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TWELFTH GADDUM MEMORIAL LECTURE

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Drug receptors and the inhibition of nerve cells

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Introduction

It would be trite indeed to say much to this audience of the contributions to pharmacology made by Sir John Gaddum. His quantitative approaches to the study of drug antagonism have found their way into most textbooks of pharmacology. I dare say that, if asked to define that aspect of the subject which is uniquely 'pharmacology'—a task of increasing difficulty in these interdisciplinary days—many Society members would think first of those quantitative methods for studying drug-receptor interactions spawned by A.J. Clark, tested with antagonists by John Gaddum, and much popularized by Heinz Schild (Clark, 1933; Gaddum, 1937; 1957; Schild, 1949). I thank the Trustees for providing me with the opportunity to add my own small tribute to the memory of John Gaddum's work; this is particularly so because—as you will see—my own research has been much influenced by his contributions.

John Gaddum worked mostly with peripheral tissues. This was convenient because the tissues were readily accessible, easy to maintain *in vitro*, and in those days devices could be made to measure the appropriate response, such as contraction, relaxation or secretion; furthermore, it was generally not important to distinguish the drug effects on the individual cells within the syncytium. The first efforts to classify receptors on nerves were also made at their terminations in the periphery, following on from the well-known observations of Lindor Brown and John Gillespie (1957). That field, the study of autonomic presynaptic receptors, has matured and has led to novel therapies. But in the ganglia of the autonomic nervous system and in the central nervous system, the individual nerve cell is the functional unit, and information about drug receptors on nerve cells can best be obtained by recording from single cells. Conversely, the demonstration and characterization of drug receptors on nerve cells can itself be used as a way of classifying the cells, particularly when taken in concert with other information regarding the ion channels expressed, the transmitters synthesized and the targets to which the cells project.

Inhibition of nerve cells

If we restrict our thoughts to those relatively immediate effects on nerve cells which control their function, then the most important measure is the frequency of discharge of action potentials, and drugs either increase or decrease this. Few nerve cells in the body are silent for long; many are endowed with ion channels which open or close at different rates when the membrane potential changes and thus generate their own activity, and others are constantly bombarded with excitatory synaptic inputs. Our early insight into inhibition in the mammalian nervous system came from the work of Sir Charles Sherrington (1932) and his one-time student Jack Eccles (1964). Such inhibition causes a reduction in the excitability of motoneurones supplying opposing muscle groups when the primary (agonistic) muscles contract; the inhibition is fast, appropriate to the control of neurones that encode their information in frequencies even greater than 100 Hz. It is brought about by the opening of membrane chloride channels; chloride enters the cell tending to hyperpolarize, and the dissipation of contemporaneous excitatory synaptic currents through the open chloride channels can also contribute to the inhibition. In these cases, at least in the vertebrate nervous system, the chloride ion channels are oligomeric proteins; one or more of the subunits serves as transmitter receptor—γ-aminobutyric acid or glycine. *Drugs* can act at these receptors or at other sites on the oligomeric complex (see Barnard *et al.*, 1987).

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Another way to increase the negativity inside the neurone and thus inhibit firing, is to open channels that allow potassium ions to diffuse from the cell down their concentration gradient. The mechanism was first invoked to explain the inhibitory action of acetylcholine on the heart (Burgen & Terroux, 1953; del Castillo & Katz, 1955), and was later shown to be responsible for synaptic inhibition in a number of molluscan and amphibian neurones (Tauc & Gerschenfeld, 1962; Hartzell *et al.*, 1977). In the remainder of my lecture I will review experiments which test the general hypothesis that many drugs act at their receptors on mammalian nerve cells to bring about an inhibition in cell firing, and that they do so by opening potassium channels in the cell membrane.

Characterization of receptors

The experimental work that I shall describe has been carried out on small pieces of nervous tissue maintained *in vitro*, submerged in a warm, flowing physiological solution of appropriate composition. The tip of a glass microelectrode is positioned in the cell interior so that the membrane potential can be measured. We can take advantage of having the tissue *in vitro* to use Gaddum's methods: both the agonist and the antagonist are applied for sufficiently long periods of time that their effects reach a steady-state, because the solution that flows over and through the tissue is simply changed to one that contains the drug. Figure 1 shows an experiment in which the opioid agonist [Met^5]enkephalin was applied to a noradrenaline-containing cell, in the rat locus coeruleus. A low concentration reduces the frequency of discharge, and a higher concentration stops the firing completely and hyperpolarizes the membrane.

These kinds of results can be plotted in the usual way. The results from a similar experiments carried out on other nerve cells are shown in Figure 2. In Figure 2a the neurone was from the guinea-pig submucous plexus. The agonist is again [Met^5]enkephalin and the first antagonist was naloxone; the naloxone was then washed from the tissue and the experiment was repeated using a N-bisallyl substituted enkephalin analogue from ICI (Figure 2b). Both antagonists caused a parallel shift in the dose-response curve. It turns out that the naloxone K_D was about 20 nM in this cell, which differs by a factor of ten from that on the locus coeruleus cell (Figure 1). On the other hand, the ICI compound can be used to distinguish between the receptors on the two neurones. In Figure 2c are presented

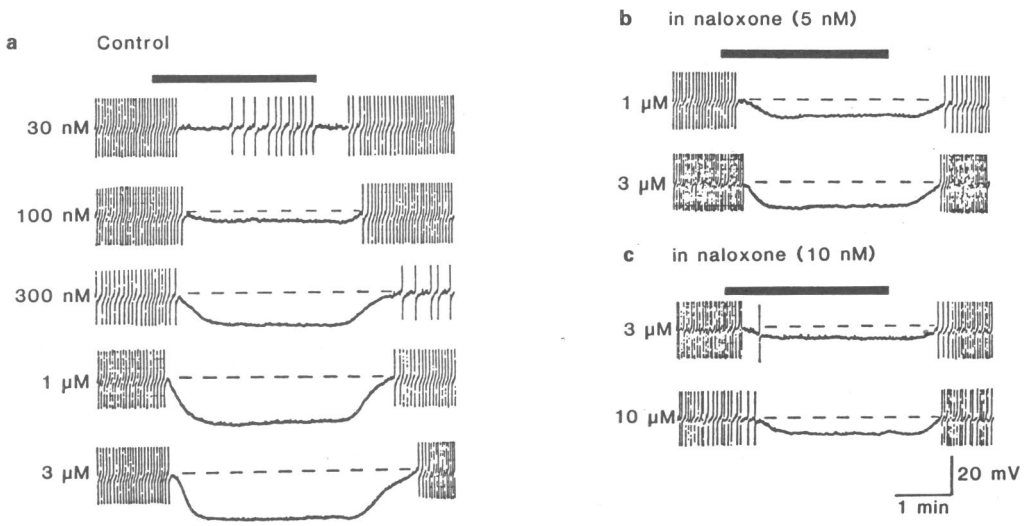


Figure 1 [Met^5]enkephalin hyperpolarizes a rat locus coeruleus neurone, and this is antagonized by naloxone. The records show the membrane potential of a spontaneously firing neurone. Upward deflections are action potentials which are not fully resolved because of the limited frequency response of the pen recorder (full amplitude was 70 mV); downward deflections are after-hyperpolarizations following the action potentials. (a) When the superfusing solution was changed to one that contained [Met^5]enkephalin (period indicated by the bar above the top trace) the firing slowed and the membrane was hyperpolarized. Higher concentrations of [Met^5]enkephalin (indicated to left of each trace) caused larger hyperpolarizations. (b & c) In naloxone, higher concentrations of [Met^5]enkephalin were required to hyperpolarize the cell. Only nine agonist applications are shown in this figure; it is often possible to apply more combinations of agonist and antagonist, but this is limited by the time for which the intracellular recording can be maintained from a single cell. Reproduced with permission from Williams & North (1984).

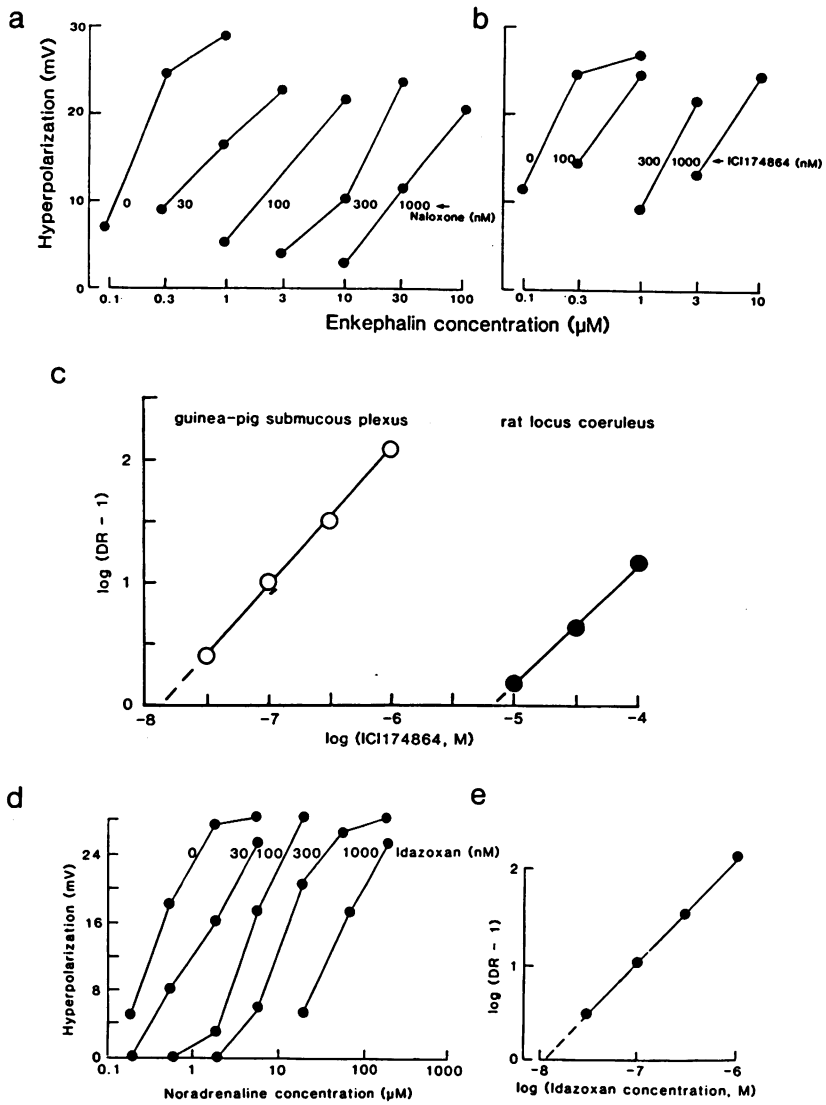


Figure 2 Examples of receptor classification on single neurones. Concentration-response curves from experiments such as those illustrated in Figure 1. (a & b), [Met^5]enkephalin hyperpolarization of a guinea-pig submucous plexus neurone: this is antagonized by naloxone (a) and by ICI174864 (*N*,*N*-bisallyl-Tyr-Aib-Aib-Phe-Leu-OH, Aib = aminoisobutyrate) (b). (c) Schild plots for the antagonism by ICI174864 of the hyperpolarizing action of [Met^5]enkephalin in a guinea-pig submucous plexus neurone (○) and a rat locus coeruleus neurone (●). The estimates of the K_D differ by almost three orders of magnitude, indicating that locus coeruleus neurones express μ-receptors and submucous plexus neurones have δ-receptors. (d) Noradrenaline hyperpolarization of a guinea-pig submucous plexus neurone. (e) Schild plot of the results in (d) indicate that the noradrenaline acts at α_2-adrenoceptors (idazoxan K_D about 10 nM). Reproduced, with permission, from Mihara & North (1986) (a & b), North (1986a) (c) and Surprenant (1986) (d & e).

the results of experiments on one neurone from each tissue; in both cases the agonist was [Met^5]enkephalin, the effect measured was hyperpolarization, and the antagonist was ICI174864. Gaddum would have concluded that these receptors are not the same: the one on the locus coeruleus is a μ-receptor, and on the submucous plexus neurone, a δ-receptor. Experiments with selective agonists such

as DAGOL (Tyr-D-Ala-Gly-MePhe-Gly-ol) and DPDPE (Tyr-D-Pen-Gly-Phe-D-Pen) that were developed later gave complementary results.

Let me provide one further example of this kind of experiment, because the receptor characterization is fundamental to much that follows. The same neurones clearly have more than one receptor that brings about hyperpolarization. Figure 2d plots the hyperpolarization caused by noradrenaline in another neurone, also from the guinea-pig submucous plexus; the antagonist was idazoxan. From the Schild plot (Figure 2e) one can obtain a fairly precise estimate of the K_D for idazoxan and we conclude that this is an α_2-adrenoceptor. Thus, submucous plexus neurones have both δ-opioid and α_2-adrenoceptors, activation of which hyperpolarizes the cell.

By such experiments we have found that not only dopamine, opioids, and noradrenaline but agonists at a number of different drug receptors result in membrane hyperpolarization and inhibition of firing on various sets of neurones (North *et al.*, 1987). Some examples of the tissues studied and the results obtained are shown in Table 1. All these neurones were acutely dissected from adult animals; under these conditions they all express at least two receptors from this *family*. We have determined antagonist dissociation equilibrium constants wherever possible, although in some cases satisfactory antagonists remain unavailable.

Potassium channels are opened

I wish to turn next to the reason why the cells hyperpolarize in the face of all these different receptor agonists. The simplest way to study this is to measure the membrane currents directly by the voltage-clamp method. Figure 3a shows an experimental recording of membrane current from a rat locus coeruleus neurone; the membrane potential is the independent variable. The control current/voltage relation (labelled 2.5) was made by slowly changing the membrane potential and recording the resulting membrane current. The fact that the line is not straight indicates that this cell membrane does not obey Ohm's law—in fact the conductance of the cell increases (or the resistance falls, or ion channels open) as the membrane is hyperpolarized. [Met^5]enkephalin causes an outward current at the resting potential, no current at about -120 mV, and an inward current at more strongly hyperpolarized potentials (outward currents are assigned positive values and depicted in the upward direction). The potential at which enkephalin causes no current, the reversal potential, is -120 mV if the extracellular potassium concentration is similar to that normally found in mammalian brain—about 2.5 mM. This reversal potential changes according to the logarithm of the extracellular potassium concentration (traces labelled 6.5), and this indicates that the action of the agonist is to open ion channels in the membrane which are selectively permeable to potassium (Williams *et al.*, 1982; 1988a). In the normal operating range of the cell this current is outward, leading to hyperpolarization.

A concentration of 100 nM enkephalin typically causes an outward current at -60 mV of 100 pA; during a 2 min application such as those illustrated, this would correspond to the loss of about 100 fmol of potassium. For a locus coeruleus neurone of about 10 μm radius, this represents about one-sixth of its potassium content. Bear in mind, however, that in order to stop the cell from firing completely, much smaller outward ionic current is normally required and the loss of cell potassium would be proproportionately less.

The properties of the set of channels opened by the opioids can be obtained by the method of subtracting the currents. The results indicate that the potassium channels opened by the opioids are probably not significantly in operation before the drug is applied; equivalent circuits for the cell (Figure 3b) indicate that the agonists open a set of channels having relatively unique properties. The conductance of the channels changes almost immediately with membrane potential, being higher at negative potentials (inward rectification); the inward current is particularly sensitive to barium, being reduced by as little as 1 μM (Williams *et al.*, 1988a). The significance of the inward rectification (in these or other potassium channels) is not well understood, but the conductance increase over the very voltage range at which the agonists are normally working tends to amplify their effects.

We know that a single neurone typically has at least two receptors that can couple to potassium channels; it is possible that each receptor is associated with, or actually comprises, its own channels. This can be readily tested by recording the membrane current from the entire neurone: if a maximal outward current is elicited by an agonist at one receptor type, then a second agonist should still be effective to open 'its own' channels through 'its own' receptor. The experiments depicted in Figure 4 show that this is not the case. The figure shows a recording of the membrane current of a neurone in the rat substantia nigra zona compacta; the concentration of baclofen was increased until it caused a maximal outward current, and at this stage the action of dopamine was occluded. A second example (Figure 4b) shows that noradrenaline and somatostatin open the same channels in a submucous plexus neurone. The outward current evoked by noradrenaline was

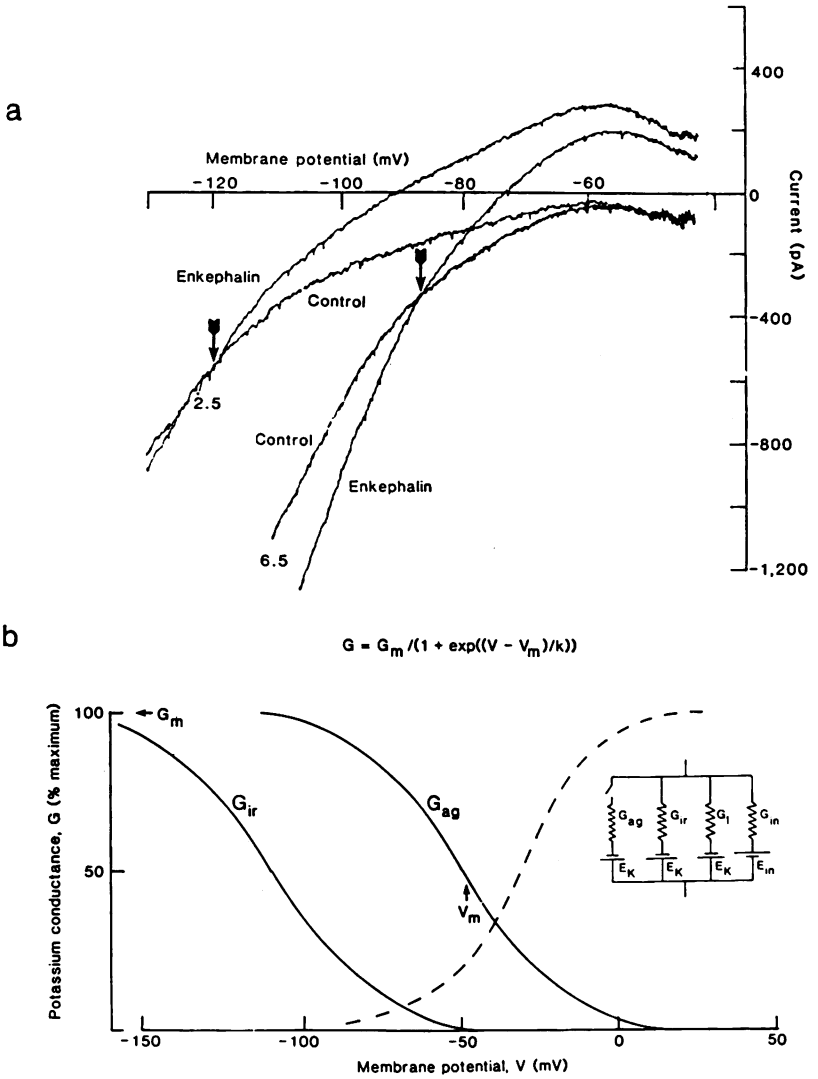


Figure 3 (a) [Met^5]enkephalin increases potassium conductance in a rat locus coeruleus neurone. The left two traces (labelled 2.5) show the membrane currents (I, inward current is downward) recorded as the membrane potential (V) was slowly changed from -130 mV to -45 mV. In control circumstances, the conductance of the cell (dI/dV) steadily falls until the potential reaches about -60 mV. (At -60 mV the net membrane current is close to zero; this would be the resting potential of the cell. The steady-state membrane current is actually still slightly inward at -60 mV, because locus coeruleus neurones fire spontaneously under these conditions. The time-dependent currents underlying the action potential are not seen.) When [Met^5]enkephalin (30 μM) is added, an additional membrane current flows which is large and outward at -60 mV, zero at about -120 mV (arrow), and reverses to inward at more negative potentials. The right pair of traces (labelled 6.5) show the results of repeating the experiment in a higher potassium concentration ($[K]_o$, 6.5 instead of 2.5 mM). The potential at which [Met^5]enkephalin causes no net current (arrow) changes to about -88 mV. In many such experiments it is found that [Met^5]enkephalin (and the other agonists listed in Table 1) cause a membrane current which reverses at a potential (E_{rev}) which is directly related to the logarithm of $[K]_o$. This indicates that the current caused by [Met^5]enkephalin results from an increase in the membrane potassium conductance. (b) Many neurones behave as though they have two potassium conductances operating in the range of voltage between -60 and -130 mV. These are a voltage-independent conductance (G_l) and an inward rectifier conductance (G_{ir}) that increases with hyperpolarization according to $G_{ir} = G_m/(1 + \exp((V - V_m)/k))$ where V_m is the potential at which G_{ir} is half its maximal value (G_m) and k represents the steepness of the voltage-dependence. Agonists such as [Met^5]enkephalin act to increase a potassium conductance (G_{ag}) which is also voltage-dependent, but the potential at which it is half-maximal (V_m, arrow) is closer to the resting potential of the cell. Adapted from Williams *et al.* (1988a).

