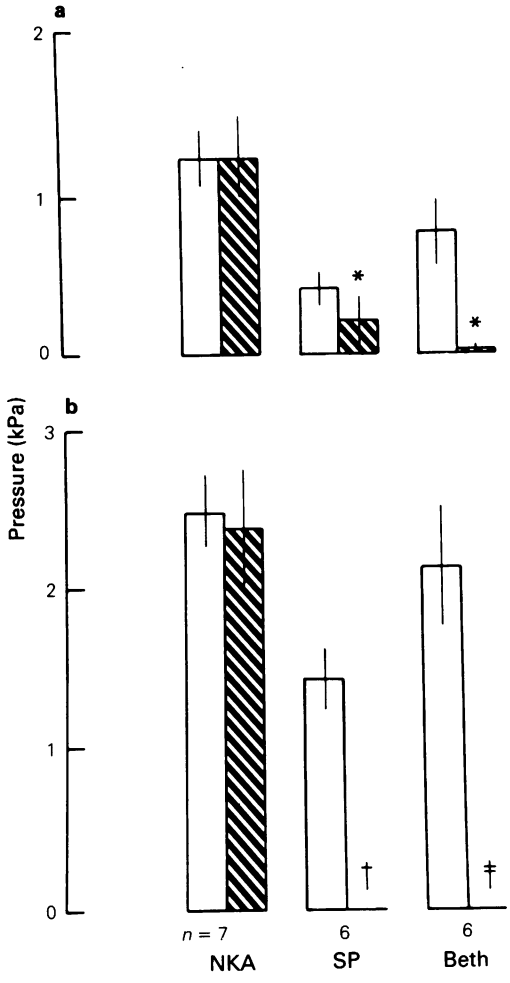

Figure 3 Effect of atropine (1 mg kg^{-1}, i.v.) on gastric contractions due to 0.6 nmol min^{-1} neurokinin A (NKA), 6 nmol min^{-1} substance P (SP) and to 60 nmol min^{-1} bethanechol (Beth). (a) Increase in baseline pressure (tonic contraction). (b) Maximum pressure at the peak of the phasic contractions. Open columns represent mean responses before atropine and hatched columns represent mean responses after atropine. Vertical lines show s.e.mean. † No phasic contractions in 4 of 6 experiments. ‡ No phasic contractions. *$P < 0.05$ vs control.

induced one only 58 ± 4% ($n = 7$), of the maximal response to bethanechol (Figure 4).

The contractions elicited by NKA were not affected by either TTX, atropine or methysergide (Tables 1 and 2). The doses of antagonists used were sufficient to reduce significantly the effect of DMPP (100 μM) or

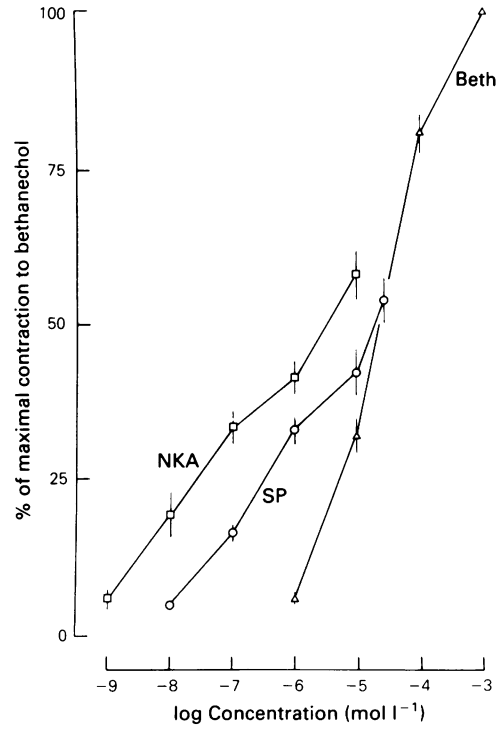

Figure 4 Dose-response curves for the effects of substance P (SP, ○), neurokinin A (NKA, □) and bethanechol (Beth, Δ) on the isolated circular muscle strips from the rat gastric corpus *in vitro*. Each point represents the mean, $n = 5$–7, and vertical lines show s.e.mean.