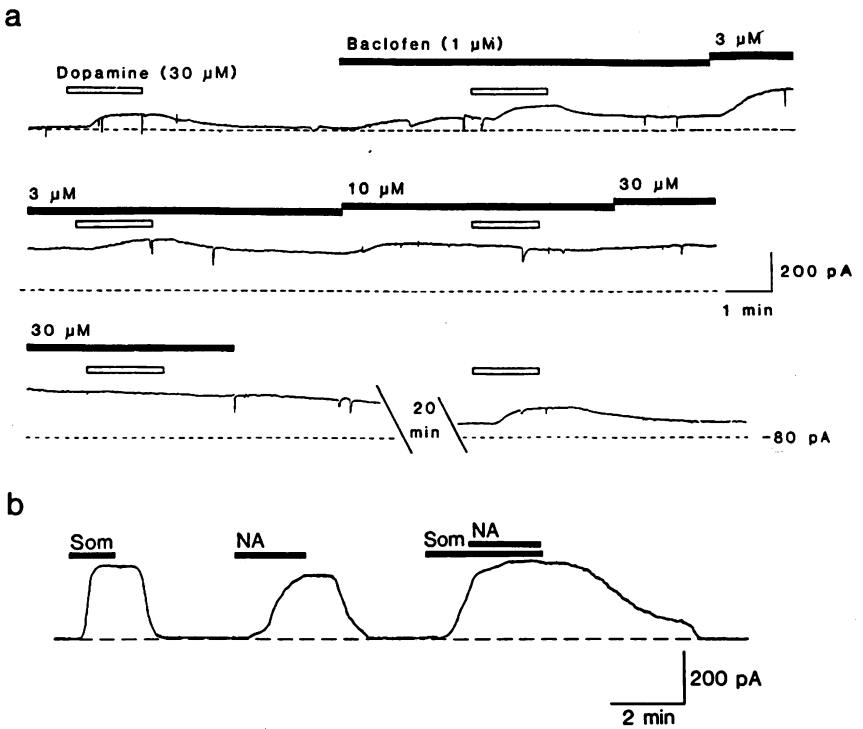


Figure 4 Several agonists increase the same potassium conductance on individual neurones. (a) Membrane current of a rat substantia nigra neurone. Dopamine (open bars) and baclofen (filled bar) were applied. The outward current evoked by baclofen became greater as the concentration was increased; when the baclofen-induced current was maximal, dopamine became ineffective. Holding potential −60 mV. (b) A similar experiment to show that somatostatin (Som, 5 nM) occludes the current caused by noradrenaline (NA, 2 μM) in a neurone of guinea-pig submucous plexus. These agonist concentrations gave maximal currents in this neurone. Holding potential −55 mV. Reproduced, with permission, from Lacey *et al.* (1987) (a) and Surprenant & North (1988) (b).

completely occluded by the outward current caused by somatostatin: a similar result was found with enkephalin. I need hardly remind you that somatostatin, enkephalin and noradrenaline act at distinct receptors: Figure 5 shows the selective antagonism of the hyperpolarizations caused by these agonists when applied to a single submucous plexus neurone.

Receptor-channel coupling

An obvious way in which agonists at distinct receptors might open the same potassium channel is by activating the same second messenger molecule. For all the receptors in the family, agonist binding affinity is reduced by adding guanosine 5′-triphosphate (GTP), and several of the receptors have been co-purified with G-proteins. If GTP hydrolysis is prevented, by providing the cell interior with a non-hydrolyzable derivative such as guanosine 5′-*O*-(3-thiotriphosphate) (GTP-γ-S), then the action of the agonists does not reverse when the application is

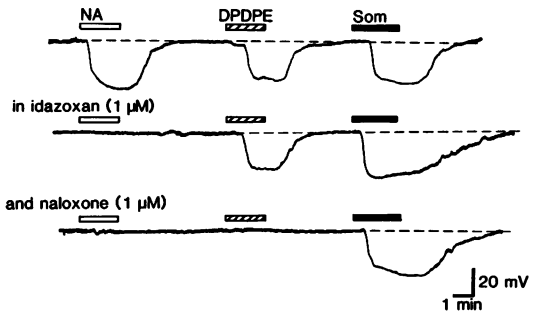


Figure 5 Activation of distinct receptors (α_2-adrenoceptor, δ-opioid and somatostatin) on the same neurone causes hyperpolarization. Three agonists [noradrenaline (NA, 2 μM), Tyr-D-Pen-Gly-Phe-D-Pen (DPDPE, 200 nM) and somatostatin (Som, 5 nM)] caused approximately the same hyperpolarization of this guinea-pig submucous plexus neurone. Idazoxan selectively blocked the action of noradrenaline, and naloxone blocked the effect of the enkephalin analogue. Reproduced, with permission, from Mihara *et al.* (1987a).

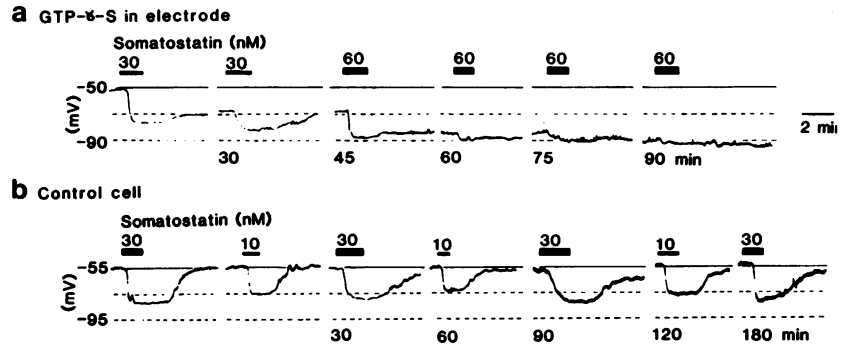


Figure 6 Hydrolysis of guanosine 5'-triphosphate (GTP) is necessary for the termination of the hyperpolarization caused by somatostatin. (a) Intracellular recording from a guinea-pig submucous neurone: the recording electrode contained potassium chloride and guanosine 5'-*O*-(3-thiotriphosphate) (GTP-γ-S, 20 mM), a non-hydrolyzable analogue of GTP. Successive applications of somatostatin caused progressive hyperpolarizations which eventually caused the membrane potential to remain close to the potassium equilibrium potential (about −95 mV, potassium concentration was 5 mM). Times indicate the time after impaling the neurone, during which GTP-γ-S could diffuse into the neurone. (b) A similar experiment in which somatostatin was applied repeatedly to another submucous plexus neurone, when the recording electrode contained only potassium chloride. The effect of the somatostatin remained fully reversible during 3 h of recording. Reproduced, with permission, from Mihara *et al.* (1987a).

discontinued, and the potassium channels remain open (Figure 6). In all the cases tested, the coupling to the potassium channel involves a *Pertussis toxin*-sensitive G-protein (Table 1). The action of the agonists can be prevented by low concentrations of *Pertussis toxin* applied to the cell interior, or by larger concentrations applied for an hour or so to the external surface. In the latter case, the lost response to the agonists can be 'reconstituted' if purified G proteins are included in the recording electrode so that they diffuse into the cell (North *et al.*, 1988).

A second kind of evidence implicates a secondary transducing molecule within the cell; this is the time course of the response to agonist. When the agonist is applied briefly, the channels open after a delay of at least 20–30 ms, commonly more. This contrasts markedly with the delay that precedes the nicotinic action of acetylcholine in the same cell, which can be made less than 1 ms. We have studied this in detail for the action of noradrenaline (Figure 7). The noradrenaline was applied by iontophoresis from a pipette tip situated within a few micrometers of the cell membrane, the application lasted 0.5 ms. Yet the hyperpolarization begins only after 30 ms. A similar application of acetylcholine to the same cell resulted in an almost immediate depolarization, which had completely disappeared before the noradrenaline response peaked.

Synaptic inhibition

The time course of the response is particularly well seen when the agonist is released as transmitter by a synchronous shock to presynaptic nerves. Focal stimulation of the nucleus raphe dorsalis in a brain slice releases 5-HT to cause synaptic potentials in adjacent neurones (Figure 8). The noradrenaline-containing sympathetic fibres can also be stimulated as they enter the submucous plexus; the released noradrenaline causes an i.p.s.p. Note the similarity in the time courses of the two synaptic potentials; the difference in decay rate may reflect differences in diffusion and reuptake mechanisms, since the brain slice offers a greater barrier to diffusion and hence opportunity for uptake than does the isolated ganglion of the submucous plexus (Surprenant & Williams, 1987).

Currents through single potassium channels

These responses are therefore considerably slower than those, such as the nicotinic action of acetylcholine, in which the receptor is itself a channel. But they are faster than many responses associated with diffusion of second messenger molecules throughout the cell cytoplasm (see Hartzell, 1981; North, 1986b). This suggests the possibility that the association between receptor and channel is fairly intimate. This has been shown directly by recording from single potassium channels (Miyake *et al.*, 1989). Figure 9 shows the currents through single channels in the membrane of a rat locus coeruleus neurone. The neurones were dissociated from neonatal rats, which we knew from a series of preliminary experiments to give robust hyperpolarizations when μ-receptor agonists were applied (Miyake *et al.*, 1989). The

Table 1 Drug receptors and increases in potassium conductances: evidence for involvement of G-protein comes either from block by pertussis toxin, or activation by GTP-γ-S

Agonist (receptor)	*Tissue*	*G-protein*	*Synaptic potential*	*Reference*
Acetylcholine (M?)	Frog heart	—	Yes	del Castillo & Katz, 1955
Acetylcholine (M?)	Guinea-pig cardiac muscle	—	—	Soejima & Noma, 1984
Acetylcholine (M?)	Guinea-pig cardiac muscle	Yes	—	Pfaffinger *et al.*, 1985
Acetylcholine (M?)	Guinea-pig cardiac muscle	Yes	—	Breitwieser & Szabo, 1985
Acetylcholine (M?)	Guinea-pig cardiac muscle	Yes	—	Yatani *et al.*, 1987a
Acetylcholine (M?)	*Helix aspersa* ganglion	—	Yes	Tauc & Gerschenfeld, 1962
Acetylcholine (M?)	*Aplysia* pleural ganglion	—	—	Kehoe, 1972
Acetylcholine (M?)	*Aplysia* abdominal ganglion	Yes	—	Sasaki & Sato, 1987
Acetylcholine (M?)	Mudpuppy cardiac ganglion	—	Yes	Hartzell *et al.*, 1977
Acetylcholine (M?)	Frog sympathetic C cells	—	Yes	Dodd & Horn, 1983
Acetylcholine (M?)	Cultured GH_3 pituitary cells	Yes	—	Yatani *et al.*, 1987b
Acetylcholine (M_2)	Rat nucleus parabrachialis	—	—	Egan & North, 1986b
Acetylcholine (M_2)	Rat thalamus reticularis	—	—	McCormick & Prince, 1986
Acetylcholine (M_1)	Guinea-pig submucous plexus	Yes	Yes	Mihara & Nishi, 1989
Noradrenaline (α_2)	Guinea-pig submucous plexus	—	Yes	North & Surprenant, 1985
Noradrenaline (α_2)	Guinea-pig submucous plexus	Yes	Yes	Surprenant & North, 1988
Noradrenaline (α_2)	Rat nucleus locus coeruleus	—	Yes	Egan *et al.*, 1983
Noradrenaline (α_2)	Rat nucleus locus coeruleus	—	—	Williams *et al.*, 1985
Noradrenaline (α_2)	Rat nucleus locus coeruleus	Yes	—	Aghajanian & Wang, 1986
Noradrenaline (α_2)	Rat substantia gelatinosa	—	—	North & Yoshimura, 1984
Noradrenaline (α_2)	Guinea-pig myenteric plexus	—	—	Morita & North, 1981
Noradrenaline (α_2)	Rat preganglionic sympathetic neurones	—	Yes	Yoshimura *et al.*, 1987
Enkephalin (μ)	Guinea-pig locus coeruleus	—	—	Pepper & Henderson, 1980
Enkephalin (μ)	Guinea-pig myenteric plexus	—	—	Morita & North, 1982
Enkephalin (μ)	Rat hippocampus interneurones	—	—	Madison & Nicholl, 1987
Enkephalin (μ)	Rat substantia gelatinosa	—	—	Yoshimura & North, 1983
Enkephalin (μ)	Mouse dorsal root ganglion	—	—	Werz & Macdonald, 1983
Enkephalin (μ)	Rat nucleus locus coeruleus	—	—	Williams & North, 1984
Enkephalin (μ)	Rat nucleus locus coeruleus	Yes	—	North *et al.*, 1987
Enkephalin (μ)	Rat nucleus locus coeruleus	Yes	—	Aghajanian & Wang, 1986
Enkephalin (μ)	Rat nucleus parabrachialis	—	—	Christie & North, 1988
Enkephalin (μ)	Guinea-pig hypothalamus	—	—	Charpak *et al.*, 1988
Enkephalin (δ)	Guinea-pig submucous plexus	Yes	—	North *et al.*, 1987
Enkephalin (δ)	Guinea-pig submucous plexus	—	—	Mihara & North, 1986
γ-Aminobutyric acid ($GABA_B$)	Rat hippocampus pyramidal cells	—	—	Gahwiler & Brown, 1985
γ-Aminobutyric acid ($GABA_B$)	Rat hippocampus pyramidal cells	Yes	—	Newberry & Nicoll, 1985
γ-Aminobutyric acid ($GABA_B$)	Rat hippocampus pyramidal cells	—	Yes	Newberry & Nicoll, 1984
γ-Aminobutyric acid ($GABA_B$)	Rat hippocampus pyramidal cells	Yes	—	Andrade *et al.*, 1986
γ-Aminobutyric acid ($GABA_B$)	Rat hippocampus pyramidal cells	Yes	Yes	Thalmann, 1989
γ-Aminobutyric acid ($GABA_B$)	Rat nucleus substantia nigra	Yes	—	Lacey *et al.*, 1988
γ-Aminobutyric acid ($GABA_B$)	Rat nucleus parabrachialis	—	—	Christie & North, 1988a
γ-Aminobutyric acid ($GABA_B$)	Rat frontal neocortex	Yes	—	Howe *et al.*, 1987
γ-Aminobutyric acid ($GABA_B$)	Rat nucleus locus coeruleus	—	—	Osmanovic & Shefner, 1987
γ-Aminobutyric acid ($GABA_B$)	Rat nucleus dorsal raphe	—	—	Colmers & Williams, 1988
Adenosine (A_1)	Mouse striatal cells in culture	—	—	Trussell & Jackson, 1985
Adenosine (A_1)	Guinea-pig cardiac cells	—	—	Kurachi *et al.*, 1986

Table 1—*continued*

Agonist (receptor)	*Tissue*	*G-protein*	*Synaptic potential*	*Reference*
Somatostatin	Guinea-pig myenteric plexus	—	—	Katayama & North, 1980
Somatostatin	Cultured GH_3 pituitary cells	—	—	Yamashita *et al.*, 1986
Somatostatin	Guinea-pig submucous plexus	—	Yes	Mihara *et al.*, 1987a
Somatostatin	Guinea-pig submucous plexus	Yes	—	Mihara *et al.*, 1987b
Somatostatin	Cultured GH_3 pituitary cells	Yes	—	Yatani *et al.*, 1987b
Somatostatin	Rat nucleus locus coeruleus	—	—	Williams *et al.*, 1987
Somatostatin	Rat nucleus locus coeruleus	Yes	—	Inoue *et al.*, 1988
Somatostatin	Cultured AtT-20 cells	Yes	—	Pennefather *et al.*, 1988
Somatostatin	Rat somatotrophs in culture	—	—	Chen *et al.*, 1989
5-hydroxytryptamine	*Aplysia* buccal and abdominal, and *Helix* pallial ganglion	—	—	Gerschenfeld & Paupardin-Tritsch, 1974
5-hydroxytryptamine	*Aplysia* R15 neurone	—	—	Benson & Levitan, 1983
5-hydroxytryptamine	Guinea-pig myenteric plexus	—	—	Johnson *et al.*, 1980
5-hydroxytryptamine	Rat nucleus dorsal raphe	—	Yes	Yoshimura & Higashi, 1985
5-hydroxytryptamine ($5HT_{1A}$)	Guinea-pig myenteric plexus	—	—	Galligan *et al.*, 1988
5-hydroxytryptamine ($5HT_{1A}$)	Rat nucleus dorsal raphe	—	—	Sprouse & Aghajanian, 1987
5-hydroxytryptamine ($5HT_{1A}$)	Rat nucleus dorsal raphe	Yes	Yes	Williams *et al.*, 1988b
5-hydroxytryptamine ($5HT_{1A}$)	Guinea-pig prepositus hypoglossi	—	Yes	Bobker & Williams, unpub.
5-hydroxytryptamine ($5HT_{1A}$)	Rat hippocampus pyramidal cells	Yes	—	Andrade & Nicoll, 1987
5-hydroxytryptamine ($5HT_{1A}$)	Rat hippocampus pyramidal cells	—	—	Colino & Halliwell, 1987
Dopamine	*Aplysia* pleural ganglion	—	—	Kehoe, 1972
Dopamine	*Aplysia* pleural ganglion	—	—	Ascher, 1972
Dopamine	*Aplysia* abdominal ganglion	Yes	—	Sasaki & Sato, 1987
Dopamine (D_2)	Human pituitary adenoma	—	—	Israel *et al.*, 1985
Dopamine (D_2)	Rat intermediate pituitary	—	Yes	MacVicar & Pittman, 1986
Dopamine (D_2)	Rat pituitary corticotrophs	—	—	Stack & Surprenant, 1988
Dopamine (D_2)	Rat nucleus substantia nigra	—	—	Lacey *et al.*, 1987
Dopamine (D_2)	Rat nucleus substantia nigra	Yes	—	Innis & Aghajanian, 1987
Galanin	Mudpuppy cardiac ganglion	—	—	Konopka *et al.*, 1989
Histamine	*Aplysia* neurones	Yes	—	Sasaki & Sato, 1987

direction of current flow is inward in these experiments, becoming larger when the electrical potential difference across the membrane patch is increased.

The kinetic behaviour of the channels allows one to resolve certain states. The channels tend to close for long periods of time (several seconds to minutes) and then begin to chatter in a 'period of activity' of similar duration. Within such periods of activity, the channels behave as though they have a single open state and two closed states. The 'periods of activity' are more frequent when the concentration of enkephalin is increased, and within them the open state of the channel appears to be stabilized by higher enkephalin concentrations. This rather surprising finding that the duration of an open state is influenced by the agonist concentration might be interpreted to indicate that the opening mechanism involves the cooperative action of several molecules or subunits, as would be expected if the channel is formed from several closely similar or identical subunits (see below).

The records in Figure 9 were obtained in the 'on-cell' or 'cell-attached' configuration in which the tight seal between the recording pipette and the cell membrane effectively isolates the compartment containing the potassium channel under study from the remainder of the cell membrane. For channel openings to be seen, it was necessary to have the opioid agonist in the recording electrode; the opioid agonist was not effective to open the potassium channels when it was applied to the remainder of the cell. We conclude from this experiment that the receptor and

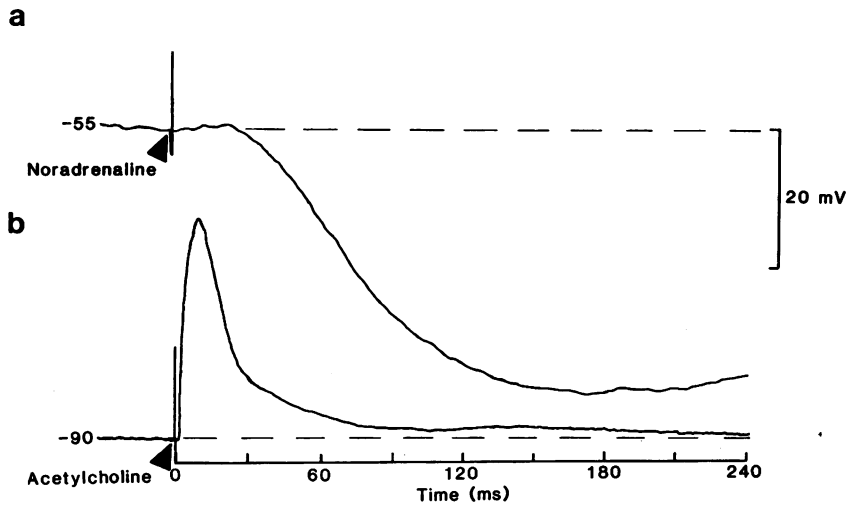


Figure 7 Potassium conductance increase evoked by noradrenaline has a latency of tens of milliseconds. Records show intracellular recording of membrane potential from a guinea-pig submucous plexus neurone. (a) Noradrenaline was applied by iontophoresis (triangle). The neurone was at the resting potential, −55 mV. The iontophoresis pipette contained noradrenaline (10 mM) and its tip was positioned within 5 μm of the neuronal membrane: a rectangular current pulse of 0.5 ms duration was used to eject noradrenaline. The hyperpolarization begins with a latency of at least 30 ms and reaches its peak in 180 ms. (b) The pipette was then removed, emptied, refilled with acetylcholine (1 M) and repositioned. The membrane potential was held at −90 mV. Ejection of acetylcholine (0.5 ms pulse) caused a depolarization of very short latency. This depolarization is blocked by hexamethonium (not shown) and is presumed to result from activation of nicotinic receptors. Unpublished observations of A. Surprenant.

the ion channel are located close to each other, within the few square micrometres of membrane isolated by the pipette tip. We interpret these findings to indicate that a freely diffusible second messenger is probably not involved in the transduction from these receptors to the potassium channel. We found that channels of very similar properties were also opened by α_2-receptor agonists and somatostatin on these cells (Miyake *et al.*, 1989). In these respects, the situation seems very similar to that first described by Soejima & Noma (1984) for the action of acetylcholine in the heart.

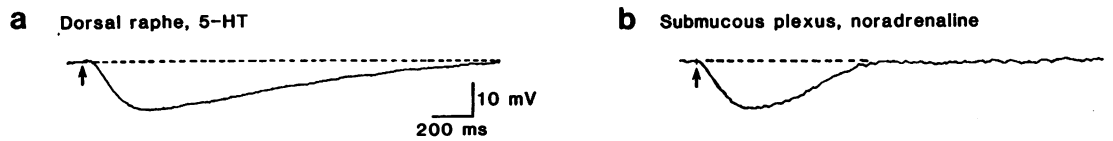


Figure 8 Synaptic inhibition of mammalian neurones mediated by potassium conductance increase. (a) Hyperpolarizing synaptic potential (i.p.s.p.) recorded from a dorsal raphe neurone in a brain slice, following a single electrical stimulus to the surface of the slice (arrow). Fast postsynaptic potentials which normally precede the i.p.s.p. have been blocked by DL-2-amino-5-phosphonovaleric acid (30 μM), 6-cyano-2,3-dihydroxy-7-nitro-quinoxaline (10 μM) and picrotoxin (100 μM). The i.p.s.p. arises with a latency of about 30 ms (not resolved at this recording speed) and reaches its peak in about 200 ms. This synaptic potential is blocked by antagonists at 5-HT_{1A} receptors. (b) A similar synaptic potential in a neurone of the guinea-pig submucous plexus. In this case, the transmitter (noradrenaline) was released by an electrical stimulus to the sympathetic nerve fibres entering the ganglion. Hexamethonium (100 μM) was present to block the nicotinic synaptic depolarization resulting from concomitant stimulation of cholinergic fibres. Note the similarity of the rising phases of the two potentials. The decay of the synaptic potential in the dorsal raphe is thought to be prolonged because of barriers to diffusion in the brain slice which are absent in the submucous ganglia (see Surprenant & Williams, 1987). Unpublished observations of J.T. Williams (a) and A. Surprenant (b).

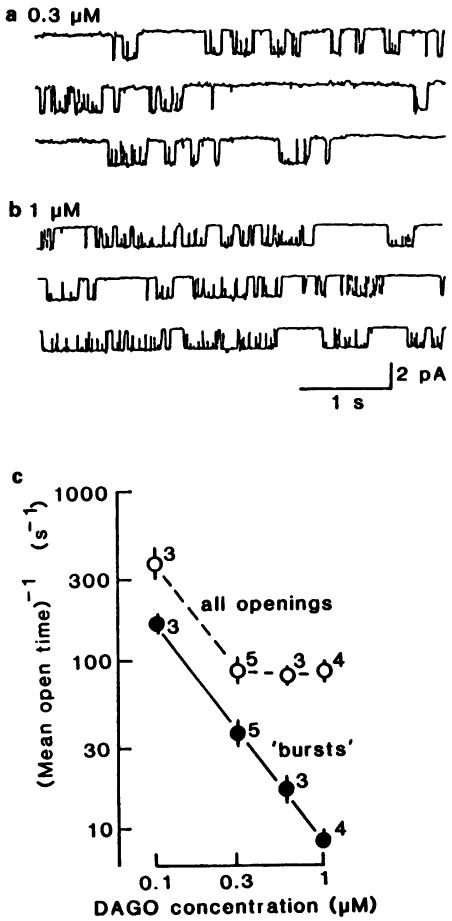


Figure 9 Currents through single potassium channels in the membrane of a rat locus coeruleus neurone. (a & b) Single channel currents: the recording electrode contained (among other things) 150 mM potassium gluconate and the enkephalin analogue Tyr-D-Ala-Gly-MePhe-Gly-ol (DAGO) at 300 nM (a) and 1 μM (b). The electrode was used to make a cell-attached (on-cell) recording from the neurone; the remainder of the neurone, not isolated by the patch electrode, was bathed in a normal physiological saline solution. The potential across the membrane patch (determined later by breaking the membrane and correcting for junction potentials) was about −46 mV. Thus, potassium currents are inward (downward) when the channel opens. (c) Mean open time increases with agonist concentration: (○) mean open times computed from all openings and closings; (●) mean burst duration (open times computed when brief closures (<4 ms) were ignored). Points show mean value (±s.e.mean) for the number of patches indicated. A relatively small change in agonist concentration has a large effect on the burst duration. This would be consistent with cooperativity in the activation of the channel by G-protein. Reproduced, with permission, from Miyake *et al.* (1989).

Divergence after the receptor

When γ-aminobutyric acid (GABA) binds to its receptor, and a chloride channel opens, and inhibition occurs, only a single molecule is primarily involved. The interpolation of a molecule *between* receptor and channel provides a means for diversification of the action of the agonist. In fact, opening potassium channels is rarely the only action that results from activation of receptors in this *family*. Let me give two examples.

In the same cell the receptors can couple to different effectors: they may use the same G-protein. This should come as no surprise because we know that all these receptors (Table 1) will inhibit the activity of adenylate cyclase through a *Pertussis*-sensitive G_i protein. We have found that noradrenaline will not only increase the potassium conductance of submucous neurones, but will inhibit calcium currents, even in the same cell. This depression of the 'calcium' current results from activation of α_2 receptors, and also requires a *Pertussis toxin*-sensitive G-protein (North *et al.*, 1988). In other words, at least two ion channels can be affected in the same neurone.

Second, the same receptors also appear able to couple to more than one G-protein, and thus to distinct effectors, in different neurones (Figure 10). For example, agonists at the cardiac type of M_2 receptor on rat parabrachial neurones lead to an opening of potassium channels and inhibition of the type that I have described (Christie & North, 1988a); the typical outward currents are shown in Figure 10b. On locus coeruleus cells, μ-opioids open the potassium channels (Williams *et al.*, 1982; North & Williams, 1985)

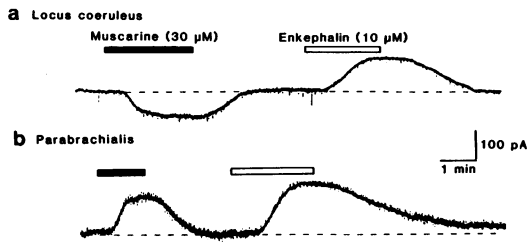


Figure 10 Contrasting actions of muscarine (acting at M_2-receptors) on neurones of the locus coeruleus (a) and parabrachialis (b) of the rat. In both nuclei [Met^5]enkephalin causes an outward potassium current, by acting at μ-opioid receptors. In the parabrachialis neurone (b), muscarine also causes an outward current; this results from opening the same population of potassium channels (see Christie & North, 1988). In the locus coeruleus neurone (a), muscarine causes an inward rather than outward current. The type of the muscarinic receptor on the two cell types can not be distinguished by AFDX-116, pirenzepine or hexahydrosiladifenidol. Unpublished observations of M.J. Christie.

but muscarine instead causes an inward current (Figure 10a) (Egan & North, 1986a). We know that the locus coeruleus neurone has the *Pertussis*-sensitive G-protein and the potassium channels, because they are used by the μ-receptors (Aghajanian & Wang, 1986; J.T. Williams & M.J. Christie, unpublished observations); and we also have evidence that the muscarinic receptor on these cells is the same (cardiac M_2) as those on the parabrachial cells (more precisely, it can not be distinguished by the antagonists pirenzepine, AFDX-116 and hexahydrosiladifenidol; Christie & North, 1988b) but the excitatory action of muscarine in the locus coeruleus is resistant to *Pertussis toxin*, suggesting that the receptor couples to a different G protein (M.J. Christie & R.A. North, unpublished observations.

The overall picture that emerges is illustrated schematically in Figure 11. The receptor might be any member of our receptor *family* (Table 1). Almost all the main transmitters are able, through one of their receptor types, to couple to potassium channels. It remains unclear what might be the functional role of the coupling to the other effectors. If a neurone is inhibited, it might also be appropriate for it to reduce slightly the level of calcium in the cell body as well as the level of cyclic AMP—both would tend to slow gene transcription and thus reduce the turnover of essential molecules, such as transmitters, receptors and channels. It is the inhibition itself that has been the primary theme of my lecture, and there is abundant evidence that the functional role here is synaptic inhibition. Of course many drugs in current therapeutic use act as agonists and antagonists at this *family* of receptors.

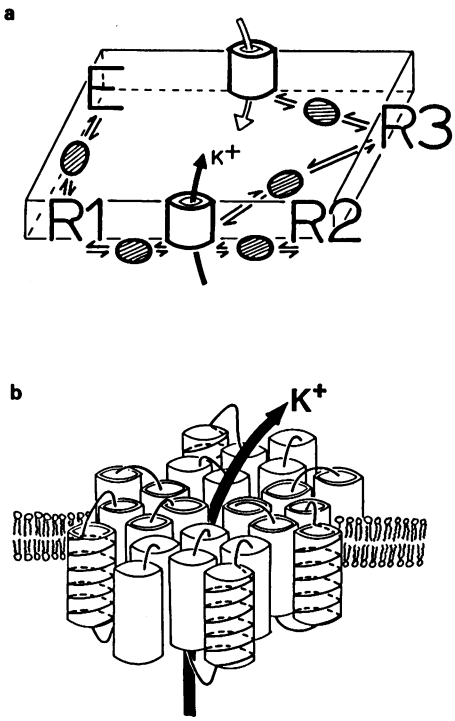


Figure 11 (a) Diagram to illustrate the way in which activation of several membrane receptors might open the same potassium channel. R1, R2 and R3 represent distinct receptors (for example, α_2-adrenoceptor, δ-opioid and somatostatin in Figure 5). The hatched egg-like symbols represent G-proteins (same or different species). The channel at the rear, and E, represent other membrane channels (e.g. calcium channels) and other effectors (e.g. adenylate cyclase) which might be affected by activated G-proteins. (b) Schematic representation of possible membrane topology of a rat brain potassium chanel (RBK1, see Christie *et al.*, 1989). Only transmembrane regions are shown, which are postulated to form pseudocylindrical α-helices. A single subunit is thought to contain six (or seven) membrane spanning regions. Analogy with the voltage-dependent sodium channel (Noda *et al.*, 1984) suggests that four such subunits may together form a potassium channel with a central conducting pore.

The molecules of inhibition

Let me close by making some remarks about the structure of the molecules themselves. The detailed structure of the potassium channel in question is unclear. However, in a collaboration with molecular biologists at the Vollum Institute we have recently isolated from the rat hippocampus a cDNA clone which we call RBK-1 (Christie *et al.*, 1989; see also Stuhmer *et al.*, 1988). The RBK-1 sequence was subcloned into a plasmid expression vector, and the mRNA transcripts synthesized *in vitro* were injected into *Xenopus* oocytes. One to four days later the oocytes expressed very large potassium currents that were not seen at all in uninjected cells. The predicted protein comprises 495 amino acids, has six or seven hydrophobic membrane spanning regions, including one with seven equally spaced positively charged residues that presumably serves as the voltage sensor (Figure 11b). This potassium channel protein is quite homologous to the central core region of the expected translation products of the *Shaker* locus of *Drosophila*, which also form potassium channels (Papazian *et al.*, 1987). It is our expectation that our isolated clone encodes the formation of one subunit, and that perhaps four such subunits (not necessarily identical) together form the potassium channel (Figure 11b). The details of the potassium conductance of oocytes injected with RBK-1 mRNA indicate that it is not likely to be the one which is

regulated by G-proteins; it is opened by depolarization rather than by hyperpolarization and has features of both the delayed rectifier and the A-types of potassium conductance. We are now working to isolate homologous clones in the hope of discovering more about potassium channels in general and perhaps finding the structure of the channel gated by activated G-protein that is used to inhibit neurones by the receptors of Table 1.

Finally, what about the receptors of this family? The structure of several has now been determined by molecular cloning [muscarinic M_2 (Peralta *et al.*, 1987), 5-HT_{1A} (Kobilka *et al.*, 1987a), platelet α_2 (Kobilka *et al.*, 1987b), kidney α_2 (Regan *et al.*, 1988), dopamine D_2 (Bunzow *et al.*, 1988)]. They all belong to the class of receptors having seven transmembrane regions, and share considerable homology with rhodopsin; those that couple to G_i preferentially seem to have large cytoplasmic loops (intracellular loop 3, linking transmembrane segments 5 and 6) and short C terminals. Rapid progress is to be expected in the determination of the tertiary structure of these and other receptors in this class.

This image of the receptor is rather different from that presented by Gaddum in 1926; his was a more mysterious view, based heavily on inference and the Law of Mass Action. Our affair with these receptors deepens daily. We shall soon know the roles of particular amino acids within them, and much more about the disposition of the individual family members. They seem unclothed before us, every bony peptide bond and proline kink intimately revealed. William Hazlitt wrote 'Though familiarity may not breed contempt, it takes the edge off admiration'. The drug receptors involved in neuronal inhibition are increasingly familiar to us, and we may admire them less. But the admiration for John Gaddum remains: without him we may never have set forth on this great romance.

The work described in this lecture has been a long term collaborative effort with many colleagues. Macdonald Christie, Mike Lacey, Annmarie Surprenant and John Williams contributed particularly to the experimental work illustrated; I am greatly indebted to them and to other past and present coworkers. The research has been supported by grants DK32979, DA03160, DA03161 and MH40416 from the U.S. Department of Health and Human Services.

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5-HT_{1D} binding sites in porcine brain can be sub-divided by GR43175

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We have examined the binding of 5-carboxamidotryptamine (5-CT) and GR43175 (3-(2-dimethylamino)ethyl-N-methyl-1H-indole-5-methane sulphonamide) to 5-HT_{1D} sites labelled with [^{3}H]-5-hydroxytryptamine ([^{3}H]-5-HT) in neonatal porcine caudate membranes. In competition studies, 5-CT produced shallow inhibition curves (K_i 138 nM, slope 0.31), indicating binding site heterogeneity, while GR43175 interacted with a single population of binding sites (K_i 251 nM, slope 0.98), producing a maximum of only 52% inhibition of [^{3}H]-5-HT binding compared to 100% for 5-HT or 5-CT. In the presence of excess GR43175 (10 μM), 5-CT produced a monophasic inhibition curve with a K_i value of 800 nM for the remaining sites (slope 0.89). These preliminary data suggest that under the conditions employed, GR43175, and to a lesser extent 5-CT, may discriminate between two sub-populations of 5-HT_{1D} binding sites in porcine brain.

Introduction The advent of a new generation of ligands for studying 5-hydroxytryptamine (5-HT) receptors has led to a large increase in the number of putative receptors (5-HT_1-like, 5-HT_2, 5-HT_3) and binding sites (5-HT_{1A}, $_{1B}$, $_{1C}$, $_{1D}$, 5-HT_2, 5-HT_3) for 5-HT (Bradley *et al.*, 1986; Fozard, 1987). As part of an ongoing study aimed at characterizing the properties of the novel 5-HT_1-like receptor agonist, GR43175 (3-(2-dimethylamino)ethlyl-N-methyl-1H-indole-5-methane sulphonamide) (Humphrey *et al.*, 1988), we have examined the effect of this compound at 5-HT_{1D} binding sites in membranes prepared from piglet brain. We now report that GR43175 appears to differentiate between two sub-populations of 5-HT_{1D} binding sites in porcine caudate nuclei.

Methods Male piglets (8–12 days old, weighing 1.5–4.5 kg) of the Large White variety were killed by captive-bolt pistol and exsanguinated. The brains were removed and the caudate nuclei used to prepare a crude membrane fraction as described by Heuring & Peroutka (1987). Binding assays were performed in triplicate by incubation for 30 min at 37°C using 100 μl of membrane suspension (2 mg protein ml^{-1}) in a total volume of 250 μl in a Tris-HCl buffer (50 mM, pH 7.4) containing L-ascorbic acid (0.1%), calcium chloride (4 mM), pargyline (10 μM) and [^{3}H]-5-HT (10 nM) (New England Nuclear, sp. act. 29.7 Ci mmol^{-1}; purity consistently greater than 95% by t.l.c.) This buffer also contained 8-hydroxy-2-di-n-propylamino tetralin (8-hydroxy-DPAT, 100 nM) and mesulergine (100 nM) to prevent labelling of 5-HT_{1A} and 5-HT_{1C} binding sites, respectively (Heuring & Peroutka, 1987; Waeber *et al.*, 1988). Assays were terminated by rapid vacuum filtration through glass-fibre filters (Whatman, GFB), and the trapped radioactivity counted by liquid scintillation spectroscopy in 10 ml of Picofluor TM30 scintillation fluid (Packard). Specific [^{3}H]-5-HT binding was defined as that inhibited by 10 μM 5-HT, and represented 40–60% of total [^{3}H]-5-HT binding. Analyses of binding data (disintegrations per min) were performed using the programmes LIGAND (Munson & Rodbard, 1980) and ALLFIT (DeLean *et al.*, 1978). Results are given as mean ± s.e.mean of at least three separate determinations. K_d and K_i are the equilibrium dissociation constants for the radioligand and inhibitor respectively, and B_{max} is the maximum binding capacity of the radioligand.

Results Under the conditions employed, [^{3}H]-5-HT appeared to label a single, homogeneous population of binding sites in piglet caudate nucleus, with a K_d of 19 ± 4 nM, B_{max} 0.36 ± 0.08 nM (corresponding to 158 ± 35 fmol mg^{-1} protein) and Hill coefficient (slope) 0.98 ± 0.01. The following compounds displayed little or no affinity (K_i values ⩾ 1000 nM) for these binding sites: 8-hydroxy-DPAT, mesulergine, spiperone, ketanserin, GR38032 and cyanopindolol. Unlabelled 5-HT produced a monophasic inhibition of specific [^{3}H]-5-HT binding (Figure 1), with a K_i value of 16 ± 5 nM and slope 0.95 ± 0.15. In marked contrast, however, 5-carboxamidotryptamine (5-CT) clearly produced shallow inhibition curves (Figure 1), with an apparent K_i of 138 ± 64 nM and slope 0.31 ± 0.24. Using the LIGAND programme, these curves could be fitted, albeit on the basis of limited data points, to a two-site model ($P < 0.01$ versus a one-site model)

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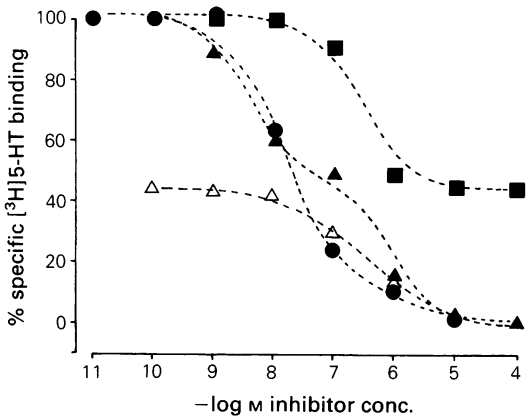


Figure 1 Inhibition of specific [^{3}H]-5-hydroxytryptamine ([^{3}H]-5-HT) binding to porcine caudate membranes by 5-HT (●), 5-CT (▲), GR43175 (■) and by 5-CT in the presence of 10 μM GR43175 (△). Data are from at least three independent determinations and the curves were computer-fitted using the LIGAND programme.

described by K_i values of 1.1 ± 0.4 nM and 2.5 ± 1.7 μM. The two sites represented $48 \pm 4\%$ and $52 \pm 4\%$ respectively of the total number of specific [^{3}H]-5-HT binding sites. GR43175 produced a monophasic inhibition of specific [^{3}H]-5-HT binding (K_i 251 ± 103 nM, slope 0.98 ± 0.17), but these curves consistently plateaued at $52 \pm 8\%$ inhibition when compared to 100% for 5-HT or 5-CT (Figure 1). When a maximally effective concentration of GR43175 (10 μM) was included in the assay, specific [^{3}H]-5-HT binding was reduced by $50 \pm 7\%$. Under these conditions, 5-CT appeared to produce a monophasic inhibition (slope 0.89 ± 0.20) with a K_i of 0.80 ± 0.71 μM.

Discussion The [^{3}H]-5-HT binding site examined in this preliminary study appeared to be of the 5-HT$_{1D}$ type on the basis of the conditions employed and the lack of affinity displayed by archetypal ligands for each of the other known 5-HT binding sites (Heuring & Peroutka, 1987). However, although 5-HT apparently recognised a single homogeneous population of binding sites, this did not appear to be the case for 5-CT, which produced shallow inhibition curves. Analyses of these curves suggested that 5-CT showed some 2000 fold selectivity for about 50% of the sites (K_i 1.1 nM) over the remainder (K_i 2500 nM). Clearly more extensive studies are needed to confirm these preliminary analyses which are based upon a limited number of data points, but nevertheless it would seem that 5-CT is binding to more than one population of sites. Notably, GR43175 appeared to inhibit the binding of [^{3}H]-5-HT to only one of these sites, with a Hill coefficient not significantly different from unity and maximum inhibition of binding of approximately 50%. Furthermore, in the presence of excess GR43175, the shallow inhibition curves (slope of 0.3) produced by 5-CT were converted to monophasic curves (slope of 0.89), yielding a K_i value for 5-CT of 800 nM. It is therefore tempting, on the basis of these early data, to suggest that neonatal porcine caudate '5-HT$_{1D}$' binding sites are comprised of two subpopulations, namely one that recognises GR43175 and has a high affinity for 5-CT, and another that shows little affinity for either 5-CT or GR43175. It remains to be seen if the same is true in the brains of other mammalian species. If so the 5-HT$_{1D}$ site as first described may actually comprise more than one population of binding sites, an observation that has previously been made (Waeber *et al.*, 1988). One might further speculate that the high affinity site for 5-CT and GR43175 may be equivalent to the 5-HT$_1$-like receptor in the dog saphenous vein (Humphrey *et al.*, 1988) while the site showing a low affinity for 5-CT could correlate with the 5-HT receptor found on vascular endothelial cells (Leff *et al.*, 1987; Sumner & Humphrey, 1988).

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Selective inhibition by vasocortin of histamine release induced by dextran and concanavalin-A from rat peritoneal cells

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Vasocortin, a glucocorticoid-induced anti-inflammatory protein, has been purified from the peritoneal lavage fluid of dexamethasone-treated rats. Vasocortin inhibited the release of histamine from rat peritoneal cells stimulated by dextran or concanavalin A but did not alter the release induced by calcium ionophore A23187 or compound 48/80. This selective effect exhibited by vasocortin mimics the glucocorticoid inhibition of histamine release from rat mast cells.

Introduction The anti-inflammatory effects of glucocorticoids depend on a common molecular mechanism through which they exert physiological effects, i.e. by inducing the synthesis of regulatory proteins (Di Rosa, 1984).

Vasocortin is a glucocorticoid-induced anti-inflammatory protein that we have recently identified in the peritoneal cavity of dexamethasone-treated rats (Carnuccio *et al.*, 1987). From its physicochemical and biological properties, vasocortin is distinct from lipocortins (Di Rosa *et al.*, 1984), a family of phospholipase A_2 inhibitory proteins that are also induced by glucocorticoids.

Both lipocortin and vasocortin are associated with the anti-inflammatory effect of glucocorticoids but their mode of action is different as some types of acute inflammation (e.g. rat dextran oedema) are inhibited by vasocortin but are unaffected by lipocortin (Di Rosa *et al.*, 1985). Since dextran oedema is predominantly mediated by the release of histamine and 5-hydroxytryptamine (Di Rosa & Willoughby, 1971), we suggested that vasocortin might interfere with the release of these mediators.

Now we show that partially purified vasocortin selectively inhibits histamine release from rat peritoneal cells induced by dextran or concanavalin A (Con A) but does not modify the release induced by calcium ionophore A23187 or compound 48/80.

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Methods Vasocortin was generated, collected and partially purified from the peritoneal lavage fluid of dexamethasone-treated rats following the procedure described by Carnuccio *et al.* (1987).

Mixed peritoneal cells (approx 5% mast cells) were recovered from male Wistar rats (200–250 g) according to Atkinson *et al.* (1979). Cells were washed and suspended in Tyrode solution (pH 7.2) having the following composition (mM): NaCl 137, KCl 2.7, $CaCl_2$ 1, $MgCl_2$ 1, $NaHCO_3$ 12, NaH_2PO_4 0.4 and glucose 5.6.