bethanechol (10 μM or 1 mM), and to abolish the action of 5-hydroxytryptamine (10 μM), respectively. The contractions induced by SP were, however, reduced by TTX, although this decrease did not reach statistical significance for the highest dose of SP examined (10 μM) (Table 1). The effect of this dose was significantly reduced by atropine. No effect of methysergide was observed (Table 2).

Discussion

The present results provide evidence that NKA, as well as SP, is able to contract the stomach of the rat, NKA being ten times more potent than SP. However, there are differences in the mode of action of the two tachykinins: NKA appears to act directly on gastric circular smooth muscle, as it is not inhibited by TTX. In contrast the effect of SP seems to be mediated, at least in part, by cholinergic excitatory interneurones

Table 1 Influence of tetrodotoxin (TTX, 1 μM) on the contractile effect of several agonists on circular muscle strips of the rat gastric corpus

	Control	*TTX*
NKA 1 nM	−8.4 ± 2.7	−15.8 ± 3.3
10 nM	−2.7 ± 1.6	−8.0 ± 3.9
100 nM	−1.2 ± 1.8	−3.1 ± 4.3
1 μM	−0.7 ± 3.1	−4.9 ± 3.6
10 μM	−1.2 ± 1.4	−6.5 ± 4.8
SP 10 nM	−1.4 ± 0.9	−5.6 ± 1.4*
100 nM	−1.2 ± 0.7	−10.3 ± 2.4**
1 μM	−0.4 ± 0.5	−16.5 ± 7.0*
10 μM	−2.2 ± 2.4	−6.7 ± 11.5
DMPP 100 μM	−3.5 ± 3.0	−14.8 ± 4.7**
Beth 1 mM	−11.1 ± 2.9	−8.3 ± 3.9

The values (mean ± s.e.mean, $n = 5–10$) denote the difference in contractile force (in mN) between the 1st and 2nd addition of a particular dose; i.e. a negative value means a decrease in sensitivity after th 2nd addition of the agonist, a positive value means an increase in sensitivity; a value of 0 denotes the same response after both additions of the agonist (for details see Methods).
NKA = neurokinin A; SP = substance P; DMPP = dimethyl-phenylpiperaziniumiodide; Beth = bethanechol.
*$P < 0.05$, **$P < 0.01$ vs control (one-sided Mann-Whitney U-test).

since it is antagonized by atropine, this antagonism being more pronounced *in vivo* than *in vitro*. This observation is similar to one described by Fox *et al.* (1983) for the dog, namely indirect, nerve-mediated effects being more pronounced *in vivo* than *in vitro*. 5-Hydroxytryptaminergic interneurones did not seem to play a role in mediating the effect of SP. In contrast to the present observations, Lidberg *et al.* (1985) described a reduction of the action of SP on pyloric and antral longitudinal strips by 10^{-8}–10^{-5} M ketanserin. In our study, however, 100 nM ketanserin had no consistent effect on the response of either longitudinal fundus or circular corpus strips to 10 or 100 μM 5-hydroxytryptamine (6 observations), which is supported by the findings of Van Nueten *et al.* (1984) that ketanserin does not affect the motility of gastrointestinal tissues *in vitro*.

SP and NKA are 10 and 100 times, respectively, more potent than bethanechol in contracting the rat stomach, although the maximal contraction due to bethanechol *in vitro* is almost double that due to the tachykinins. It was not possible to test dose-response relationships for muscarinic agonists on the stomach *in vivo* since acetylcholine itself, possibly due to its fast metabolism, did not elicit any gastric contractions even after an infusion of extremely high doses. Also, if doses of bethanechol higher than 60 nmol min^{-1} were tested, they elicited intense systemic effects which would have rendered the gastric recordings unreliable.