Aliquots of cell suspension (to a final volume of 1 ml) were allowed to equilibrate at 37°C in a metabolic shaker with gentle agitation and histamine release was initiated by the addition of the following agents (final concentration ml^{-1}): dextran, mol. wt. 70,000 (1 mg), Con A (5 μg), calcium ionophore A23187 (0.25 μg), compound 48/80 (3 μg).

Experiments with dextran and Con A were carried out in presence of phosphatidylserine 50 $\mu g\,ml^{-1}$. Vasocortin was added to the cell suspension 10 min before the inducer. The release was terminated after 10 min by addition of 2 ml ice-cold Tyrode solution.

Cells and supernatants were recovered by centrifugation (10 min, 150 *g*, 4°C) and histamine concentrations in solutions and cells were quantified fluorimetrically (Shore *et al.*, 1959).

Histamine release was calculated as a percentage of the total cellular content of the amine. All values were corrected for the spontaneous release occurring in the absence of the inducer (approx 5%). Results are expressed as a percentage of the control release (that occurring in the absence of vasocortin).

Results The effect of partially purified vasocortin on histamine release from rat peritoneal cells stimulated by various agents is shown in Figure 1.

Histamine release induced by dextran was moderately reduced (15%) by 12.5 $\mu g\,ml^{-1}$ vasocortin while

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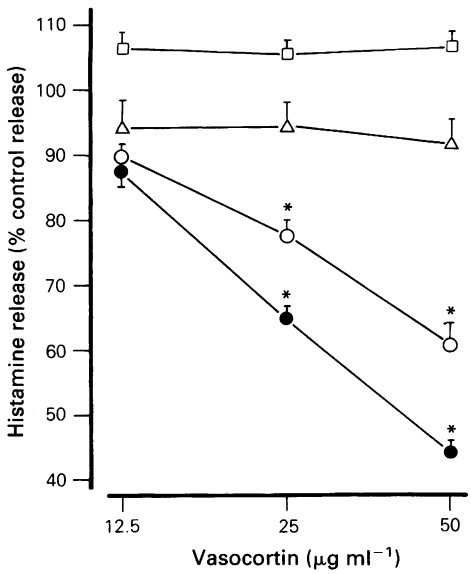


Figure 1 Effect of vasocortin on the histamine release from rat peritoneal cells induced by dextran 1 mg ml^{-1} (●), Con A 5 μg ml^{-1} (○), calcium ionophore A23187 0.25 μg ml^{-1} (△) and compound 48/80 3 μg ml^{-1} (□). Cells were preincubated (10 min, 37°C) with vasocortin and then challenged. The points are the means of three experiments and vertical bars show s.e. mean where *$P < 0.01$. Control histamine releases (% total histamine in absence of vasocortin) were: dextran 42.6 ± 4.2, Con A 29.2 ± 1.1, calcium ionophore A23187 44.6 ± 2.2, compound 48/80 44.1 ± 2.3.

25 and 50 μg ml^{-1} caused a substantial inhibition of the release (35% and 56% respectively, $P < 0.01$).

Vasocortin 12.5 μg ml^{-1} slightly inhibited (by 10%) Con A-induced histamine release and this was significantly reduced by 25 and 50 μg ml^{-1} (23% and 40% respectively, $P < 0.01$). In contrast vasocortin did not significantly alter the release of histamine induced by either calcium ionophore A23187 or compound 48/80.

In further experiments carried out without phosphatidylserine in the incubation medium, vasocortin (12.5–50 μg ml^{-1}) exhibited a comparable concentration-related degree of inhibition of the lower degree of histamine release induced by Con A ($n = 2$). Furthermore the histamine release induced by calcium ionophore A23187 was not affected by vasocortin (50 μg ml^{-1}) when phosphatidylserine was present in the incubation medium ($n = 3$).

Discussion Glucocorticoids inhibit histamine release from human basophils and rat mast cells activated by immunological stimuli (Schleimer *et al.*, 1981; Heiman & Crews, 1984). In both systems the inhibition is time-dependent and the inhibitory potencies exhibited by various glucocorticoids parallel their affinities for the cytosolic receptor suggesting that the induction of specific proteins is an important component of the glucocorticoid inhibition of histamine release.

Recently, we identified vasocortin, a glucocorticoid-induced protein distinct from lipocortin, which inhibits rat dextran oedema following both local or systemic administration (Carnuccio *et al.*, 1987). The present study demonstrates that vasocortin inhibits histamine release from rat peritoneal cells induced by dextran or Con A. This inhibition is concentration-related and is selective since it does not occur when cells are stimulated by calcium ionophore A23187 or compound 48/80.

Similar selective inhibition of histamine release from rat mast cells is exhibited by glucocorticoids,, which are able to reduce the histamine release induced by IgE-type secretagogues like Con A, while not altering the release induced by calcium ionophore A23187 or compound 48/80 (Heiman & Crews, 1984). Thus vasocortin mimics the glucocorticoid inhibition of histamine release from rat mast cells.

Both dextran and Con A appear to act by cross-linking specific receptors on the cell membrane, are dependent on extracellular calcium and potentiated by phosphatidylserine (Siraganian & Siraganian, 1975; Baxter & Adamik, 1978; Moodley *et al.*, 1982). Furthermore Con A stimulates phospholipid methylation and phosphatidylserine decarboxylation in rat mast cells suggesting that the synthesis and degradation of methylated lipids are an intrinsic part of the biochemical mechanism that modulates histamine release from these cells (Hirata *et al.*, 1979).

The present results and our previous studies (Di Rosa *et al.*, 1985) strongly support the concept that the anti-inflammatory effects of glucocorticoids may depend on the induction of (at least) two species of regulatory proteins: lipocortin which inhibits arachidonic acid availability and vasocortin which prevents histamine release. The mechanism of the selective inhibition exhibited by vasocortin on the histamine release by dextran and Con A, as well as its role in allergic reactions deserve further investigation.

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Increase in gastric secretion induced by 2-deoxy-D-glucose is impaired in capsaicin pretreated rats

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Gastric acid secretion was determined following intravenous administration of 2-deoxy-D-glucose (2-DG; 60 mg kg^{-1}) or electrical stimulation of the vagus nerve in urethane-anaesthetized rats pretreated when newborn with either capsaicin or the vehicle. The secretory response to 2-DG was substantially reduced in the capsaicin pretreated rats, while that induced by electrical vagal stimulation (1 mA, 1 ms, 3 Hz) was unaffected.

These results suggest that capsaicin-sensitive fibres are involved in the afferent branch of the reflex response activated by 2-DG to stimulate gastric acid secretion.

Introduction There is morphological and functional evidence indicating that the stomach receives an afferent innervation sensitive to the neurotoxin capsaicin and the physiological role of these nerves has recently received considerable attention (see Maggi & Meli, 1988, for review).

Sensory denervation by capsaicin did not influence carbachol- (Alfoldi *et al.*, 1986) or bethanechol- (Raybould & Taché, 1988) induced increase in gastric acid secretion (GAS) while controversial results have been reported for histamine- (Alfoldi *et al.*, 1986; Raybould & Taché, 1988) and pentagastrin-induced (Alfoldi *et al.*, 1986; Dugani & Glavin, 1986; Raybould & Taché, 1988) increase in GAS. When GAS was stimulated by gastric distension, an indirect vago-vagal reflex was activated and sensory denervation induced by capsaicin inhibited the response (Raybould & Taché, 1988).

In view of these findings, it appeared worthwhile to determine the influence of systemic capsaicin sensory denervation on the increase in GAS induced by a central stimulant 2-deoxy-D-glucose (2-DG), that is considered to act through vagal stimulation (Hirschowitz & Sachs, 1965; Colin-Jones & Himsworth, 1970). The stimulation of GAS by electrical stimulation of the vagus nerve was also studied to determine whether the vagal efferent branch was sensitive to capsaicin.

Methods Male and female albino rats, Sprague-Dawley Nossan strain, were housed at constant room temperature (21 ± 1°C), relative humidity (60%) and with 12 h light-dark cycle (light on 06 h 00 min). The animals were deprived of food for 20 h before the experiments but allowed free access to tap water.

Capsaicin was administered subcutaneously (50 mg kg^{-1}), under ether anaesthesia, on the second day of life. This dose was reported to cause a permanent degeneration of unmyelinated afferent neurones (Maggi & Meli, 1988) and a marked depletion of calcitonin gene-related peptide-like immunoreactivity in the stomach (Evangelista & Renzi, unpublished data; see also Sternini *et al.*, 1987). Control animals received equal volumes of vehicle (10% ethanol, 10% Tween 80 and 80% saline, v/v/v). The animals were used two months later.

Acid secretion was determined according to Ghosh & Schild (1958) in urethane (1.5 g kg^{-1} s.c.)-anaesthetized rats, maintained at 36–37°C by means of a heating lamp. The animals were tracheotomized and polyethylene tubes inserted into the stomach lumen through the œsophagus and the duodenum. The stomachs were flushed with 50 ml of warm saline to remove any solid material and thereafter perfused at a rate of 0.8–0.9 ml min^{-1} by means of a peristaltic pump (De Saga, Heidelberg, F.R.G.). A period of 45 min was allowed for equilibration after all surgical procedures had been completed. After collection of two samples, acid secretion was stimulated by intravenous (via the jugular vein) administration of 2-deoxy-D-glucose (Sigma; 60 mg kg^{-1}), dissolved in saline in a volume of 1 ml kg^{-1} or by electrical stimulation of the vagus nerve. In this latter case, the animals were artificially ventilated by means of a ventilator (Basile, Varese, Italy) for small rodents (60 strokes min^{-1}, 0.8 ml 100 g^{-1} body wt). The vagi were cut bilaterally at cervical level and the peripheral end of the left vagus was placed on a bipolar hook-shaped platinum electrode and stimulated by means of an electronic stimulator (WPI, New Haven, CT, U.S.A.) with square wave pulses of 1 ms duration, at 3 Hz and 1 mA.

Acid output was determined at 15 min intervals by titration of the perfusate with 0.005 N NaOH to pH 7 using a digital pH meter (Radiometer, Copenhagen, Denmark).

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Statistical analysis was performed by means of Student's t test for unpaired data.

Results Mean basal acid output of controls or that from capsaicin pretreated rats did not differ significantly (Figure 1a, b). Intravenous 2-DG (60 mg kg^{-1}) produced a significant increase in GAS as compared to pre-injection values which reached maximal values between 75–105 min after administration (Figure 1a).

In capsaicin pretreated rats, 2-DG elicited only a slight increase in GAS, which was not significantly different from pre-injection values. The increase in GAS induced by 2-DG was significantly different from that in control animals at all time periods (Figure 1a).

As shown in Figure 1b, the increase in GAS induced by electrical vagal stimulation (1 mA, 1 ms, 3 Hz) reached its maximal values 30 min after the beginning of the stimulation. The increase in GAS produced by vagal stimulation in capsaicin-pretreated rats was not significantly different from that observed in vehicle-treated animals at any time tested (figure 1b).

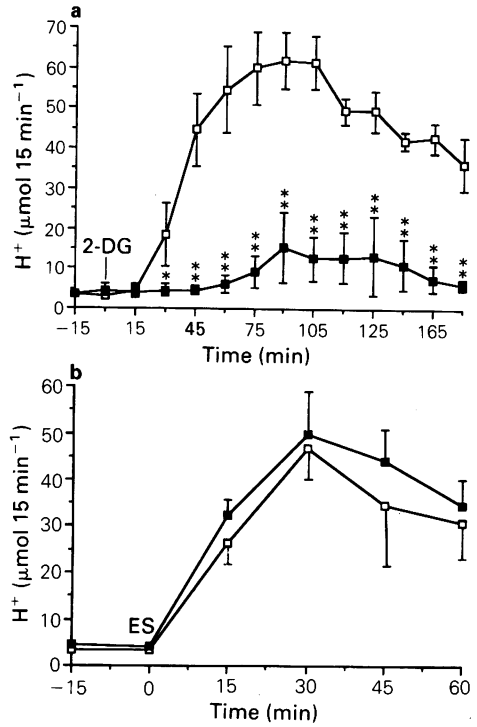


Figure 1 Effect of 2-deoxy-D-glucose (2-DG, 60 mg kg^{-1} i.v., a) or electrical stimulation (ES) of the vagus nerve (b) on gastric acid secretion (mean of at least 5 animals, s.e. indicated by vertical bars) in rats pretreated with capsaicin (■) or the vehicle (□). Statistically significant differences from the control groups are shown as * $P < 0.05$; ** $P < 0.01$.

Discussion The influence of the central nervous system in regulating GAS is well known. Electrical or pharmacological stimulation of selective brain areas, in particular the hypothalamus can induce changes in GAS mediated through the vagus nerve (see Taché, 1987, for review). Our results show that capsaicin pretreatment abolished 2-DG-induced increase in GAS but not increase in GAS induced by direct vagal stimulation. The increase in GAS elicited by 2-DG administration has been reported to be due to activation of chemoreceptors in the lateral hypothalamic area (Colin-Jones & Himsworth, 1970) which initiate and sustain a vagally-mediated response (Hirschowitz & Sachs, 1965). Neurotoxic damage at hypothalamic level has been described after sensory denervation with capsaicin (Ritter & Dinh, 1988), although a specific effect on the lateral hypothalamic area has not been reported. In conclusion, the present findings indicate that capsaicin-sensitive nerves play a crucial role in the reflex increase in GAS produced by chemically induced glucopenia brought about by 2-DG. Further studies are needed to assess whether the site of action of capsaicin in preventing the GAS response to 2-DG is central or might also have a peripheral component. In either case, the present data indicate that the efferent vagal control of GAS is unaffected by capsaicin pretreatment.

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Prostacyclin mediates antiaggregatory and hypotensive actions of endothelin in anaesthetized beagle dogs

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The effects of endothelin on blood pressure and *in vivo* aggregation of platelets were studied in anaesthetized beagle dogs. Intravenous administration of endothelin (0.03–0.3 nmol kg^{-1}) resulted in a dose-dependent transient hypotension followed by a long-lasting hypertension and inhibition of platelet aggregation. These changes were accompanied by dose-dependent elevation of plasma 6-keto prostaglandin $F_{1\alpha}$ levels. Pretreatment of the animals with acetylsalicylic acid significantly attenuated both the vascular and antiaggregatory responses to endothelin. These data provide evidence for *in vivo* release of prostacyclin by endothelin in anaesthetized dogs.

Introduction Endothelin isolated from porcine aortic endothelial cells (Yanagisawa *et al.*, 1988) has been demonstrated to be a potent constrictor of vascular smooth muscle in a variety of species *in vivo* and *in vitro* (Yanagisawa *et al.*, 1988; DeNucci *et al.*, 1988; Goetz *et al.*, 1988; Miller *et al.*, 1989). Endothelial cells, on the other hand, release both prostacyclin (Moncada *et al.*, 1976) and endothelium-derived relaxing factor (Furchgott & Zawadzki, 1980), which induce relaxation of the vascular smooth muscle. Thus, the production by the endothelium of substances with opposing vascular effects suggest it has a complex role in the regulation of vascular tone. In addition, endothelin inhibits *ex vivo* platelet aggregation in rabbits and this antiaggregatory effect of endothelin is blocked by indomethacin suggesting the involvement of prostanoids (Thiemermann *et al.*, 1988). In the present paper we present evidence that the *in vivo* antiaggregatory and hypotensive actions of endothelin are mediated by prostacyclin release.

Methods Experiments were performed on anaesthetized (sodium pentobarbitone, 30 mg kg^{-1}, i.v.) beagle dogs of either sex. Preparation of the animals has been described in detail elsewhere (Hermán *et al.*, 1986). Briefly, catheters were inserted into the left femoral artery and vein for monitoring mean arterial blood pressure and blood collection, respectively. An arteriovenous bypass was formed between the right femoral artery and vein. The arterial blood was directed through an extracorporeal filter with 30 μm pores by a roller pump. A few minutes after pumping the blood through the filter the perfusion pressure, measured proximal to the filter, started to increase linearly as a result of spontaneous platelet aggregation (Hermán *et al.*, 1986). Mean arterial blood pressure and filter pressure were monitored continuously by electromanometers (EM 31, Medicor, Budapest, Hungary) using Statham P 50 and P 23dB strain gauges (Hato Rey, Puerto Rico), respectively. After control measurements, porcine endothelin (Peninsula, 0.03–0.3 nmol kg^{-1} body wt) was given intravenously as a bolus. Four minutes after administration of endothelin 5 ml of blood was collected into plastic tube containing indomethacin (final concentration 10 μM).

Blood was mixed with [^{3}H]-prostaglandin $F_{1\alpha}$ ([^{3}H]-$PGF_{1\alpha}$) (4000 d.p.m.) for monitoring procedural losses and then prostanoids were extracted according to the method of Green *et al.* (1978). 6-keto-$PGF_{1\alpha}$ levels were measured by radioimmunoassay (IZINTA, Hungary). The antiserum had less than 0.9% cross reactivity with other prostanoids. Intraassay coefficient of variation was 4.6%. Values were corrected for individual recovery.

Results are expressed as means ± s.e.mean. Statistical analysis of the data was performed by one way analysis of variance using ranks (Friedman's test) followed by Wilcoxon–Wilcox's test to identify differences between groups (i.e. control versus different doses of endothelin).

Results Intravenous administration of endothelin resulted in a dose-dependent transient hypotension followed by long-lasting elevation in mean arterial blood pressure. The maximum falls in mean arterial blood pressure were 2 ± 1, 21 ± 15 and 29 ± 13 mmHg ($n = 4$) corresponding to 0.03, 0.1

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Table 1 Effects of endothelin on mean arterial blood pressure (MABP), filter occlusion rate (FOR), filter pressure (FP) and venous 6-keto prostaglandin $F_{1\alpha}$ (6-keto $PGF_{1\alpha}$) concentrations in anaesthetized beagle dogs

	n	*For* (mmHg s^{-1})	*Decrease in FP* (mmHg)	*MABP* (mmHg)	*6-keto $PGF_{1\alpha}$* (ng ml^{-1} plasma)
Vehicle	6	—	—	—	0.68 ± 0.14
ET, 0.03 nmol kg^{-1}	4	0.25 ± 0.04	0	2 ± 1	0.60 ± 0.10
ET, 0.1 nmol kg^{-1}	4	0.70 ± 0.1*	9.5 ± 4*	21 ± 15*	1.04 ± 0.25
ET, 0.3 nmol kg^{-1}	6	1.41 ± 0.1*	34.1 ± 13*	29 ± 13*	1.35 ± 0.16*
ASA plus ET, 0.3 nmol kg^{-1}	4	0.12 ± 0.03§	0§	3 ± 1§	0.23 ± 0.15§

Animals were prepared as described under Methods. Four min after intravenous injection of various doses of endothelin (ET) blood was collected for determination of plasma 6-keto $PGF_{1\alpha}$ levels. Acetylsalicylic acid (ASA) was administered i.v. 30 min prior to injection of endothelin. Values are means ± s.e.mean. *n*, number of independent experiments. *$P < 0.05$ (compared to vehicle), §$P < 0.05$ (compared to ET, 0.3 nmol kg^{-1}).

and 0.3 nmol kg^{-1} of endothelin and the hypotensive effect lasted for 5–15, 10–90 and 40–180 s, respectively. These changes were accompanied by changes in filter pressure. The lowest dose of endothelin decreased the slope of the elevation of filter pressure on average for 52 s, 0.1 nmol kg^{-1} of endothelin stabilized filter pressure for 96 s, whereas the highest dose of endothelin employed reversed the spontaneous increase in filter pressure (average decrease was 19 mmHg), suggesting a disaggregatory action. This effect lasted on average for 182 s. In addition, endothelin enhanced venous 6-keto-$PGF_{1\alpha}$ concentrations in a dose-dependent fashion (Table 1).

When administration of endothelin (0.3 nmol kg^{-1}) was repeated in dogs pretreated with acetylsalicylic acid (Aspisol, Bayer, FRG, 25 mg kg^{-1}, i.v. 30 min before administration of endothelin) neither hypotension nor alteration of time course of changes in filter pressure were observed (Table 1).

Discussion The present study shows that in addition to its well established vasoconstrictor property, endothelin can exert hypotensive and *in vivo* antiaggregatory effects in anaesthetized beagle dogs. Several lines of evidence suggest that these actions of endothelin can be attributed to enhanced prostacyclin formation rather than to its direct effect. First, the hypotensive and antiaggregatory actions of endothelin were accompanied by a parallel rise in plasma 6-keto-$PGF_{1\alpha}$ levels and endothelin has been reported to release prostacyclin from guinea-pig and rat isolated lungs (DeNucci *et al.*, 1988). Secondly, acetylsalicylic acid at a dose that blocked prostacyclin formation, significantly attenuated the hypotensive and completely prevented the antiaggregatory responses to endothelin. Whether the slight decrease in mean arterial pressure after injection of endothelin in acetylsalicylic acid-pretreated animals was due to release of endothelium-derived relaxing factor is not known. Thirdly, the concentrations of prostacyclin measured in the present experiments were similar to those seen after administration of exogenous prostacyclin necessary to stabilize and/or reverse the spontaneous increase in filter pressure (Hermán *et al.*, 1986). Fourthly, endothelin (up to a concentration of 250 nM) added *in vitro* to platelet-rich plasma prepared from beagle dogs failed to modify the aggregatory response to ADP, platelet-activating factor or arachidonic acid (Filep *et al.*, unpublished observations). Fifthly, endothelin activates phospholipase A_2 with subsequent metabolism of arachidonic acid (Resink *et al.*, 1989).

In conclusion, the present findings provide direct evidence for *in vivo* release of prostacyclin by endothelin and strongly suggest that endothelin-induced immediate hypotension and antiaggregatory effects may be mediated through release of prostacyclin in anaesthetized beagle dogs.