A striking observation was that, contrary to the *in vitro* experiments, the dose-response curves for SP *in vivo* were flatter than those for NKA. This result might be explained in several ways: firstly, it was observed that SP, being more potent than NKA in reducing blood pressure (Nawa *et al.*, 1984a), also caused a short-lasting hypotension. Therefore, it might have led to a reflex increase in sympathetic nerve activity, which, in turn, would have reduced the contractility of gastric smooth muscle. This possibility was examined by repeating the experiments in rats pretreated with guanethidine. In these animals, however, instead of approximating the dose-response curve for SP to that of NKA, the effects of both SP and NKA were markedly increased without change in the difference in potency between the two tachykinins. This can be explained by the observation that laparotomy as such is known to cause a massive increase in activity of the sympathetic nervous system with concomitant inhibi-

Table 2 Influence of atropine (0.6 μM) and methysergide (0.1 μM) on the contractile effect of neurokinin A (NKA), substance P (SP) and bethanechol (Beth) on circular muscle strips of the rat gastric corpus

		Control	*Atropine*	*Methysergide*
NKA	100 nM	−0.6 ± 0.3	−2.4 ± 0.9	−0.8 ± 0.2
	10 μM	+0.1 ± 0.2	−0.8 ± 0.4	0.0 ± 0.6
SP	1 μM	−1.9 ± 0.5	−2.5 ± 0.6	−1.5 ± 0.2
	10 μM	−0.6 ± 0.5	−3.2 ± 0.6**	−0.9 ± 0.4
Beth	10 μM	+0.1 ± 0.2	−6.2 ± 1.8*	—
	1 mM	0.0 ± 0.3	−4.5 ± 1.7**	—

The values (mean ± s.e.mean, $n = 6–7$) denote the difference in contractile force (in mN) between the 1st and 2nd addition of a particular dose (for further details see Table 1 and Methods).
*$P < 0.05$, **$P < 0.01$ vs control (one-sided Mann-Whitney U-test).

tion of gastrointestinal motility (Furness & Costa, 1974). Thus, guanethidine appears simply to have abolished the effects of laparotomy.

A second reason for the smaller efficacy of SP compared with NKA *in vivo* might be because it is metabolized by enzymes which do not cleave NKA. This view is supported by the observation of Holzer (1985) that the hypotensive effect of NKA after intraperitoneal administration lasts longer than that of SP. Acetylcholinesterase has been shown to hydrolyse SP (Chubb *et al.*, 1980). Whether it fails to hydrolyse NKA has not been examined, but this possibility is not totally improbable, since it was demonstrated recently that angiotensin converting enzyme cleaves SP but not NKA (Turner & Hooper, 1987). In contrast, in a study on rabbit blood pressure and rat salivation *in vivo*, after intravenous injections of the tachykinins, no differences in the slope of the dose-response curves were observed (Holzer-Petsche *et al.*, 1985). In fact, the results indicated, if anything, that NKA was the tachykinin being metabolized faster than the other.

A third explanation might come from the finding of Burcher *et al.* (1986) that the number of binding sites for NKA in the rat stomach is greater than that of SP. This could be a possible basis for the greater efficacy of NKA compared to SP, although it is not clear why this should determine the action of the tachykinins only *in vivo* but not *in vitro*.

Fourthly, one major difference between the preparations *in vivo* and *in vitro* is the presence or lack of the gastric mucosa and, possibly, also the submucous plexus. The possibility has to be considered that, in addition to its direct effect on gastric smooth muscle, one of the tachykinins tested has an indirect action via some submucous or mucosal structure, thus reducing the effect of SP or potentiating the effect of NKA.

Finally, since SP was recently shown to be able to stimulate visceral sensory receptors (Lew & Longhurst, 1986), it might, in the *in vivo* preparations, evoke a vagal reflex response, thus superimposing relaxation on the SP-induced contraction. It is not yet known, though, whether or not NKA shares this property with SP.

In conclusion, it has been shown that, in the rat stomach, NKA acts directly on circular smooth muscle and is more potent than SP, which appears to act partly via cholinergic interneurones. The role of tachykinins derived from intrinsic neurones and that of tachykinins from sensory neurones remain to be established.

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Effects of oxytocin-antagonist injections on luteal regression in the goat

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1 Intra-arterial administration of 0.25 ml physiological saline to the non-pregnant goat between days 12 and 20 of the oestrous cycle did not affect luteal regression, which was characterized by decreasing peripheral plasma progesterone concentration, beginning on day 13 of the oestrous cycle, and an increase in the plasma concentration of 13,14-dihydro-15-keto-prostaglandin $F_{2\alpha}$ (PGFM) as oestrus approached on about day 20.

2 Intra-arterial administration of oxytocin antagonist (OA) in saline at a dose of 0.2 $\mu g\,kg^{-1}$ body weight to goats between days 12 and 20 of the cycle significantly ($P < 0.001$) delayed luteal regression beyond day 20 (to day 26).

3 Injection of OA maintained plasma progesterone secretion at 4–5 $ng\,ml^{-1}$ till day 23 of the cycle and suppressed the increase in PGFM concentration.

4 Corpus luteum extract (100 μl) of OA-treated animals released a significant ($P < 0.001$) amount of $PGF_{2\alpha}$ from rat uterus *in vitro* as did authentic oxytocin. This oxytocic material failed to release $PGF_{2\alpha}$ during luteolysis in the goat, suggesting that oxytocin receptors for $PGF_{2\alpha}$ release may be occupied by OA.

5 It is concluded that oxytocin-receptor interaction in the uterus may be the stimulus for $PGF_{2\alpha}$ release which triggers luteal regression in the goat.

Introduction

There is evidence now to suggest that the corpus luteum of non-pregnant women, cows, sheep and goats contains oxytocin (Wathes & Swan, 1982; Flint & Sheldrick, 1982; Wathes *et al.*, 1982; 1983; Homeida, 1986). Oxytocin (OT) given exogenously in pharmacological doses interrupted the oestrous cycle of the goat and cow and caused luteolysis (Armstrong & Hansel, 1959; Cooke & Knifton, 1981). The physiological role of luteal OT, though uncertain, may be to facilitate the control of luteal function; the best evidence to date for such a role is that obtained from sheep and goats immunized against OT in which luteal regression is delayed (Sheldrick *et al.*, 1980; Cooke & Homeida, 1985). However, the response to OT immunization is quite variable both within and between the animals, and antisera produced might have significant cross-reactions with related compounds (Pavel *et al.*, 1979; Sheldrick *et al.*, 1980; Schams *et al.*, 1983; Cooke & Homeida, 1985). For this reason an attempt was made to inhibit the action of endogenous OT on the release of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) from the uterus and hence prevent luteolysis, in the goat by the use of an oxytocin antagonist (OA).

Methods

Animals and treatments

The 12 non-pregnant Nubian goats that were used were 3–4 years old, clinically normal and experiencing regular cycles (19–21 days). On day 5 of the oestrous cycle, the animals were anaesthetized with pentobarbitone sodium (Sagatal, May & Baker Ltd. England), injected intravenously at a dose of 0.45 $g\,kg^{-1}$ body weight, and maintained with halothane (Fluothane, ICI Ltd, England) and oxygen in a closed system. At laboratomy, a femoral artery was catheterized with a polyvinyl catheter. The catheter was fed 20 cm into the vessel so that the tip lay in the abdominal aorta 6–10 cm above the origin of the uterine artery.