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Inhibition by neuropeptides of interleukin-1β-induced, prostaglandin-independent hyperalgesia

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The cytokine interleukin-1β (IL-1β) is a potent hyperalgesic agent in the rat whereas IL-1α is relatively inactive (Ferreira *et al.*, 1988). IL-1β induced a dose-dependent increase in the sensitivity of rat paws to mechanical stimulation following intra-plantar injection but this effect was not reduced by indomethacin ($1.0\,mg\,kg^{-1}$, p.o.), at a dose known to inhibit completely prostaglandin synthesis in the rat (Salmon *et al.*, 1983). Prostaglandin (PG)E_2 enhanced sensitivity to both mechanical pressure and increased temperature but IL-1β enhanced only sensitivity to pressure. These observations indicate that IL-1β sensitized pressure-sensitive but not temperature-sensitive sensory neurones, through a prostaglandin-independent mechanism. Hyperalgesia induced by IL-1β but not PGE_2, was inhibited by the neuropeptide melanocyte-stimulating hormone (αMSH) and its analogue [Nle^4, D-Phe^7] αMSH which are known to antagonize IL-1 responses in other systems (Holdeman & Lipton, 1985; Cannon *et al.*, 1986). IL-1β-induced hyperalgesia was also reduced by the putative IL-1β antagonist Lys-D-Pro-Thr (Ferreira *et al.*, 1988) but αMSH and its analogue were 10–50 times more potent.

Introduction Interleukin-1 has been implicated as a key mediator of inflammation and host defence mechanisms in infection. It is a potent pyretic agent (Bernheim *et al.*, 1980) which also enhances lymphocyte proliferation (Gery *et al.*, 1972), stimulates prostaglandin E_2 (PGE_2) production (Dayer, 1985), causes the accumulation of inflammatory leukocytes and induces proteoglycan loss from articular cartilage (Pettipher *et al.*, 1986). In each of these properties, IL-1α and IL-1β are approximately equi-active. More recently, however, it has been shown that IL-1β is a potent nociceptive agent and that IL-1α does not share this property (Ferreira *et al.*, 1988). We have now investigated the hyperalgesia induced by IL-1β and have shown that it is unlikely to be mediated solely through prostaglandin synthesis and that IL-1β may act directly to stimulate certain sensory nerves but not others. Furthermore, IL-1β-induced hyperalgesia is inhibited by the neuropeptide melanocyte-stimulating hormone (αMSH), providing further evidence for the link between immune responses and the central nervous system (Blalock *et al.*, 1985).

Methods Increased sensitivity to pressure was measured following intra-plantar (i.pl.) injections of IL-1α, IL-1β or PGE_2 into the hind paws of male Wistar rats (110–130 g). In a modified Randall and Sellito test (Ferreira *et al.*, 1988), the time taken for each hind paw to respond to a constant pressure of 20 mmHg was measured before and 1 h after injection. Injections (100 μl i.pl.) were given to groups of 6 male Wistar rats (110–130 g), the right hind paw receiving IL-1 or PGE_2 and the left hind paw, the same volume of saline. Hyperalgesia was expressed as the difference in reaction time before and after injection. Recombinant human IL-1α and IL-1β were obtained from the National Institute of Biological Standards and Control, London or were prepared in the Wellcome Research Laboratories and dissolved in sterile saline containing 0.01% low endotoxin foetal calf serum (Hyclone, Utah) before injection.

The effects of PGE_2 and IL-1β on sensitivity to heat were measured by a hot-plate test (Woolfe & MacDonald, 1944). IL-1β or PGE_2 were injected in volumes of 20 μl into each hind paw of female CD1 mice (20–30 g) in groups of 5. The animals were then placed on the surface of the copper base of a Perspex box partially immersed in a water bath at 50°C and observed for signs of discomfort for a maximum period of 30 s. Response times were recorded before and after intra-plantar injections of IL-1β or PGE_2.

In some experiments, groups of animals were dosed orally with indomethacin 1 h before intra-plantar injection or intravenously with αMSH, [Nle^4,D-Phe^7]αMSH or Lys-D-Pro-Thr dissolved in saline and administered immediately prior to intra-plantar injection. Indomethacin was obtained from

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Merck Sharpe and Dohme, αMSH and [Nle^4,D-Phe^7]αMSH were obtained from Sigma and the tripeptide Lys-D-Pro-Thr was synthesized at the Wellcome Research Laboratories.

Results Intraplantar injection of IL-1α (0.5–500 pg) or IL-1β (0.005–5.0 pg) caused a dose-dependent decrease in the time taken for rats to respond to a constant pressure of 20 mmHg applied to the injected paws. Intraperitoneal administration of IL-1β (0.5–50 pg kg^{-1}) caused a dose-dependent and bilateral hyperalgesia in both hind paws but intraplantar administration of IL-1β to a single paw did not cause a bilateral effect. A half-maximal response was obtained with 0.05 pg IL-1β i.pl. which was more than 1000 times more potent than IL-1α. Oral administration of indomethacin at a dose (1 mg kg^{-1}) which is known to inhibit completely prostaglandin synthesis in the rat (Salmon *et al.*, 1983), had no significant effect on IL-1β-induced hyperalgesia. Higher doses (3–10 mg kg^{-1}) reduced the hyperalgesia by up to 35%.

Hyperalgesia induced by IL-1β (0.5 pg) was reduced dose-dependently by the peptide Lys-D-Pro-Thr administered intravenously immediately before intra-plantar injection of IL-1β (Figure 1). The neuropeptide αMSH and its analogue (Nle^4,D-Phe^7]-αMSH had a similar effect but were up to 50 times more potent. High doses of each of these peptides completely abolished the hyperalgesia induced by IL-1β.

Injections of PGE_2 (1–100 ng i.pl.) also caused a dose-dependent hyperalgesia which was not significantly reduced by indomethacin (10 mg kg^{-1}, p.o.), αMSH (0.5 mg kg^{-1}, i.v.) or Lys-D-Pro-Thr (3 mg kg^{-1}, i.v.). The dose-dependent inhibition of IL-1β-induced hyperalgesia by Lys-D-Pro-Thr (0.1–3 mg kg^{-1}, i.v.) was not modified by the opiate antagonist naloxone (1 mg kg^{-1}) indicating that Lys-D-Pro-Thr is not acting through an opioid receptor.

In groups of mice placed on a 50°C hot plate, the mean response time of 24.9 ± 1.05 s ($n = 10$) was not significantly changed by prior injection of the hind paws with saline. PGE_2 (0.03–1.0 μg i.pl.) caused a dose-dependent decrease in the time to respond which at a dose of 1.0 μg was 17.4 ± 0.67 s ($P < 0.005$). IL-1β (0.5–50 pg) did not significantly change the times to respond. αMSH (3 mg kg^{-1}, i.v.) had no effect on the hyperalgesia caused by PGE_2 in the hot plate test.

Discussion These results indicate that IL-1β stimulated pressure but not temperature sensory neurones

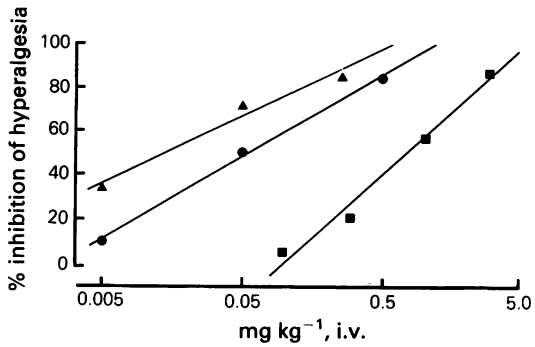


Figure 1 Inhibition of interleukin-1β (IL-1β)-induced hyperalgesia in rat paws by [Nle^4,D-Phe^7]αMSH (▲), αMSH (●) or Lys-D-Pro-Thr (■). Hyperalgesia was induced by intraplantar-injection of 0.5 pg IL-1β. The mean response time in paws before injection was 35.10 ± 0.33 s ($n = 6$) and this was reduced to 19.87 ± 0.36 s 1 h after injection of 0.5 pg IL-1β. Inhibition for each treatment was calculated as a percentage of the response obtained from vehicle-treated control groups. The dose-response data for each peptide were subjected to linear regression analysis and the line of best fit calculated. Each point is the mean of 6 measurements and the ED_{50} values with 95% confidence limits are as follows: [Nle^4,D-Phe^7]αMSH = 0.015 (0.007–0.030) mg kg^{-1}; αMSH = 0.052 (0.026–0.11) mg kg^{-1}; Lys-D-Pro-Thr = 0.72 (0.53–1.01) mg kg^{-1}.

to produce hyperalgesia. In a previous study, local administration of indomethacin (100 μg, i.pl.) markedly reduced IL-1β-induced hyperalgesia but had a much smaller effect on the contralateral paw (Ferreira *et al.*, 1988). This dose is equivalent to 0.7 mg kg^{-1} and is sufficiently large to have systemic effects (Salmon *et al.*, 1983). It is possible, therefore, that the rather small effect in the contralateral paw was due to the inhibition of prostaglandin synthesis and that the greater reduction of hyperalgesia in the injected paw was due to a non-specific high dose phenomenon. This interpretation, coupled to the weak activity of indomethacin reported in the present study, suggests that IL-1β hyperalgesia is largely prostaglandin-independent.

The fever associated with infection is mediated by IL-1 release (Bernheim *et al.*, 1980) and it is also known that during fever, the αMSH levels in the septum region of the brain are elevated (Samson *et al.*, 1981). Since αMSH is anti-pyretic, it has been suggested that production of αMSH in the central nervous system could be an endogenous control mechanism in response to pyrexia. We have now shown that αMSH selectively reduces IL-1β-induced hyperalgesia. It is possible that this property also

represents an important endogenous regulatory mechanism. If production of IL-1β during the acute phase response to infection and inflammation is responsible for the increased irritability and malaise associated with these conditions, then drugs which inhibit the action of IL-1β could have therapeutic benefit. The activity of αMSH, its analogue and the tri-peptide previously described (Ferreira *et al.*, 1988) represent structural leads to novel pharmaceutical agents in this area.

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Evidence for tonic activation of prejunctional β-adrenoceptors in guinea-pig pulmonary arteries by adrenaline derived from the adrenal medulla

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1 The effects of (±)-carteolol 10^{-8} M to 10^{-6} M, a non-selective β-antagonist, applied cumulatively, on stimulation-evoked ^{3}H-release at 1 Hz were studied in pulmonary arteries isolated from guinea-pigs. The guinea-pigs were subjected to either bilateral adrenalectomy, adrenalectomy followed by injections of deoxycorticosterone acetate (DOCA) and hydrocortisone, bilateral adrenodemedullation or a sham operation, and then loaded *in vitro* with [^{3}H]-noradrenaline.

2 Carteolol inhibited ^{3}H-output in arteries from sham-operated animals in a concentration-dependent manner. This inhibitory effect was not found in pulmonary arteries from animals subjected to adrenalectomy or adrenodemedullation. However, DOCA and hydrocortisone pretreatment, did not prevent the disappearance of the carteolol-induced inhibition of ^{3}H-release.

3 Adrenalectomy and adrenodemedullation depleted or markedly reduced the endogenous contents of adrenaline in pulmonary arteries without altering the levels of dopamine and noradrenaline.

4 It is concluded that adrenaline, mainly derived from the adrenal medulla, acts as an endogenous agonist for tonically functioning prejunctional β-adrenoceptors in guinea-pig pulmonary arteries, probably by being taken up and co-released with noradrenaline.

Introduction

It has been proposed that there is a positive feedback regulation of the release of the transmitter noradrenaline via prejunctional β-adrenoceptors (Adler-Graschinsky & Langer, 1975; Langer 1977; Dahlöf, 1981; Majewski, 1983; Misu & Kubo, 1986). These adrenoceptors were thought to be activated by circulating adrenaline (Stjärne & Brundin, 1975) and/or adrenaline taken up and co-released with noradrenaline (Majewski *et al.*, 1980; 1981) rather than by noradrenaline itself released from noradrenergic nerve endings (Adler-Graschinsky & Langer, 1975). However, the endogenous agonist has not been identified. Guimarães *et al.* (1978) showed that in dog saphenous veins propranolol reduced the evoked ^{3}H-overflow from strips loaded with [^{3}H]-adrenaline but not from those loaded with [^{3}H]-noradrenaline. Majewski *et al.* (1980, 1981) found that in guinea-pig atria and rat atria preincubated with unlabelled adrenaline, and then loaded with [^{3}H]-noradrenaline, propranolol and other β-adrenoceptor antagonists alone decreased the evoked ^{3}H-release, while this decrease was not seen after preincubation with unlabelled noradrenaline. From these findings, they proposed that adrenaline is taken up, stored, released as a co-transmitter with noradrenaline, and then activates prejunctional β-adrenoceptors. We have shown that carteolol, a non-selective β-adrenoceptor antagonist (Nakagawa *et al.*, 1974), readily and stereoselectively inhibits the evoked release of noradrenaline from guinea-pig pulmonary arteries in the absence of any agents (Kuwahara *et al.*, 1986). In the present experiments, by use of carteolol as a tool, we have attempted to clarify the identity of the endogenous agonist for prejunctional β-adrenoceptors in pulmonary arteries, from guinea-pigs subjected to adrenalectomy, adrenodemedullation or sham operation.

Methods

Animals and operative procedures

Bilateral adrenalectomy, bilateral adrenodemedullation or sham operations were carried out under ether anaesthesia on male guinea-pigs weighing 200 to 250 g. Some adrenalectomized guinea-pigs were injected s.c. with deoxycorticosterone acetate

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Table 1 Effects of (±)-carteolol (10^{-8} M, 10^{-7} M and 10^{-6} M), applied cumulatively, on stimulus-evoked ^{3}H-release in pulmonary arteries isolated from guinea-pigs 5 days after either bilateral adrenalectomy or sham operation

Group	n	S_1	S_2 (S_2/S_1)	S_3 (S_3/S_1)	S_4 (d.p.m. mg^{-1}) (S_4/S_1) (%)
Sham-operated					
Control	10	1059.6 ± 69.6	1092.2 ± 56.2 (104.6 ± 3.9)	1079.8 ± 61.6 (102.7 ± 2.2)	1021.6 ± 52.8 (97.7 ± 3.4)
Carteolol	8	903.0 ± 109.6	864.6 ± 99.4 (97.4 ± 4.6)	825.2 ± 103.1**† (91.3 ± 2.2¶¶)	770.3 ± 99.8**† (84.7 ± 3.6¶§)
Adrenalectomized					
Control	11	971.8 ± 111.3	972.3 ± 100.6 (102.5 ± 4.0)	970.8 ± 101.2 (102.3 ± 3.8)	912.2 ± 86.8 (97.2 ± 4.3)
Carteolol	8	837.9 ± 49.2	839.6 ± 58.6 (100.2 ± 3.0)	823.4 ± 61.3 (98.2 ± 4.1)	791.6 ± 51.0* (94.4 ± 2.5)

Strips were incubated for 1 h in 10^{-7} M [^{3}H]-noradrenaline, washed, set up and then superfused with Krebs medium. Supramaximal transmural stimuli were applied 4 times at 1 Hz for 100 s, 90, 105, 120 and 135 min after the start of superfusion (S_1 to S_4). Above-mentioned concentrations of carteolol were applied cumulatively immediately after the S_1, S_2 and S_3 period of stimulation, respectively. Data shown are mean ± s.e.mean of n absolute values of evoked ^{3}H-release at S_x and of release ratios, S_x/S_1 expressed as percentage in parentheses. Significantly different: *$P < 0.05$, **$P < 0.01$, compared to S_1 (paired t test); †$P < 0.05$, compared to corresponding control (Student's t test); ¶$P < 0.05$, ¶¶$P < 0.01$, compared to corresponding control, and §$P < 0.05$, compared to carteolol in arteries from adrenalectomized animals (unpaired Wilcoxon's rank-sum test).

(DOCA) 0.2 mg per animal daily plus hydrocortisone 1.0 mg per animal daily for 4 days.

Strips of pulmonary arteries

Guinea-pigs were killed by a blow on the head 5 days post-operatively and spiral preparations of the pulmonary arteries were prepared and incubated at 37°C for 1 h with oxygenated Krebs-bicarbonate solution containing 10^{-7} M [^{3}H]-noradrenaline (specific activity 38.6 Ci mmol^{-1}, The Radiochemical Centre, Amersham/Searle, U.S.A.) and ascorbic acid 100 mg l^{-1} as described previously (Misu *et al.*, 1981; 1983). After washing for 10 min with noradrenaline-free medium, the preparations were mounted vertically between a pair of platinum stimulating electrodes, of 0.5 mm diameter and separated by 2 mm along the whole length of the strips (approximately 15 mm, weighing 5 to 6 mg), and superfused with Krebs solution at a constant rate of 1 ml min^{-1} by means of microtube pumps (MP-3A, Tokyo Rikakikai, Japan). The Krebs solution had the following composition (mM): NaCl 120.7, KCl 5.9, $CaCl_2$ 2.5, $NaHCO_3$ 15.5, NaH_2PO_4 1.2 and glucose 11.5, and was maintained at 37°C, pH 7.2 to 7.4, and bubbled with 95% O_2 plus 5% CO_2. A 90 min equilibration period was allowed, and then supramaximal transmural field stimulation (2 ms, 10 V at 1 Hz for 100 s) was applied with an electrical stimulator and an isolator (SEN 3201 and SS-201J, Nihon Kohden, Japan) 4 times (S_1 to S_4 periods) at 15 min intervals.

Carteolol 10^{-8} M, 10^{-7} M and 10^{-6} M was applied cumulatively immediately after the S_1, S_2 and S_3 periods of stimulation, respectively. Superfusate was collected for 2 min intervals before, during and after the period of nerve stimulation, 2 ml of ACS-II solution was added and total ^{3}H-activities, expressed as disintegration per min (d.p.m.), were determined by a liquid scintillation spectrometer (Packard 2660, U.S.A.). The stimulus-evoked release of ^{3}H (S) was calculated as the difference between resting efflux before stimulation and total efflux, detected in 3 successive samples during and after stimulation. The resting efflux before stimulation was regarded as spontaneous release (Sp). The effect of each concentration of carteolol on the spontaneous and evoked release was evaluated from absolute values of Sp_x and S_x and also by % ratios of Sp_x/Sp_1 and S_x/S_1, for the standardization of data obtained. These experiments were done in the absence of cocaine or an α_2-adrenoceptor antagonist.

Measurement of catecholamine content of pulmonary arteries

In another series of experiments, the pulmonary arteries were isolated 5 days after the operation, homogenized in 2 ml of 0.1 N $HClO_4$, centrifuged and total aliquots of the supernatant transferred to polypropylene test tubes (10 ml) containing activated alumina 10 mg, disodium EDTA 10 mg and dihydroxybenzylamine 50 pmol as an internal standard. To the test tubes, 1 ml of 1.5 M Tris-HCl buffer (pH 8.6) was added and the preparations were placed

Table 2 Endogenous catecholamine contents of pulmonary arteries isolated from guinea-pigs 5 days after bilateral adrenalectomy, bilateral adrenodemedullation or sham operation

Group	n	*Dopamine* (pmol mg^{-1})	*Noradrenaline* (pmol mg^{-1})	*Adrenaline* (fmol mg^{-1})
Sham operation	9	0.32 ± 0.06	13.0 ± 1.5	65.3 ± 9.7
Adrenalectomy	7	0.25 ± 0.04	14.3 ± 2.5	6.4 ± 1.1**
Adrenodemedullation	7	0.44 ± 0.03	13.7 ± 1.2	16.8 ± 8.9**

Data shown are mean ± s.e.mean of n values. Significantly different: $^{**}P < 0.01$, compared to values from sham-operated animals (Student's t test).

in a shaker for 30 min. The supernatant was discarded and the alumina washed three times with diluted water. Adsorbed catecholamines were eluted with 60 μl of 0.1 N HCl and endogenous catecholamines were measured by high performance liquid-chromatography with electrochemical detection (Yanaco, Japan). Details of the chromatographic data were as follows (Goshima *et al.*, 1985); column, Cosmosil $_5C_{18}$ (4.6 × 150 mm, particle size 5 μm): mobile phase, 0.1 M phosphate-potassium buffer (pH 5.8) containing 10% methanol, 0.02% (−)-heptanesulphonate sodium and 1 mM disodium EDTA; applied potential, 600 mV vs Ag AgCl^{-1}; and flow rate, 1.5 ml min^{-1}.

Drugs used

(±)-Carteolol hydrochloride (Otsuka, Japan) was dissolved in distilled water. DOCA (Nakarai, Japan) and hydrocortisone (Sigma, U.S.A.) was suspended in olive oil.

Analysis

Experiments were performed according to a paired design, whenever possible. Data shown are mean ± s.e.mean. Data significance was calculated by paired t test, Student's t test and unpaired Wilcoxon's rank-sum test.

Results

In control pulmonary arteries isolated from sham-operated guinea-pigs, then loaded with [^{3}H]-noradrenaline, the spontaneous ^{3}H-release and the stimulation-evoked ^{3}H-release at 1 Hz were 394.7 ± 15.7 d.p.m. mg^{-1} 2 min^{-1} and 990.0 ± 63.1 d.p.m. mg^{-1} (n = 18), respectively, 90 min after the start of superfusion. There was no decline of the evoked release in response to the S_1 to S_4 stimulations at 15 min intervals (Table 1). Carteolol 10^{-8} M, 10^{-7} M and 10^{-6} M, applied cumulatively, inhibited the evoked ^{3}H-release in a concentration-

Table 3 Effects of (±)-carteolol (10^{-8} M, 10^{-7} M and 10^{-6} M), applied cumulatively, on the stimulus-evoked ^{3}H-release from pulmonary arteries isolated from guinea-pigs 5 days after either bilateral adrenalectomy, followed by injections of DOCA plus hydrocortisone, or after bilateral adrenodemedullation

Group	n	S_1	S_2 (S_2/S_1)	S_3 (S_3/S_1)	S_4 (d.p.m. mg^{-1}) (S_4/S_1) (%)
Adrenalectomized plus DOCA and hydrocortisone treatment					
Control	4	924.3 ± 182.3	897.8 ± 184.4 (97.3 ± 11.4)	848.1 ± 174.9 (92.4 ± 11.5)	798.6 ± 172.1 (86.4 ± 12.3)
Carteolol	5	778.3 ± 234.4	789.0 ± 207.9 (105.4 ± 4.8)	717.1 ± 170.2 (99.3 ± 7.4)	662.4 ± 179.7 (88.0 ± 7.5)
Adrenodemedullated					
Control	12	1151.4 ± 124.4	1053.2 ± 124.9* (90.6 ± 3.0)	1000.8 ± 114.1** (86.6 ± 2.7)	965.9 ± 107.9** (83.6 ± 3.2)
Carteolol	11	1095.0 ± 94.1	1018.0 ± 87.1* (93.3 ± 2.4)	922.5 ± 76.8** (85.9 ± 2.8)	837.7 ± 67.2** (78.0 ± 2.1)

Deoxycorticosterone acetate (DOCA) 0.2 mg per animal daily and hydrocortisone 1.0 mg per animal daily were injected s.c. for 4 days. Significantly different: $^*P < 0.05$, $^{**}P < 0.01$, compared to S_1 (paired t test). Other details are as in Table 1. Note the absence of a significant difference between the carteolol-treated groups and their equivalent, time-matched controls.