On day 12 of the cycle the animals were divided randomly into two equal groups. Animals in group A received 0.25 ml saline (9 g NaCl per litre) injected through the femoral arterial catheter over a period of 90 s daily between days 10 and 20 of cycle. Animals in group B were treated exactly as in group A but with an oxytocin antagonist (OA), (1-(B-mercapto-B, B-

diethyl-propionic acid), 2-O-ethyl tyrosine, 8-ornithine) vasotocin (dEt_2 Tyr(Et).OVT, code KB-IV-24; a gift from Professor Manning). The synthesis and pharmacological properties have been described by Bankowski *et al.* (1980). OA was given at a dose of 0.2 $\mu g\,kg^{-1}$ body weight in 0.25 ml saline (a total dose of about 8 μg). This dose was selected because in a preliminary study it inhibited the release of $PGF_{2\alpha}$ from the rat uterus *in vitro*, delayed luteal regression and produced no ill effects in the goats. The solution of OA was prepared fresh and kept in ice until used.

Corpora lutea (CL) were removed under halothane anaesthesia from three animals from each group on day 18 of the cycle. CL extracts were prepared as previously described (Homeida, 1986).

Jugular vein blood (5 ml) was collected every 2 h between 07 h 00 min and 19 h 00 min by venepuncture using 23 gauge needles between days 12 and 23 of the cycle. All blood samples were collected into chilled heparinized nylon syringes, stored in ice for a short period and centrifuged at 1500 *g* for 10 min. Plasma samples were stored at −30°C until analysis.

All females were checked for oestrus (day 0) at least twice daily and considered to be in oestrus when they allowed mounting by a male goat.

Hormone assays

The concentration of progesterone and the $PGF_{2\alpha}$ metabolite, 13,14-dihydro-15-keto prostaglandin $F_{2\alpha}$ (PGFM) in the plasma were estimated by radioimmunoassay (RIA) as previously described (Cooke & Homeida, 1985; Homeida, 1986). The intra- and inter-assay coefficients of variation were 4.6% ($n = 12$) and 10.6% ($n = 15$), respectively for progesterone and 8.0% ($n = 9$) and 11.2% ($n = 11$), respectively for PGFM. Assay sensitivity was 75 pg ml^{-1} for progesterone and 45 pg ml^{-1} for PGFM.

Bioassay

The freeze-dried luteal extracts were pooled and reconstituted in Dejalon's solution. These were tested on *in vitro* rat uterus on day 1 post partum. The experimental protocol $PGF_{2\alpha}$ extraction and RIA were as described elsewhere (Homeida, 1986). $PGF_{2\alpha}$ intra- and inter-assay coefficients of variation were 5.2% ($n = 12$) and 8.4% ($n = 14$), respectively.

Data were analysed by Student *t* test (Snedecor & Cochran, 1967).

Results

The mean (± s.e.mean) length of the oestrous cycle in the 3 goats injected with OA (group B) was 26 ± 1.3 days ($n = 3$) and this was significantly ($P < 0.001$) longer than that of 19.2 ± 0.5 days ($n = 3$) in goats injected with saline (group A).

Plasma concentration of progesterone and PGFM are summarized in Table 1.

Progesterone concentration declined on day 15 of the cycle and thereafter till the day of oestrus (19–20 days after the first oestrus) in group A, whereas progesterone concentration was maintained at about 4–5 ng ml^{-1} throughout the sampling period (day 23)

Table 1 Mean (± s.d.) plasma concentration of progesterone and 13,14-dihydro-15-keto-prostaglandin $F_{2\alpha}$ (PGFM) in goats injected with saline (group A) or oxytocin antagonist (OA, group B) between days 12 and 23 of the oestrous cycle