Table 4 Effects of (±)-carteolol (10^{-8} M, 10^{-7} M and 10^{-6} M), applied cumulatively, on the spontaneous ^{3}H-release (Sp) in pulmonary arteries isolated from guinea-pigs 5 days after either bilateral adrenalectomy, adrenalectomy followed by injections of DOCA plus hydrocortisone, bilateral adrenodemedullation or sham operation

Group	n	Sp_1	Sp_2 (Sp_2/Sp_1)	Sp_3 (Sp_3/Sp_1)	Sp_4 (d.p.m. mg^{-1}) (Sp_4/Sp_1) (%)
Sham-operated					
Control	10	415.8 ± 14.9	353.2 ± 14.7**	320.8 ± 10.0**	309.0 ± 14.5**
			(85.0 ± 2.1)	(77.4 ± 1.4)	(74.2 ± 1.8)
Carteolol	8	368.3 ± 28.4	340.3 ± 29.1**	327.9 ± 30.5**	313.5 ± 30.1**
			(92.2 ± 1.9)	(88.6 ± 3.4¶)	(84.7 ± 4.6¶)
Adrenalectomized					
Control	11	402.5 ± 29.6	356.6 ± 26.8**	319.1 ± 23.3**	302.6 ± 22.9**
			(88.8 ± 2.2)	(79.6 ± 1.8)	(75.6 ± 2.1)
Carteolol	8	429.9 ± 64.3	389.7 ± 45.7	348.7 ± 42.9*	330.4 ± 41.9**
			(93.1 ± 3.5)	(83.1 ± 2.8)	(78.5 ± 2.5)
Adrenalectomized plus DOCA and hydrocortisone treatment					
Control	4	373.8 ± 65.9	304.3 ± 25.0	275.3 ± 20.2	256.6 ± 19.6
			(84.7 ± 6.5)	(77.2 ± 7.5)	(71.6 ± 5.9)
Carteolol	5	338.5 ± 41.2	287.9 ± 22.9	261.3 ± 30.4*	250.1 ± 26.9*
			(81.2 ± 4.0)	(77.6 ± 4.3)	(74.6 ± 3.8)
Adrenodemedullated					
Control	12	337.1 ± 25.3†	321.0 ± 22.6	285.4 ± 18.3**	267.8 ± 16.5**
			(95.9 ± 2.2)	(85.3 ± 1.9)	(80.3 ± 1.9)
Carteolol	11	331.2 ± 21.6	304.7 ± 16.7	286.2 ± 15.8**	277.5 ± 15.5**
			(93.0 ± 3.4)	(87.4 ± 3.4)	(84.9 ± 3.4)

Significantly different: *$P < 0.05$, **$P < 0.01$, compared to Sp_1 (paired t test); ¶$P < 0.05$, compared to corresponding control (unpaired Wilcoxon's rank-sum test); †$P < 0.05$, compared to control in arteries from sham-operated animals (Student's t test). Other details are as in Tables 1 and 3. Note the absence of a significant difference between the carteolol-treated groups and their equivalent, time-matched controls, except for the sham-operated groups.

dependent manner, compared to both the release before its application in the same preparations and the corresponding, time-matched control.

Bilateral adrenalectomy depleted the endogenous content of adrenaline in the pulmonary arteries, compared to that in the arteries of sham-operated animals (Table 2). However, there were no changes in the equivalent noradrenaline and dopamine values. Adrenalectomy produced no changes in either the spontaneous ^{3}H-release (414.0 ± 31.2 d.p.m. mg^{-1} 2 min^{-1}) or the stimulation-evoked ^{3}H-release (915.4 ± 68.0 d.p.m. mg^{-1}) at S_1 ($n = 19$), compared to the respective values of arteries from sham-operated animals. Any tonic action of endogenous adrenaline on the pulmonary arteries was not reflected in the absolute values of the spontaneous and evoked release. There was also no decline of the ^{3}H-release evoked by repetitive stimulation in arteries from adrenalectomized guinea-pigs (Table 1). Carteolol 10^{-6} M inhibited the evoked release, compared to the release before its application. However, this inhibition was not significantly different from the corresponding time-matched control in terms of either absolute value or release ratio. Furthermore, in arteries from guinea-pigs subjected to adrenalectomy there was no carteolol-induced inhibition of evoked ^{3}H-release (as occurred in sham-operated animals).

Injections of DOCA 0.2 mg per animal day^{-1} plus hydrocortisone 1.0 mg per animal day^{-1} for 4 days to adrenalectomized guinea-pigs, before the isolation of pulmonary arteries, did not modify the spontaneous and evoked ^{3}H-release at S_1, compared to the equivalent release obtained in arteries from adrenalectomized animals (Tables 1, 3 and 4). There was no decline of the control ^{3}H-release evoked by repetitive nerve stimulation (Table 3). Hence, DOCA and hydrocortisone pretreatment did not prevent the loss of the carteolol-induced inhibition of evoked ^{3}H-release.

Bilateral adrenodemedullation also markedly reduced the endogenous content of adrenaline without affecting the noradrenaline and dopamine content (Table 2). Adrenodemedullation did not affect the evoked ^{3}H-release at S_1, compared to the equivalent value for release in arteries from sham-

operated controls (Tables 1 and 3). In this series of experiments, a progressive decline in the ^{3}H-release evoked by repetitive nerve stimulation was seen even in preparations not exposed to carteolol (compare S_2 to S_4 with S_1, Table 3). Although this decline was also seen in carteolol-treated preparations, there were no significant differences between the carteolol-treated groups and the corresponding, time-matched controls in terms of either absolute values or release ratios (Table 3). Thus, adrenodemedullation appeared to prevent the carteolol-induced inhibition of the evoked release. On the other hand, the spontaneous ^{3}H-release at S_1 in arteries from adrenodemedullated animals was decreased, compared to the corresponding release in arteries from sham-operated animals (Table 4). This decrease might be due to some differences arising from both groups of experiments, which were not done in a paired design.

The spontaneous ^{3}H-release gradually declined with time in almost every series of experiments (Table 4). Carteolol 10^{-8} M to 10^{-6} M, applied cumulatively, appeared to prevent the fall in the spontaneous release ratios, compared to the corresponding control, in arteries from sham-operated animals. However, carteolol had no consistent effect in the other series of experiments.

Discussion

(±)-Carteolol 10^{-8} M to 10^{-6} M inhibited the evoked release of noradrenaline in pulmonary arteries from sham-operated guinea-pigs in a concentration-dependent manner. The carteolol-induced inhibition is consistent with our previous findings in the same arteries from intact animals (Kuwahara *et al.*, 1986). Furthermore, we demonstrated that the same concentrations of (+)-carteolol produced no inhibition of the evoked release of noradrenaline. These results indicate that prejunctional β-adrenoceptors function tonically and some endogenous agonist is released and activates these adrenoceptors to facilitate the further release of noradrenaline.

Bilateral adrenalectomy and adrenodemedullation prevented the occurrence of the carteolol-induced inhibition of the evoked release of noradrenaline and depleted or markedly reduced the endogenous concentration of adrenaline in the pulmonary arteries, without affecting that of noradrenaline and dopamine, compared to sham-operated preparations. Furthermore, DOCA and hydrocortisone pretreatment did not prevent the loss of the carteolol-induced inhibition of release in arteries from adrenalectomized animals. These results indicate that adrenaline within the noradrenergic nerve endings is mainly derived from the adrenal medulla and suggest that adrenaline is taken up and co-exists with noradrenaline. Thus, released adrenaline, but not noradrenaline and dopamine, appears to act as an endogenous agonist for prejunctional β-adrenoceptors. This idea is consistent with the characterization of prejunctional β-adrenoceptors as the β_2 subtype (Misu *et al.*, 1983; Majewski, 1983; Misu & Kubo, 1986). Adrenodemedullation-induced modifications of the catecholamine content of guinea-pig pulmonary arteries are consistent with those in rat portal veins (Dahlöf *et al.*, 1979). Both [^{3}H]-noradrenaline and [^{3}H]-adrenaline are bound in dense core vesicles in the noradrenergic nerve endings (Wolfe & Potter, 1963) and are localized in small particles associated with the microsomes of various noradrenergic tissues in rats (Potter & Axelrod, 1963). [^{3}H]-adrenaline taken up is released by noradrenergic nerve stimulation (Rosell *et al.*, 1964; Guimarães *et al.*, 1978; Majewski *et al.*, 1980; 1981). The present findings support the adrenaline-mediated positive feedback loop hypothesis proposed by Majewski *et al.* (1980, 1981), i.e. adrenaline is taken up, stored and released as a co-transmitter with noradrenaline and then activates prejunctional β-adrenoceptors.

The spontaneous release in almost every group of experiments and even the evoked release in some experiments gradually declined with time. Such a decline is, to some extent, unavoidable as [^{3}H]-noradrenaline pooled within noradrenergic nerve endings and available for release is limited in its amount in preload experiments such as these. This is one of the reasons for doing the experiment in a paired design whenever possible. It is not clear at present why there was a decline of the evoked ^{3}H-release only in the adrenodemedullation group of experiments. However, the extent of the decline in the carteolol-applied preparations was similar to that in control preparations. From these findings, adrenodemedullation appears to prevent the carteolol-induced inhibition of the evoked release just as in the case of adrenalectomy.

Carteolol (10^{-8} M to 10^{-6} M) slightly increased the spontaneous release in some pulmonary arteries from sham-operated guinea-pigs, but not in those from animals with either adrenalectomy, adrenalectomy followed by the injections of DOCA plus hydrocortisone or adrenodemedullation. However, this increase was not seen in intact guinea-pig pulmonary arteries (Kuwahara *et al.*, 1986), or in renal and mesenteric arteries from young and adult spontaneously hypertensive rats and age-matched Wistar Kyoto rats (Misu *et al.*, 1987). Higher concentrations of carteolol (10^{-5} M and 10^{-4} M) increase the spontaneous ^{3}H-release in guinea-pig pulmonary arteries and this increase is partially inhibited by the combined application of cocaine (5×10^{-5} M) and 3-*O*-methylnoradrenaline (10^{-5} M) (unpublished data). It

is possible that carteolol may have a slight indirect sympathomimetic action and/or a blocking effect on neuronal or extraneuronal uptake.

In conclusion, the endogenous agonist for tonically functioning prejunctional β-adrenoceptors is adrenaline, which is mainly derived from the adrenal medulla and is probably taken up and co-released with noradrenaline in guinea-pig pulmonary arteries.

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Voltage-dependence of ryanodine-sensitive component of contraction in A23187- and isoprenaline-treated cardiac muscles

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1 The ryanodine-sensitive component (RSC) of contraction electrically induced in the presence of A23187 (10^{-6} M) and isoprenaline (5×10^{-8} M) was investigated in guinea-pig papillary muscles that had been partially depolarized to various membrane potentials by increasing $[K^+]_o$, and current and voltage clamp techniques.

2 Two components of contraction were produced by A23187 and isoprenaline at a stimulation frequency of 0.2 Hz. The early component was sensitive to ryanodine (10^{-6} M) (corresponding to RSC), while the late component was sensitive to nifedipine (10^{-6} M).

3 In the presence of nifedipine, RSCs produced by these drugs were selectively observed. Under these conditions, RSCs were voltage-dependently inhibited by a reduction in resting potential that was brought about by increasing $[K^+]_o$ (10–26 mM). The onset of inhibition of RSCs appeared at a resting potential between −75 and −70 mV, and almost complete inhibition was observed at a potential between −55 and −50 mV, indicating that RSCs produced by these drugs have the same voltage-dependence.

4 In the absence of nifedipine, this voltage-dependent inhibition was also observed on the early component of contraction produced by A23187 without affecting the late component.

5 RSC produced by A23187 was not affected by blocking Na channel activation with tetrodotoxin (6×10^{-6} M).

6 In current and voltage clamp experiments, this RSC was inhibited by reducing the resting and the holding potentials in much the same way as observed on the preparations depolarized by increasing $[K^+]_o$. Such dependence on voltage was not observed with either the slow action potential or the slow Ca inward current.

7 These results suggest that the dependence of RSC on resting potentials is not due to the changes in the fast Na and the slow Ca inward currents. The present RSC via ryanodine-sensitive internal Ca release may be mainly triggered by a mechanism different from Ca influx through slow channels.

Introduction

The sources of Ca for cardiac excitation-contraction coupling are thought to be mainly attributed to transmembrane Ca influx through slow Ca channels and Ca release from internal stores, sarcoplasmic reticulum (SR). The activation of contraction has also been indicated to be divided into two distinct components, the initial rapid and the following maintained components, in the absence (Morad & Goldman, 1973; Allen *et al.*, 1976; King & Bose, 1983) or the presence of an adrenoceptor agonist (Reuter, 1974; Beresewicz & Reuter, 1977; Honore *et al.*, 1987). With regard to these two components, many studies have suggested that the initial rapid component of contraction depends on Ca release from the SR, while the following maintained component depends on Ca influx via slow Ca channels.

It has been suggested that Ca release from the SR in cardiac muscles is mediated through a Ca-induced Ca release mechanism. From the recent studies on the voltage-dependence of contractions (London & Krueger, 1986) and $[Ca^{2+}]_i$ transients (Barcenas-Ruiz & Wier, 1987) observed in voltage-clamped guinea-pig cells, it has been suggested that SR Ca release was induced by Ca ions carried by the slow

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inward current. On the other hand, Fabiato (1985a), and Morad & Cleemann (1987) have proposed that Ca release may be triggered by Ca influx through fast Ca channels (corresponding to T-type of Ca channels), different from slow channels which have been described by Hume & Giles (1983), Bean (1985), Nilius *et al.* (1985), and Mitra & Morad (1986). Thus, although Ca-induced Ca release may be a prevailing theory of cardiac excitation-contraction coupling, the exact role of Ca inward currents on the Ca-induced Ca release process in intact cardiac muscle is the subject of some controversy.

Recently, the Ca ionophore, A23187, has been reported to produce two components of contraction each component of which could be separately observed by use of specific pharmacological interventions (Kondo, 1986). An agent decreasing internal Ca release by depletion of the releasable Ca of the SR, ryanodine (Sutko & Kenyon, 1983; Chamberlain *et al.*, 1984; Marban & Wier, 1985; Fabiato, 1985b; Meissner, 1986; Lattanzio *et al.*, 1987; Rousseau *et al.*, 1987), blocked the fast component of contraction, while the late one was inhibited by the slow Ca channel blocker, nifedipine. Thus, A23187 is a useful agent for developing the two components of contraction which could be pharmacologically distinguished. Furthermore, one of these two components, the ryanodine-sensitive one, was found to disappear without inhibiting the nifedipine-sensitive one when the resting potential was depolarized by increasing $[K^+]_o$; it has been proposed that in the depolarized condition, internal Ca release contributes less to the cardiac contraction (Kondo, 1986). Similar inhibition of contraction by resting potential depolarization has also been observed when the two components of contraction were induced under the influence of the β-adrenoceptor agonist, isoprenaline (Honore *et al.*, 1987). In the present study, therefore, the effects of resting potential depolarization on the biphasic contractions electrically induced in the presence of A23187 were examined, to evaluate the involvement of Ca ions carried by nifedipine-sensitive Ca currents in excitation-contraction coupling. Some similar experiments were also carried out on the biphasic contraction produced by isoprenaline.

Methods

Guinea-pigs weighing 250 to 300 g were killed by a blow on the head. The hearts were quickly removed and a papillary muscle, 2 to 3 mm in length and less than 1 mm in diameter, was isolated from the right ventricle. These preparations were mounted in a tissue bath and superfused continuously with Krebs Ringer solution at 30°C equilibrated with 95% O_2 and 5% CO_2. The composition of the Krebs Ringer solution was as follows (mM): NaCl 120.3, KCl 4.8, $CaCl_2$ 1.2, $MgSO_4 \cdot 7H_2O$ 1.3, KH_2PO_4 1.2, $NaHCO_3$ 24.2 and glucose 5.5 (pH 7.4). In some experiments the K^+ concentration of the solution was raised to 10, 13, 16, 19 or 26 mM by substituting KCl for NaCl on an equimolar basis to reduce the resting potential. The tendinous end of the muscle was connected to a force displacement transducer for recording isometric tension. The preparations were constantly stimulated at 0.2 Hz. Pulses employed for stimulation were 1 ms in duration and twice the diastolic threshold in intensity. Membrane action potentials were recorded through glass microelectrodes filled with 3 M KCl (10–30 MΩ), and displayed simultaneously with the signal from the force-displacement transducer on a storage oscilloscope (Tektronix 7613). The mechanical tension was also recorded on a polygraph (Nihon Kohden TB612T).

In voltage clamp experiments, papillary muscles of 0.3–0.4 mm in diameter were mounted in a three-compartment, single sucrose gap chamber. Each compartment of the chamber was separated by rubber sheets having a hole permitting the preparation to be passed through it. The width of the middle compartment was 1.5–2.0 mm. The tendinous end of the preparation, which was attached to the force-displacement transducer, occupied the test compartment, and the other end, the KCl compartment. The length of the preparation in the test compartment was limited to 0.3 to 0.4 mm to obtain a homogeneous potential distribution. The sucrose gap was established by perfusing the middle compartment with sucrose solution (304 mM sucrose, 5 mM glucose and 0.01 mM $CaCl_2$) gassed with 100% O_2. The KCl compartment was perfused by a KCl-Krebs Ringer solution (NaCl replaced by KCl). The KCl compartment potential (AP_o) was recorded continuously to allow determination of transgap resistance (New & Trautwein, 1972). Clamp control was obviously poor in preparations where the ratio of transgap potential (AP_o/intracellular action potential, AP_i) was less than 0.9 (McDonald & Trautwein, 1978); such preparations were rejected and presumed to have been damaged during dissection or mounting. All the voltage clamp experiments were performed within 1 h because AP_o/AP_i decreased gradually with time. To establish a voltage clamp, the measured intracellular potential is compared with a command signal, and difference is used to drive a high-gain negative feedback amplifier (DAGAN 8500) which passes current through an Ag-AgCl wire electrode in the KCl compartment across the gap. Membrane currents, voltage and contraction were recorded on FM-tape and edited by a storage oscilloscope. The frequency of application of voltage clamp pulses was 0.2 Hz and the duration of

the clamp step was usually 500 ms. The other procedures were the same as those described in a previous study (Kondo, 1986).

In current clamp experiments, all procedures were the same as those in the voltage clamp experiments described above. The amplifier (DAGAN 8500) was used with the CURRENT CLAMP mode. The currents were supplied through Ag-AgCl electrode in the KCl compartment.

A23187 (Calbiochem-Behring Corp.) was dissolved in dimethylsulphoxide at a concentration of 10^{-3} M, and then added to the Krebs Ringer solution to achieve a concentration of 10^{-6} M. The vehicle control (dimethylsulphoxide) at the concentration used in this study had no effect on the electromechanical response of the present preparations. Other drugs employed were as follows: nifedipine, ryanodine (S.B. Penick Company), tetrodotoxin (TTX; Sankyo Co., Ltd).

Results

Nifedipine- and ryanodine-sensitive components of contraction produced by A23187 and isoprenaline

A23187 (10^{-6} M) caused a biphasic positive inotropic effect in normal medium as shown in Figure 1. This inotropic effect has been shown to be pharmacologically divided into two distinct components; an initial rapid component (early component) of contraction that was inhibited by ryanodine, an agent decreasing internal Ca release, while a subsequent maintained component (late component) was inhibited by the Ca channel blocker, nifedipine (Kondo, 1986). The present cardiotonic effect of A23187 was also clearly separated into these two components, a nifedipine sensitive component (NSC) and a ryanodine-sensitive one (RSC). In preparations treated with nifedipine, RSC was selectively observed (Figure 1c).

Isoprenaline (5×10^{-8} M) also produced a biphasic contraction, although two components were less distinct in the absence of nifedipine and ryanodine. In the presence of nifedipine, RSC could be selectively observed, but the amplitude of this RSC was much smaller than that produced by A23187 (not shown).

Relationship between resting potential and RSC

The effects of various $[K^+]_o$ (10, 13, 16, 19 or 26 mM) on the resting potential and RSCs produced by A23187 and isoprenaline are summarized in Figure 2. In the presence of nifedipine (10^{-6} M), A23187 and isoprenaline produced only RSC, while NSC was completely inhibited under these conditions. An increase in $[K^+]_o$ inhibited RSCs together with the resting potential. The inhibition of these RSCs was not observed at 10 mM $[K^+]_o$, but partially inhibited at 13 and 16 mM. At $[K^+]_o$ of more than 19 mM, RSCs failed. By using these data, the amplitude of RSCs was plotted as a function of the resting potential (inset of Figure 2): the voltage-dependent inhibition of RSC was shown between −75 mV and −50 mV; at a resting potential of −50 mV, RSC was almost completely inhibited. RSCs produced by A23187 and isoprenaline depend on the resting potential in much the same way.

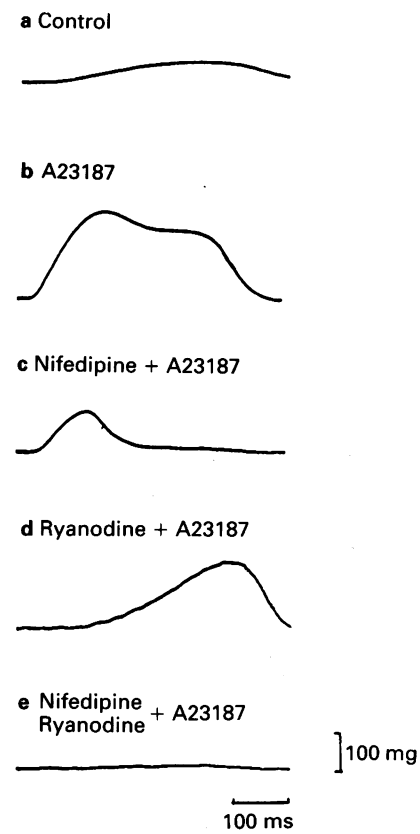


Figure 1 Mechanical response to A23187 in the presence or the absence of nifedipine and ryanodine: (a) control; (b) 10 min after application of A23187 (10^{-6} M); (c) effect of A23187 after pretreatment with nifedipine (10^{-6} M) for 25 min; (d) effect of A23187 after pretreatment with ryanodine (10^{-6} M) for 25 min; (e) effect of A23187 after combined pretreatment with ryanodine and nifedipine for 25 min.

Membrane action potential and RSC at resting potentials depolarized by high $[K^+]_o$

In the presence of nifedipine, the effects of resting potential depolarization by high $[K^+]_o$ (13 and

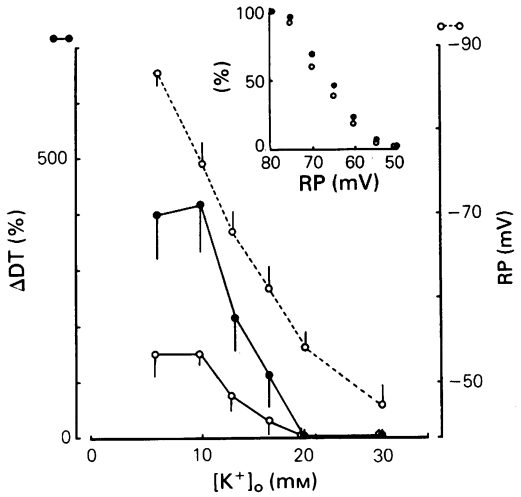


Figure 2 Effects of various $[K^+]_o$ on the resting potential (○---○) and ryanodine-sensitive components of contraction produced by A23187 (●—●) and isoprenaline (○—○) in the presence of nifedipine. The resting potentials and ryanodine-sensitive components produced by A23187 (10^{-6} M) and isoprenaline (5×10^{-8} M) in the presence of nifedipine (10^{-6} M) were plotted as a function of $[K^+]_o$. Horizontal axis shows the concentrations of K^+ (mM) in the medium. Each vertical axis indicates the developed tension increased by A23187 and isoprenaline (ΔDT) as % of contraction obtained in the presence of nifedipine (left axis), and the resting potential (RP; mV) (right axis). Vertical bars show s.e. ($n = 6$). By using these data, the amplitude of ryanodine-sensitive components produced by A23187 (●) and isoprenaline (○) was plotted as a function of the resting potential (inset). Vertical axis shows % of the amplitude of ryanodine-sensitive component produced by each drug in normal $[K^+]_o$. Horizontal axis indicates the resting potential (mV).

19 mM) were examined on RSC and the action potential. When $[K^+]_o$ was increased to 13 mM, the resting potential was reduced to -67.5 ± 2.9 mV ($n = 4$) and the slow action potential (APs) was induced by electrical stimuli. Under these conditions, nifedipine decreased APs and the contraction. In such preparations, A23187 produced RSC and slightly augmented APs. This RSC was characterized by a marked enhancement of the maximum rate of force development (1250 ± 350% of value in the presence of nifedipine, $n = 4$) resulting from an increase in the early component of contraction. After additional application of ryanodine (10^{-6} M), RSC was completely inhibited without significantly affecting APs (Figure 3).

At 19 mM $[K^+]_o$, the resting potential was reduced to -54.1 ± 2.3 mV ($n = 4$). After additional application of nifedipine, APs and the contraction were markedly inhibited. In this preparation, A23187 did not cause RSC, although APs was slightly augmented by this drug. However, by returning $[K^+]_o$ to the normal concentration (6 mM K^+) in the presence of A23187 and nifedipine, RSC was restored (425 ± 70% of contraction before returning to normal medium, $n = 4$) (Figure 4). This restoration of RSC was inhibited by application of ryanodine (not shown).

Effects of resting potential changes induced by high $[K^+]_o$ on RSC and NSC

In normal medium (6 mM K^+) with nifedipine, A23187 selectively increased RSC. When RSC increased by A23187 reached a steady state, the resting potential was gradually reduced to near -50 mV by perfusing with a medium containing high $[K^+]$ (26 mM), nifedipine and A23187 (Figure 5a). By this procedure, RSC was voltage-dependently inhibited and almost complete inhibition was observed at -50 mV (open circles in Figure 5d).

In the preparation pretreated with ryanodine, a similar experiment was carried out (Figure 5b). In the presence of ryanodine, RSC (the early component) was completely inhibited while the late component corresponding to NSC became apparent. The late component was not affected by reducing resting potential to -50 mV with increase in $[K^+]_o$. The relationship between this component and the resting potential is shown in Figure 5d (open triangles).

In the absence of nifedipine and ryanodine, A23187 produced the two components of contraction. Then, by perfusing with the medium containing high $[K^+]$, the early component was markedly inhibited, while the late component was slightly affected (Figure 5c). However, at a resting potential of -50 mV, a small early component still remained (19% of contraction of this component). The relationship between the resting potential and the early component is shown in Figure 5d (closed circles). These inhibitory effects induced by increasing $[K^+]_o$ were removed by returning to the normal level (6 mM) of $[K^+]_o$ (not shown).

Effects of tetrodotoxin on RSC

The involvement of Na channels in RSC produced by A23187 was examined in four preparations (Figure 6). The resting potential was slightly depolarized by increasing $[K^+]_o$ to 10 mM to obtain an effective block of Na channels by tetrodotoxin (TTX). In the medium containing 10 mM K^+, the resting potential was slightly depolarized to -76.9 ± 2.8 mV ($n = 4$), resulting in a decrease in the maximum upstroke velocity of the action poten-

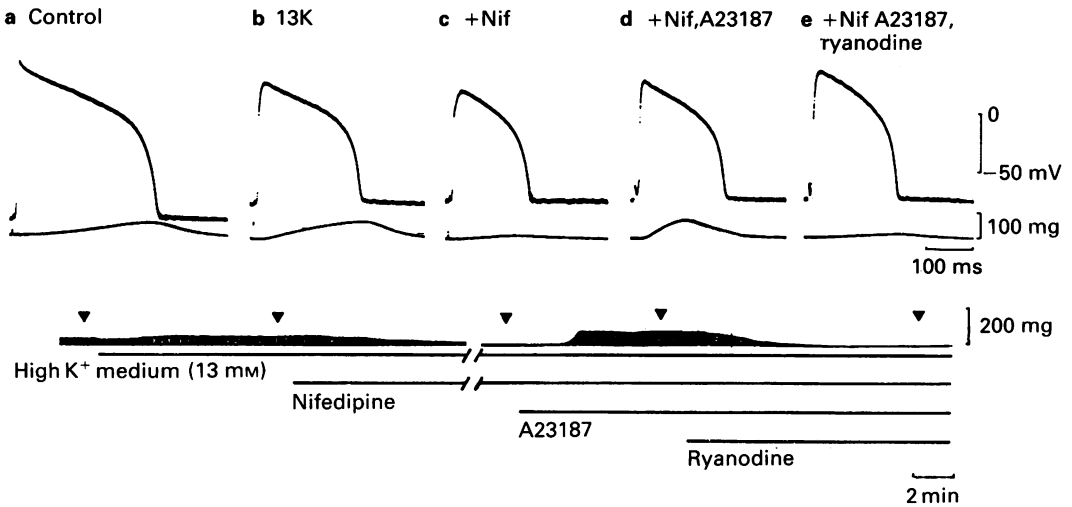


Figure 3 Effects of high $[K^+]_o$ (13 mM) and ryanodine on the electromechanical response to A23187 in the presence of nifedipine. Upper panels show the membrane action potential and simultaneously recorded contraction: (a) control; (b) 10 min after exposure to high K^+ medium (13 mM); (c) 25 min after pretreatment with nifedipine (Nif) (10^{-6} M) in high K^+ medium; (d) 10 min after application of A23187 (10^{-6} M) in high K^+ medium with nifedipine; (e) 12 min after additional application of ryanodine (10^{-6} M). Lower trace shows a continuous record of isometric tension. Arrowheads indicate recording points of the electromechanical responses.

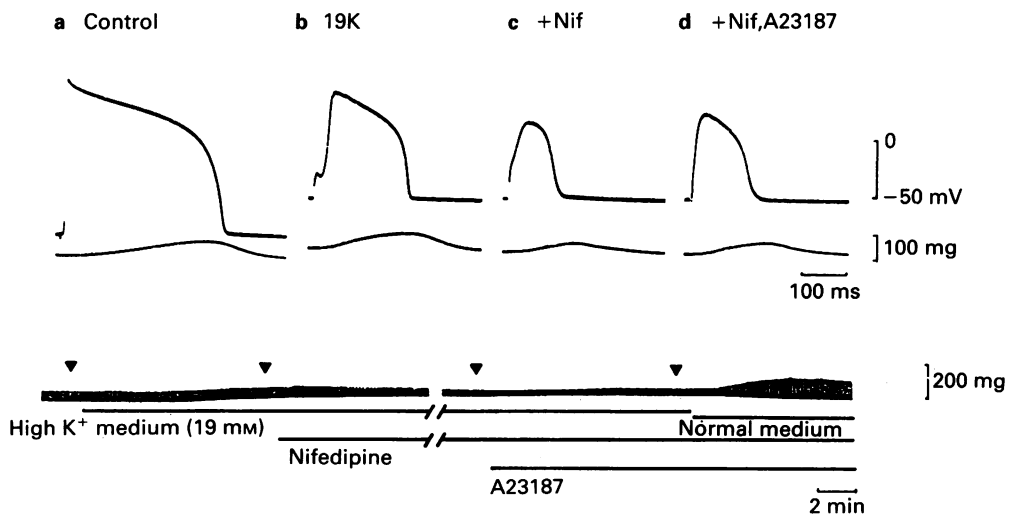


Figure 4 Effects of high $[K^+]_o$ (19 mM) and subsequent return to normal $[K^+]_o$ on the electromechanical response to A23187 in the presence of nifedipine. Upper panels show the membrane action potential and simultaneously recorded contraction: (a) control; (b) 10 min after exposure to high K^+ medium (19 mM); (c) 25 min after pretreatment with nifedipine (10^{-6} M) in high K^+ medium; (d) 10 min after application of A23187 (10^{-6} M) in high K^+ medium with nifedipine. Eleven minutes after application of A23187, medium was returned to normal K^+ medium with A23187 and nifedipine. Lower trace shows a continuous record of isometric tension. Arrowheads indicate recording points of the electromechanical responses.

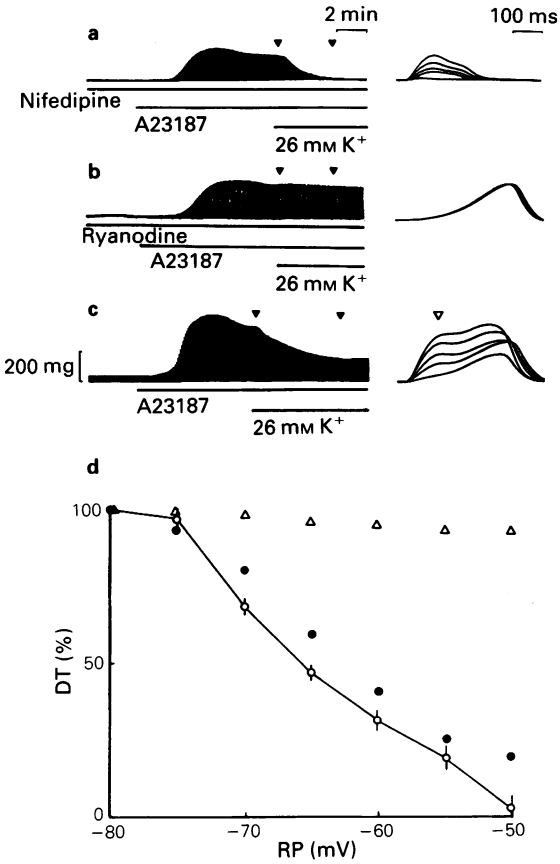


Figure 5 Effects of increasing $[K^+]_o$ to 26 mM on contraction increased by A23187 in the absence or the presence of nifedipine and ryanodine. Upper traces (a–c) show the effects of increasing $[K^+]_o$ to 26 mM on contraction increased by A23187. Nine minutes after application of A23187 (10^{-6} M) in the presence of nifedipine (10^{-6} M) (a) and ryanodine (10^{-6} M) (b), the preparation was depolarized by increasing $[K^+]_o$. In (c), 7 min after application of this drug in the absence of these inhibitors, the same experiments were carried out. Right superimposable traces (a)–(c) indicate the contraction obtained at resting potentials of −80, −70, −65, −60, and −50 mV between closed arrowheads. The relationship between the resting potential (RP) and the developed tension (DT) in the absence (●) or the presence of nifedipine (○) and ryanodine (△) was shown in (d). Vertical axis shows % of contraction before increasing $[K^+]_o$. Vertical bars show s.e. ($n = 4$). The amplitude of the early component of contraction in the absence of the inhibitors was measured at the point indicated by open arrowhead in (c) (140 ms after onset of stimuli).

tial to about 50%. In this preparation, TTX (6×10^{-6} M) markedly inhibited the maximum upstroke velocity (10–15 V s^{-1}), indicating almost complete abolition of the activation of Na channels. Additional treatment with nifedipine decreased APs and contraction. In such preparations pretreated with TTX and nifedipine, A23187 still caused RSC (487 ± 7.3% of contraction before application of A23187, $n = 4$). This RSC was blocked by ryanodine (not shown).

Effects of reducing resting potential by the current clamp method

Current clamp experiments were conducted to examine the effects of the resting potential depolarization on two components of contraction produced by A23187 in three preparations (Figure 7). The current clamp was carried out by use of a single sucrose gap technique. The resting potential was reduced from −80 mV to −60 mV by increasing the current supply. At resting potentials more positive than −75 mV, the early component of contraction was selectively inhibited in a voltage-dependent manner, while the late component was slightly affected by reducing the resting potential. Under these conditions, a large APs induced by electrical stimuli was not affected by resting potential depolarization. The voltage-dependence of the early component is shown in Figure 7b.

Effects of reducing holding potential by the voltage clamp method

By use of voltage clamp techniques, effects of reducing the holding potential on the early component of contraction corresponding to RSC produced by A23187 were investigated in three preparations pretreated with TTX and nifedipine (Figure 8). In the presence of TTX (10^{-5} M) and nifedipine (10^{-6} M), the fast Na channels were blocked and the slow Ca inward current was markedly inhibited when the depolarization step having a duration of 500 ms was applied from the holding potential of −80 mV to −5 mV (not shown). However, even after treatment with TTX and nifedipine, a small and rapidly inactivated inward current remained. Under these conditions, A23187 produced the early component of contraction (400% of control) without significantly affecting the residual inward current. In this preparation, depolarization steps from various holding potentials to −5 mV were applied at a frequency of 0.2 Hz. The early component was inhibited by reducing the holding potential between −75 and −50 mV. By this procedure, the peak of the residual inward current was decreased, and at a more positive holding potential the rate of activation of this inward current was reduced (Figure 8b). The relationship between the early component and the holding potential is shown in Figure 8c.

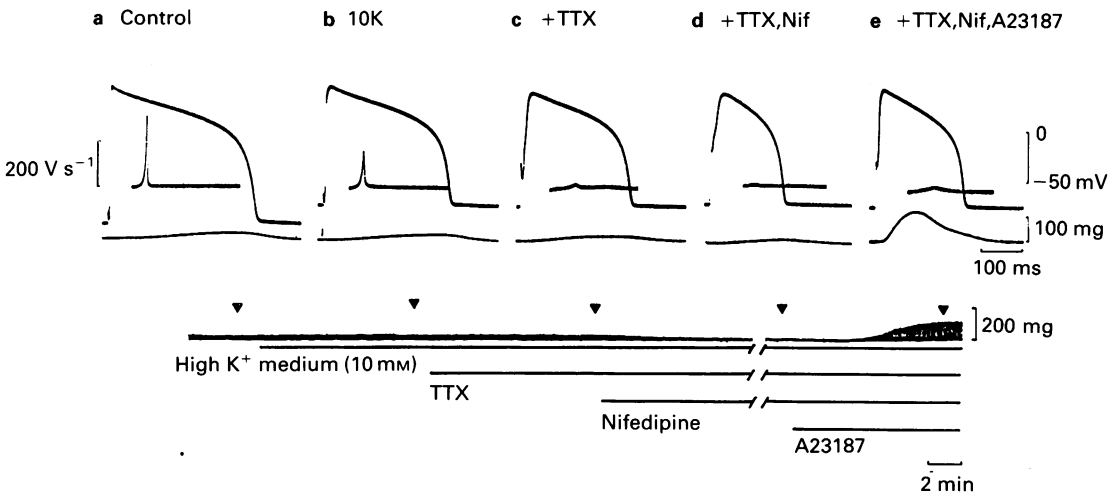


Figure 6 Effects of tetrodotoxin (TTX) on the electromechanical response to A23187 in slightly depolarized preparations in the presence of nifedipine. Upper panels show the action potential, the first derivative of the action potential and simultaneously recorded contraction: (a) control; (b) 10 min after exposure to high K^+ medium (10 mM); (c) 10 min after pretreatment with TTX (6×10^{-6} M) in high K^+ medium; (d) 25 min after additional treatment with nifedipine (Nif) (10^{-6} M); (e) 9 min after application of A23187 (10^{-6} M) in high K^+ medium with TTX and nifedipine. Lower trace shows a continuous record of isometric tension. Arrowheads indicate recording points of the electromechanical responses.

Discussion

It has been demonstrated that A23187 produced the two components (early and late) of contraction in cardiac muscles electrically stimulated at constant frequency (Kondo, 1986). The early component was inhibited by ryanodine, an agent decreasing internal Ca release, and the late one was blocked by nifedipine, a slow Ca channel blocker. Furthermore, when external Ca was replaced by strontium which can flow into cells through slow Ca channels but cannot substitute for Ca in the function of the sarcoplasmic reticulum (SR), the nifedipine-sensitive component (NSC) was fully activated, while the ryanodine-sensitive component (RSC) was not (Kondo, 1987). These previous observations indicate that the RSC corresponding to the early component is attributable to Ca release from the SR, while the NSC corresponding to the late component is due to Ca influx via slow Ca channels. The involvement of Na-Ca exchange in these two components has been excluded because of the complete abolition of these components by the combined treatment with nifedipine and ryanodine (Kondo, 1986; 1987). In the present study, such RSC produced by A23187 was found to depend on the membrane resting potential.

The biphasic contraction was also caused in the presence of isoprenaline, a β-adrenoceptor agonist, and RSC could be pharmacologically separated from NSC by pretreatment with nifedipine. This RSC showed voltage-dependence similar to that of RSC produced by A23187. Honore *et al.* (1987) have also reported two components of contraction in a K^+-rich solution containing isoprenaline and the first component of these has been shown to be more sensitive to resting potential depolarization than the second component. Therefore, the present voltage-dependence is a feature common to RSC corresponding to the early component of contraction. However, the contraction developed by A23187 is more advantageous than that induced by isoprenaline for analysis of the two components because of the clear distinction between the two components (RSC and NSC) produced by A23187 and the large amplitude of its RSC.

The presently defined RSC was inhibited by increasing $[K^+]_o$ which depolarized the resting potential. Similar dependence was observed on the early component of contraction corresponding to RSC, when the resting potential was directly depolarized by current clamp techniques. Under voltage clamp conditions, this component was also inhibited by depolarizing the holding potentials in the same potential range as observed in the current clamp study. Thus, the inhibition of RSC is due to a reduction of the resting potential, but not the direct effect of K^+, suggesting that Ca release from the SR resulting in RSC depends on membrane potential.

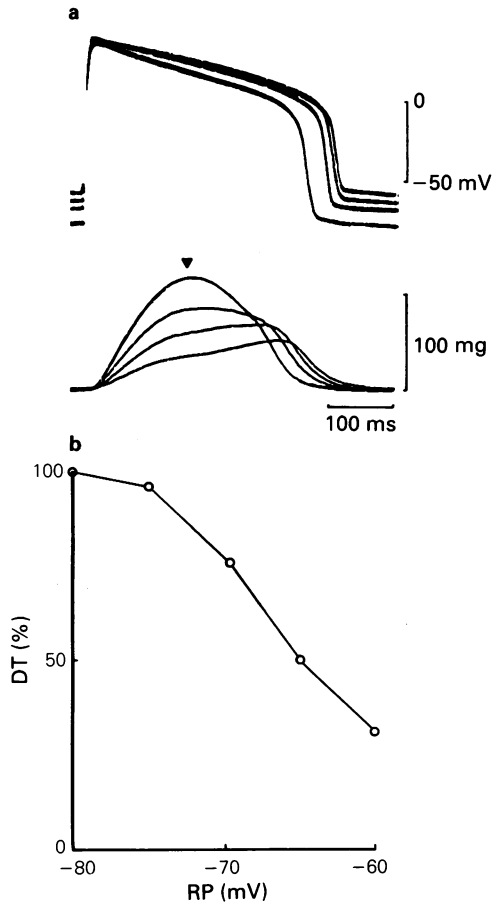


Figure 7 Effects of reducing resting potential by current clamp techniques on electromechanical response to A23187. Upper panel (a) shows the superimposable traces of the action potential and contraction obtained at resting potential of −80, −70, −65, and −60 mV. The amplitude of the early component was measured at the point indicated by arrowhead (160 ms after onset of stimuli). Current clamp experiment was performed under the condition where the contraction increased by A23187 (10^{-6} M) reached a steady state. In (b) is shown the relationship between the resting potential (RP) and the developed tension (DT) measured at the arrowhead (% of the amplitude of early component before supplying current).