Days of oestrous cycle	*Progesterone (ng ml^{-1})*		*PGFM (pg ml^{-1})*	
	Group A (n = 3)	*Group B* (n = 3)	*Group A*	*Group B*
12	5.3 ± 0.3	5.0 ± 0.4	108 ± 10	110 ± 11
13	4.8 ± 0.4	5.4 ± 0.4	150 ± 10	118 ± 11
14	4.4 ± 0.3	4.8 ± 0.4	320 ± 18	201 ± 12*
15	3.3 ± 0.3	4.6 ± 0.3*	390 ± 20	115 ± 11*
16	2.4 ± 0.2	5.1 ± 0.4*	328 ± 28	110 ± 11*
17	1.2 ± 0.2	4.8 ± 0.3*	510 ± 32	125 ± 12*
18	0.5 ± 0.2	4.3 ± 0.3*	560 ± 30	130 ± 14*
19	0.1 ± 0.1	4.7 ± 0.3*	410 ± 26	115 ± 13*
20	0.1 ± 0.1	4.6 ± 0.4*	320 ± 19	108 ± 13*
21	0.1 ± 0.1	4.8 ± 0.4*	230 ± 14	116 ± 12*
22	0.5 ± 0.2	4.4 ± 0.3*	125 ± 10	120 ± 12
23	1.2 ± 0.4	4.5 ± 0.3*	103 ± 10	103 ± 11

*$P < 0.001$ significantly different from controls.
Numbers in parentheses are the number of animals.

Table 2 Release of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) from rat uterine horns ($ng\,g^{-1}$ wet wt. h^{-1}) after treatment with Dejalon's solution (control), 1 μg oxytocin (OT) or corpus luteum (CL) extract of non-pregnant goat

Control	*OT-treated*	*CL-group A* (n = 3)	*CL-group B* (n = 3)
28 ± 3.1	63 ± 6* (8)		
29 ± 3.2		27 ± 2.9 (11)	
26 ± 2.7			72 ± 8* (9)

Values are mean ± s.e.mean.
*$P < 0.001$ significantly different from controls.
Numbers in parenthesis are the number of experiments.

in group B. The values of progesterone in group B were significantly higher ($P < 0.001$) on days 15 to 23 than in group A.

Basal levels of PGFM varied between 100–125 $pg\,ml^{-1}$. Peaks greater than 300 $pg\,ml^{-1}$ were observed during days 14 to 20 in group A and were significantly ($P < 0.001$) higher than in group B. The concentration of PGFM released between days 12 and 23 in group B remained at the basal level.

The effects of oxytocin and CL extract on the release of $PGF_{2\alpha}$ from the rat uterus are summarized in Table 2.

The addition of 100 μl of CL extract from group B released significant ($P < 0.001$) levels of $PGF_{2\alpha}$ from the rat uterus compared to control or group A.

Discussion

This study demonstrates that systemic administration of OA to goats around luteolysis maintained a functional CL, as indicated by the high level of peripheral plasma concentration of progesterone, and suppressed the increase in PGFM concentration. Similar effects using immunization against oxytocin have been reported in goats and sheep (Sheldrick *et al.*, 1980; Schams *et al.*, 1983; Cooke & Homeida, 1985). However, in contrast to the immunization studies, very little variation in the degree of cycle length was observed in this study.

Although oxytocin was present in CL of goats treated with OA on day 18 of the cycle, its ability to release uterine $PGF_{2\alpha}$, as indicated by its metabolite PGFM, was impaired. Similar effects were demonstrated *in vitro* when oxytocin co-administered with OA failed to release $PGF_{2\alpha}$ from rat uterus (Homeida, 1986). OA used in this study was also shown to be a potent antagonist to oxytocin in rat uterus and milk ejection responses (Bankowski *et al.*, 1980) and antagonists other than dEt_2 Tyr(Et)-OVT given intravenously to rats have been shown to inhibit both the uterotonic and prostaglandin-releasing action of oxytocin (Chan *et al.*, 1982). It has been reported that the degree of stimulation of $PGF_{2\alpha}$ synthesis by the endometrium of ewe is paralleled by the increased concentration of oxytocin binding sites or receptors (Soloff *et al.*, 1977). OA competes specifically with oxytocin for receptors in proportion to their biological activity *in vitro* (Soloff, 1979; Bankowski *et al.*, 1980); these receptors are the main factor controlling the uterine responsiveness to oxytocin. It would seem likely that the oxytocin-receptor interaction plays a major stimulus for the synthesis and release of uterine $PGF_{2\alpha}$, the luteolysin, and triggers luteolysis in the goat, since exogenous administration of oxytocin induced luteal regression via $PGF_{2\alpha}$ release (Cooke & Homeida, 1982; 1983) and active immunization of goats against oxytocin (Cooke & Homeida, 1985) and injection of OA suppressed $PGF_{2\alpha}$ release and delayed luteal regression. It should be noted that the role of oestrogen and progesterone in the synthesis of oxytocin receptors in the uterus is a prerequisite for the effects of oxytocin on that organ (Roberts *et al.*, 1975; Poyser, 1984; Sheldrick & Flint, 1985). However, oxytocin has also been shown to have a local role in the control of steroidogenesis (Tan *et al.*, 1982).

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European College of Neuropsychopharmacology meeting. Denmark. (Dr. P. Beck, Department of Psychiatry, Frederiksborg General Hospital, DK-3400 Hillerod, Denmark.)

10–16 May 1987

International Neurotoxicology Association. First meeting. Lunteren, Holland. (Dr. J. Hooisma, Medical Biological Laboratory, Lange Kleiweg 137, PO Box 45, 2280 AA, Rijswijk, Holland.)

4–19 June 1987

Sixth International Catecholamine Symposium. Jerusalem, Israel. (Secretariat, POB 983, Jerusalem 91009, Israel.)

22–24 July 1987

Brain Research Association & Alzheimers Disease Society joint meeting on Alzheimers Disease. Southampton, U.K. (Dr. J. Hardy, Dept of Biochemistry, St. Mary's Hosp. Medical School, London W2 1PG.)

2–7 August 1987

Fifth World Congress on Pain. Hamburg, W. Germany (Fifth World Congess on Pain, c/o Hamburg Messe und Congress GmbH, Postfach 30 24 80, 2000 Hamburg 36, W. Germany.)

16–21 August 1987

IBRO Second World Congress of Neuroscience. Budapest, Hungary. (Congress Secretariat, H-1476 Budapest, 100 POB 40, Hungary.)

17–21 August 1987

7th International Symposium on Microsomes and Drug Oxidations. Adelaide, Australia. (Secretariat, Dept. of Clinical Pharmacology, Flinders Medical Centre, Bedford Park 5042, Adelaide.)

19–21 August 1987

Satellite Symposium of IUPHAR Congress. Amino Acid Transmitters, Canberra, Australia. (Prof. D.R. Curtis, Dept of Pharmacology, JCSMR, GPO Box 334, Canberra City, A.C.T. 2601, Australia.)

23–28 August 1987

IUPHAR 10th International Congress of Pharmacology, Sydney, Australia (The Secretariat, 10th IUPHAR Congress, GPO Box 2609, Sydney, NSW 2001, Australia.)

30 August–2 September 1987

6th International Symposium on Vascular Neuroeffector Mechanisms, Melbourne, Australia. (Mr. H. Majewski and Dr. D. Story. Department of Pharmacology, University of Melbourne, Parkville, Victoria 3052, Australia.)

31 August–2 September 1987

Satellite Symposium of IUPHAR Congress. Dopamine '87: Dopamine systems and their regulation, Hunter Valley, Australia. (Dr. P.M. Beart, Dept of Medicine, Austin Hospital, Heidelberg, VIC 3084, Australia.)

31 August–2 September 1987

Satellite Symposium of IUPHAR Congress. International Symposium on Physiology and Pharmacology of Adenosine and Adenine Nucleotides. Auckland, New Zealand. (Prof. D.M. Paton, Dept. of Pharmacology, University of Auckland, Private Bag, Auckland, New Zealand.)

31 August–4 September 1987

International Narcotics Research Conference. Adelaide, Australia. (Dr. S. Johnson, c/o 1987 INRC Secretariat, PO Box 153, Nairne, S. Australia.)

31 August–5 September 1987

Xth International Symposium on Neurosecretion. Bristol, U.K. (Neurosecretion Symposium Secretary, Department of Anatomy, Medical School, University of Bristol, Bristol BS8 1TD, U.K.)

4–6 September 1987

Satellite Symposium of IUPHAR Congress. International Symposium on Nicotine. Gold Coast, Queensland, Australia. (Prof. M. Rand, Dept. of Pharmacology, University of Melbourne, Victoria 3052, Australia.)

4–6 September 1987

Satellite Symposium of IUPHAR Congress. International Symposium on Serotonin. Sydney, Australia. (Dr. E.J. Mylecharane, Dept. of Pharmacology, University of Sydney, NSW 2006, Australia.)

6–10 September 1987

Symposium on Recent Advances in Receptor Chemistry. Camerino (MC), Italy. (Prof. C. Melchiorre, Dept of Chemical Sciences, University of Camerino, 63032, Camerino (MC), Italy.)

16–18 September 1987

British Pharmacological Society Autumn Meeting. Oxford, U.K. (Closed meeting for Members and Guests.)

30 September 1987

How Good are Human Volunteers as Models for Drug Action. London, U.K. (Mrs. B. Cavilla, SDR Secretariat, 20 Queensbury Place, London SW7 2DZ, U.K.)

6–8 January 1988

British Pharmacological Society Winter Meeting. London U.K. (Closed meeting for Members and Guests.)

12–17 June 1988

European Society for Neurochemistry, Seventh General Meeting. Göteborg, Sweden. (ESN Meeting Secretariat, Kongresshuset AB, Östra Hamngaton 45, S-411 10 Göteborg, Sweden.)

INSTRUCTIONS TO AUTHORS

Papers will be considered for publication in all fields of pharmacology.
Before submitting a manuscript, authors must note the following points since failure to comply with instructions will lead to either delay or rejection of the manuscript. Overseas authors may find it helpful to seek advice from a Corresponding Editor in their country before submitting a manuscript for consideration.

1. **Manuscripts** (2 copies) should be sent to Dr G.M. Lees, Editorial Office, University of Aberdeen, Marischal College, Aberdeen AB9 1AS. The manuscript must be typed in double spacing on one side of paper, not larger than A4 (210 × 297 mm). There should be a separate title page with the names of authors (in alphabetical order) and their appropriate addresses. One first name of each author may be included.

2. **Style** Papers must be submitted in the style of the journal (see any recent issue of the journal) and in accordance with the Instructions to Authors published in *Br. J. Pharmac.* (1984), **81,** 3–10. (Copies of these instructions may be obtained from Dr Lees.)

3. **Statement** The manuscript must be accompanied by the following statement: 'This manuscript has not been nor will be published in whole or in part by any other journal.' The statement may be included as part of the accompanying letter which must be signed by all authors. No paper will be accepted without this statement and the signature of each author.

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5. **Short title** A short (running) title of not more than 50 letters and spaces should be submitted. This may be typed on the title page.

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Photographs and photomicrographs should be printed on glossy paper and should be larger than the size required for reproduction. Each figure legend should be typed on a separate sheet of paper.

7. **Tables** These should generally have not more than 85 characters to a line (counting spaces between columns as 4 characters) and never more than 110 characters to a line. Each table should be typed on a separate sheet of paper.

8. **References** These must be collected in alphabetical order at the end of the paper. They should include the authors' names, year of publication (in brackets), title of article, title of publication (abbreviated in accordance with the most recent edition of the *World List of Scientific Periodicals*), volume number and first and last page numbers. References to books should, in addition, include the names of editors, the edition number (where appropriate), the town of origin then the name of publisher. Where citation is made to a paper 'in press' the paper should if possible, accompany the manuscript, as an aid to the editor and referee.

9. **Short Communications** These will be accepted if they merit priority publication and do not require revision. They may contain up to 1,200 words of text including references and only one figure or table. The summary should be a single paragraph. A statement must be included.

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