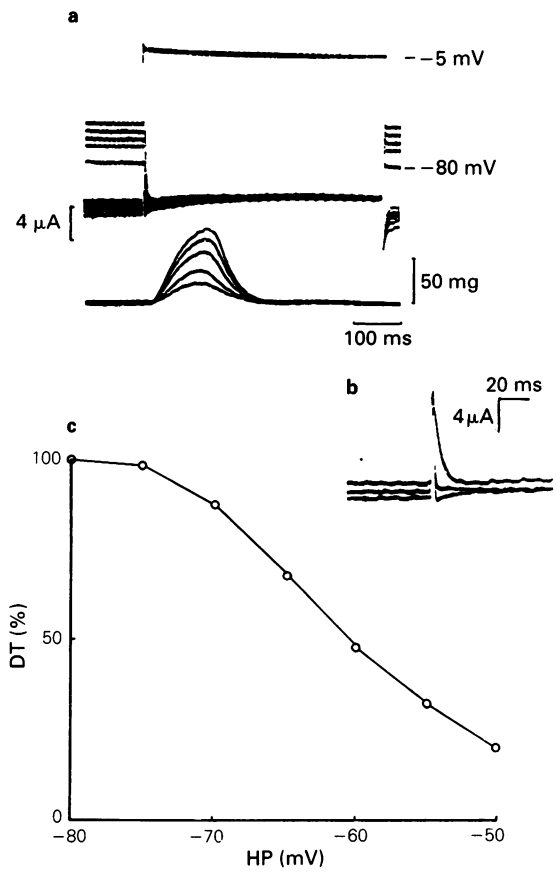


Figure 8 Effects of reducing holding potential by voltage clamp techniques on the early component of contraction produced by A23187 and currents in the presence of tetrodotoxin (TTX) and nifedipine. Upper panel (a) shows the superimposable traces of the voltage step (top trace), membrane current (middle trace) and the contraction (bottom trace). Holding potential was changed under the conditions where the contraction increased by A23187 (10^{-6} M) reached a steady state in the presence of TTX (10^{-5} M) and nifedipine (10^{-6} M). Depolarization steps of 500 ms duration from a holding potential of −80, −70, −65, −60 and −55 mV to −5 mV were applied at a frequency of 0.2 Hz. Inset (b), current traces at holding potentials of −80, −65 and −55 mV were superimposed at a high sweep speed. Lower graph (c) shows the relationship between the holding potential (HP) and the developed tension (DT) (% of DT before depolarizing the holding potential).

Ca release from the SR on cardiac excitation-contraction coupling is thought to be mainly mediated by a Ca-induced Ca release mechanism (Endo, 1977; Fabiato, 1985a,c). It has been suggested that Ca for triggering Ca release flows through slow Ca channels (London & Krueger, 1986; Barcenas-Ruiz & Wier, 1987) and induces Ca release in a concentration-dependent manner (Fabiato, 1985c). Therefore, it is possible to assume that the present voltage-dependence of RSC is due to the reduction of Ca influx for triggering Ca release by inactivating slow Ca channels at more positive resting potentials. However, this possibility may be minor because of

the following reasons: (1) in all the present experiments, the inhibition of RSC was observed in a potential range more negative than the threshold of inactivation of the slow Ca channels (from −75 to −50 mV); (2) RSC was inhibited by depolarizing the resting potential by high $[K^+]_o$ without inhibiting the NSC; (3) In current and voltage clamp experiments, the voltage-dependent inhibition of the early component of contraction corresponding to RSC occurred without affecting the slow action potentials and nifedipine-sensitive inward currents. These observations suggest that the voltage-dependence of RSC via internal Ca release is not attributable to that of the slow Ca channels. However, the possibility cannot be excluded that Ca influx through the slow Ca channels contributes to some extent to inducing RSC probably through triggering Ca release, since when the late component of contraction was fully activated in the absence of nifedipine, a small early component remained even after depolarizing the resting potential to −50 mV.

Recently, in cardiac muscles, it has been proposed that Ca-induced Ca release is triggered by Ca flowing through Ca channels different from the slow channels (Fabiato, 1985a; Morad & Cleemann, 1987). This channel has been defined as a T-type of Ca channel (Hume & Giles, 1983; Bean, 1985; Nilius *et al.*, 1985; Mitra & Morad, 1986). From these studies, a T-type of Ca channel is characterized by the low-activation threshold, the rapid inactivation, and the inactivation at low holding potential (almost abolished at a holding potential of −50 mV). It is not sensitive to dihydropyridine derivatives. These characteristics are consistent with a voltage-dependence of RSC and its pharmacological sensitivity shown in the present study. Furthermore, in the present voltage clamp experiments after blocking fast Na and slow Ca channels by TTX and nifedipine, a small residual inward current induced by positive clamp step was inhibited along with the early component of contraction by depolarizing the holding potential within a low voltage range below the threshold of the inactivation of the slow Ca channels. It is reasonable to assume that Ca influx through T-type Ca channels triggers RSC via ryanodine-sensitive Ca release. This assumption may explain the selective inhibition of RSC at depolarized resting potential (near −50 mV) without inhibiting the NSC via the slow Ca channels.

Another Ca release mechanism, depolarization-induced release, has been suggested in skeletal (Costantin & Podolsky, 1967; Kasai & Miyamoto, 1973; Endo, 1977; Martonosi, 1984) and cardiac (Isenberg *et al.*, 1985) muscles. Hence, involvement of surface membrane depolarization in the voltage-dependence of RSC may be considered. However, in the present experiments with high K^+ medium and the current clamp techniques, RSC and the early component of contraction was selectively inhibited even when a large membrane depolarization was induced by the slow action potential. When membrane depolarization was kept at a constant level by voltage clamp techniques, the dependence of the early component on the holding potential was observed. Thus, depolarization-induced release mechanism may not be a major role in activation of RSC. However, this possibility cannot be completely ruled out, since voltage clamp experiments with TTX and nifedipine, a small residual early component was still observed at a holding potential held at −50 mV.

The voltage-dependence of RSC is similar to that of Na channel inactivation. However, the involvement of the Na channel is eliminated, since RSC was not substantially affected by blocking Na channels with TTX.

Based on the above discussion, it seems reasonable to conclude that RSC via Ca release from the cardiac SR is possibly triggered by Ca flowing through Ca-channels different from the slow channels, most likely T-type of channels. However, the present results do not exclude the possibility that Ca-induced Ca release triggered by the slow Ca inward current and depolarization-induced Ca release may contribute to some extent to induction of RSC. These present results fit with the previous proposition (Morad & Cleemann, 1987; Honore *et al.*, 1987) that Ca influx through T-type Ca channels triggers Ca release, while that through the slow channels increases $[Ca^{2+}]_i$ for activation of contraction.

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Alveolar macrophages and eicosanoids but not neutrophils, mediate bronchoconstriction induced by FMLP in the guinea-pig

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1 N-formyl-methionyl-leucyl-phenylalanine (FMLP), when administered by aerosol to guinea-pigs, induced a dose-dependent bronchoconstriction (BC) with no overt effect on platelet and leukocyte blood counts. Repeated administration of FMLP by aerosol was followed by desensitization.

2 Electron microscopy studies showed that administration of FMLP by aerosol is accompanied by alveolar macrophage activation, accumulation and aggregation in the alveolar lumens. Non-degranulated eosinophils were observed in the lungs and a few platelet micro-aggregates in the pulmonary microvasculature.

3 No significant accumulation of ^{131}I-labelled albumin, ^{111}In-labelled neutrophils or ^{111}In-labelled platelets was detected in the lungs after the administration of FMLP by aerosol, whereas the intravenous administration was accompanied by an increase of extravascular albumin and significant neutrophil sequestration in the lungs.

4 Aspirin administered intravenously or by aerosol reduced significantly the BC induced by an aerosol of FMLP. By contrast, intravenous indomethacin reduced only BC induced by the submaximal dose of FMLP as an aerosol whereas, when administered by inhalation, it inhibited BC induced by FMLP administered either intravenously or by aerosol at all the concentrations tested.

5 FMLP induced a dose-dependent contraction of the guinea-pig trachea, which was not inhibited by indomethacin.

6 The dual cyclo-oxygenase/lipoxygenase inhibitor compound BW755C suppressed the BC induced by an aerosol of FMLP at all the concentrations used, whereas the histamine H_1-antagonist mepyramine was inactive.

7 Leukocyte depletion with vinblastine failed to reduce BC induced by intravenous or an aerosol of FMLP.

8 Our studies indicate that: (a) FMLP administered by aerosol induces dose-dependent BC followed by desensitization, indicating that local mechanisms account for BC; (b) BC induced by i.v. FMLP, but not by its inhalation, is accompanied by albumin extravasation and neutrophil sequestration in the lungs; (c) BC by either i.v. or an aerosol of FMLP is not due to neutrophil activation; (d) inhalation of FMLP induces BC accompanied by accumulation of activated alveolar macrophages, non-degranulated eosinophils and a few platelet microaggregates in the lung; (e) both cyclo-oxygenase and lipoxygenase metabolites are involved in the BC induced by an aerosol of FMLP.

Introduction

We have demonstrated that the leukocyte secretagogue N-formyl-methionyl-leucyl-phenylalanine (FMLP) injected intravenously to guinea-pigs induces platelet-independent bronchoconstriction (BC) and leukopenia. This suggested that the activation of circulating leukocytes might be involved in BC, but since isolated parenchymal lung strips were contracted by FMLP, the participation of polymor-

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phonuclear leukocytes (PMN) was doubtful. Furthermore, i.v. indomethacin failed to prevent FMLP-induced BC, whereas aspirin, another cyclo-oxygenase inhibitor, was very effective (Boukili *et al.*, 1986).

Leukocytes and particularly neutrophils constitute the main cellular defence system of higher organisms against bacteria and are implicated in lung injury during shock, as indicated by depletion studies (Henson, 1972; Staub *et al.*, 1982; Worthen *et al.*, 1986; Chang *et al.*, 1987). In addition, PMN play a major role in tracheal oedema induced by an aerosol of toluene diisocyanate, which is also suppressed by leukocyte depletion (Sheppard *et al.*, 1986). Furthermore, this depletion prevents albumin extravasation into rabbit skin caused by local injections of FMLP (Wedmore & Williams, 1981; Weisdorf & Jacob, 1987). This led us to investigate the mechanism of the BC induced by FMLP, and particularly the role of PMN, using PMN-depleted guinea-pigs and the isolated trachea. Since airborne substances of toxicological importance, allergens and therapeutic agents may be delivered to the respiratory tract by inhalation (Schanker *et al.*, 1986), we also determined whether FMLP retains its ability to induce BC when administered by aerosol. Since this was so, we studied the effects of indomethacin and aspirin or the dual cyclo-oxygenase/lipoxygenase inhibitor BW755C on the FMLP-induced BC, in order to determine the role of cyclo-oxygenase or lipoxygenase arachidonate metabolites.

Methods

Guinea-pig in vivo *preparation*

Hartley guinea-pigs of either sex (400–600 g) were anaesthetized with sodium pentobarbitone (40 mg kg^{-1} i.p.) and spontaneous breathing was suppressed with pancuronium (2 mg i.v.). The trachea was cannulated and guinea-pigs ventilated with a Palmer miniature pump at 60 strokes min^{-1} with 1 ml of air per 100 g of body weight. In these experiments, guinea-pigs were treated with propranolol (1 mg kg^{-1} i.v., plus 3 mg kg^{-1} i.p.). Both carotid arteries were cannulated to record arterial blood pressure and for blood sampling. Drugs were injected through the jugular vein. Bronchial reactivity was tested with 5-hydroxytryptamine (5-HT, 1–3 μg i.v.). BC was evaluated according to the pulmonary resistance to inflation and arterial blood pressure was recorded on a Beckman Dynograph R 511.

FMLP and drug administration

One group of animals was injected intravenously with FMLP (0.3, 1, 3 and 10 μg kg^{-1} at 45 min intervals), another group was exposed to an aerosol of FMLP at 10, 100 and 1000 μg ml^{-1} for 2 min in the nebuliser cuvette.

Solutions of FMLP (10, 100 and 1000 μg ml^{-1} during 2 min), aspirin (300 mg ml^{-1} during 30 min) and indomethacin (100 mg ml^{-1} during 20 min) or the appropriate solvents, i.e., saline for FMLP (the stock solution of FMLP was prepared in DMSO at final concentration of 50 mg ml^{-1} and then diluted in saline to obtain 1000 μg ml^{-1}) and aspirin, or NaOH (pH 7.8–8.0) for indomethacin (Boukili *et al.*, 1986), were placed in the reservoir of a nebuliser connected to an air compressor, and the flow was directed into the outflow tube of the respiratory pump as described by Lagente *et al.* (1988). FMLP was delivered 5 and 15 min after administration of aspirin and indomethacin intravenously or by aerosol, respectively. Finally, compound BW755C and mepyramine diluted in distilled water and in saline, respectively, were injected i.v. 3 and 5 min before FMLP administration.

Measurements of extravascular albumin accumulation and neutrophil or platelet sequestration in the lungs

The basic procedure was described by Bureau *et al.* (1987). Briefly, erythrocytes and albumin labelled with sodium pertechnetate ^{99m}technetium and ^{131}iodine respectively were injected i.v. to guinea-pigs 30 min before the experiment. Neutrophils were prepared from the pleural cavity of donor guinea-pigs, radiolabelled with ^{111}indium tropolonate and injected 24 h before the experiment. In separate experiments, platelets were isolated and radiolabelled according to Page *et al.* (1982). External monitoring of lung radioactivity was performed with a collimated gamma detector and blood samples (200 μl) were collected from the cannulated carotid artery in tubes containing 0.38% sodium citrate to evaluate the percentages of albumin lung content and of circulating neutrophils and platelets sequestered in the lungs. Radiolabelled erythrocytes were used, in this procedure, as a vascular reference tracer.

Histological techniques

FMLP was administered to guinea-pigs by inhalation at 100 μg ml^{-1} for 2 min and lungs were removed 1 min later, which corresponds to the maximum of the BC. For light microscopy, lung tissue was fixed in 10% buffered formalin and embedded in paraffin. Sections were stained with hematoxylin-eosin-safranin or Biebrich scarlet for eosinophil granules as described by Lellouch-Tubiana *et al.* (1988). For electron microscopy, tissue fragments were fixed in 2.5% glutaraldehyde at 4°C.

The small blocks were rinsed in phosphate buffer, postfixed in 2% osmic acid, dehydrated in graded alcohols and embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate before examination in a Philips EM 300 transmission electron microscope.

Treatment of guinea-pigs with vinblastine and quantification of circulating leukocytes

Guinea-pigs were injected with 0.75 mg kg^{-1} of vinblastine or its solvent (saline) into the saphenous vein. Seventy h later, under pentobarbitone anaesthesia, blood (200 μl) was collected from the carotid artery and added to 10 ml of a balanced electrolyte solution (Isoton II). Erythrocytes were then lysed with Zap Oglobin and the total leukocyte number evaluated with a Coulter Counter ZBI in control and vinblastine-pretreated guinea-pigs.

Studies on the isolated trachea

Tracheae were removed from anaesthetized guinea-pigs, cut spirally (Prancan *et al.*, 1982) and mounted in 16 ml organ baths containing Krebs solution, which was continuously gassed with a mixture of 95% O_2/5% CO_2 and maintained at 37°C. The composition of the Krebs solution was (mM): NaCl 118, KCl 4.7, $CaCl_2$ $2H_2O$ 2.5, $MgSO_4$ $7H_2O$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 5.6. One extremity of each trachea was connected to a Statham force-displacement transducer under a resting tension of 2 g. One hour was allowed for equilibration and acetylcholine, the standard bronchoconstrictor agonist used, was then added cumulatively (0.1, 1 and 10 μM) at 3 min intervals. After three washings and 15 min later, FMLP was added, at final concentrations of 3, 10, 30, 100 and 300 nM at 20 min intervals.

Statistical analysis

Statistical significance was evaluated by unpaired Student's t test. All data are expressed as mean ± s.e.mean.

Drugs and chemicals

Sodium pentobarbitone (Clin Midy, France), pancuronium (Pavulon, Organon, France); propranolol (ICI, U.K.); acetylcholine, arachidonic acid, 5-hydroxytryptamine (5-HT), indomethacin, tropolone (2-hydroxy-2,4,6-cyclo-heptatrienone) and N-formyl-L-methyionyl-L-leucyl-L-phenylalanine (FMLP) (Sigma, U.S.A.); dimethylsulphoxide (DMSO) (Merck, R.F.A.); aspirin as lysine acetyl salicylate (Aspegic, Laboratoires Egic, France); 3-amino-1-(*m*-(trifluoromethyl) phenyl)-2-pyrazoline (BW755C) (The Wellcome Research Laboratories, Beckenham, U.K.); mepyramine maleate (Rhône-Poulenc Santé, France); vinblastine sulphate (Velbé, Lilly, France). Zap Oglobin and Isoton II (Coultronics, France). Sodium citrate (3.8% w/v, Biosedra, France). ^{131}I-albumin (Sorin Biomedica, Italy); [^{111}In]-oxinate and [^{111}In]-chloride (Amersham, U.K.); sodium pyrophosphate, decahydrate and stannous chloride (CEA-OIRIS, France); sodium pertechnetate ^{99m}technetium (gift from Prof. G. Vallée, Service Central de Radio-isotopes, Hôpital Necker, Paris, France).

Results

Effects of administration of FMLP by aerosol

FMLP given by aerosol at 45 min intervals was followed by a dose-dependent BC (Figure 1) with no overt effects on platelet and leukocyte blood counts. Indeed, BC was 4.73 ± 0.56, 9.08 ± 1.37 and 20.17 ± 2.43 cmH_2O at 10, 100 and 1000 μg ml^{-1}, respectively. Repeated administration of FMLP by aerosol (1000 μg ml^{-1} for 2 min) induced 29.3 ± 5.2, 46.5 ± 9.7 and 70.3 ± 3.2% desensitization after the second, the third and the fourth administration, respectively. No significant difference in desensitization was noted when FMLP (1 μg kg^{-1}) was injected i.v. before the repeated administration of FMLP by aerosol (data not shown). BC induced by an aerosol of FMLP was accompanied by systemic arterial hypotension of 27.2 ± 3.2, 30.9 ± 6.6 and 38.6 ± 6.4% (peak values) at 10, 100 and 1000 μg ml^{-1}, respectively.

Electron microscopic study

The main microscopic finding in the lungs from animals administered FMLP by aerosol (100 μg ml^{-1} for 2 min, n = 4), was the accumulation of activated macrophages in the alveolar lumens.

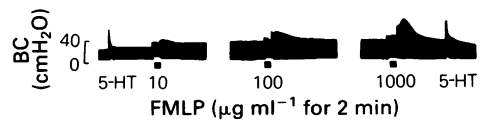


Figure 1 Representative tracing of bronchoconstriction (BC, in cmH_2O) produced by N-formyl-methionyl-leucyl-phenylalanine (FMLP) administered by aerosol at 10, 100 and 1000 μg ml^{-1} for 2 min at 45 min intervals. The beginning and the end of the administration of FMLP are indicated by the black box below each tracing. 5-Hydroxytryptamine (5-HT, 2 μg i.v.) was used to evaluate the responsiveness of the airways. The figure is representative of 5 experiments.

Some of them were aggregated (Figure 2a). Endothelial cells of the alveolar capillaries showed numerous pinocytotic blebs (Figure 2b). Moreover, eosinophil infiltration was observed in the lung parenchyma (Figure 2b). Surprisingly, platelet accumulation (Figure 2b) and a few microaggregates of non-degranulated platelets were present in the pulmonary microvasculature (Figure 2c). Alveolar macrophages from control animals ($n = 3$) which received an aerosol of saline for 2 min, were found in isolated places in the tissue and were almost inactive (Figure 2d).

Measurements of extravascular albumin accumulation and neutrophil or platelet sequestration in guinea-pig lungs

The extravascular lung content of ^{131}I albumin and ^{111}In (from labelled neutrophils and platelets, respectively) was not modified after an aerosol of FMLP ($100\,\mu g\,ml^{-1}$ for 2 min, Figures 3 and 4; not shown for platelets). The aerosol of saline and FMLP induced the same effect, i.e., a slight increase of extravascular albumin content, probably due to the trauma following the clamping of the canulla needed to perform the administration by aerosol. By contrast, clear increases of extravascular albumin content and neutrophil sequestration were recorded when FMLP was injected i.v. at $10\,\mu g\,kg^{-1}$. Indeed, the accumulation of extravascular albumin reached $17.4 \pm 3.8\%$ vs $-0.1 \pm 4.0\%$ for the control group at its peak, i.e. at 30 min ($P < 0.05$). In addition, neutrophil sequestration reached $6.8 \pm 1.3\%$ 10 min after the i.v. injection of FMLP, as compared to control (saline-injected) animals, in which accumulation was of $2.1 \pm 0.8\%$ ($P < 0.05$).

Effect of mepyramine, aspirin, indomethacin and compound BW755C on the bronchoconstriction induced by an aerosol of FMLP

The H_1-histamine antagonist mepyramine at $0.2\,mg\,kg^{-1}$, a dose which antagonizes BC by large amounts of histamine (Garcez do Carmo *et al.*, 1986), was ineffective against BC due to FMLP administered by inhalation (data not shown). By contrast, i.v. (Figure 5) and inhaled (Figure 7) aspirin significantly inhibited the BC induced by an aerosol of FMLP, whereas i.v. indomethacin was only effective against a sub-maximal amount of FMLP ($100\,\mu g\,ml^{-1}$ for 2 min, Figure 5). Furthermore, the i.v. administration of indomethacin failed to prevent BC induced by i.v. FMLP administered at 3 and $10\,\mu g\,kg^{-1}$, as shown previously (Boukili *et al.*, 1986). By contrast, i.v. indomethacin inhibited the BC induced by i.v. arachidonate; the BC was 16.6 ± 3.1 ($n = 5$) and 1.9 ± 0.4 ($n = 4$) cmH_2O in control and indomethacin-treated guinea-pigs, respectively ($P < 0.01$). When indomethacin was administered by inhalation at $100\,mg\,ml^{-1}$ for 20 min, it significantly reduced both BC due to the i.v. (3 and $10\,\mu g\,kg^{-1}$, Figure 6) and to the aerosol (10, 100 and $1000\,\mu g\,ml^{-1}$, Figure 7) administration of FMLP. Furthermore, indomethacin administered by aerosol suppressed the BC induced by i.v. arachidonate ($0.5\,mg\,kg^{-1}$; data not shown). Finally, the mixed cyclo-oxygenase and lipoxygenase inhibitor compound BW755C ($30\,mg\,kg^{-1}$, i.v.) suppressed the BC induced by all the concentrations of FMLP administered by aerosol; the inhibition was 100% ($n = 3$) at 10 and $100\,\mu g\,ml^{-1}$ and $85 \pm 20\%$ at $1000\,\mu g\,ml^{-1}$ ($n = 4$, $P < 0.01$). As previously described (Boukili *et al.*, 1986), i.v. BW755C strongly inhibited BC induced by i.v. FMLP.

Effect of leukocyte depletion with vinblastine on the bronchoconstriction induced by FMLP

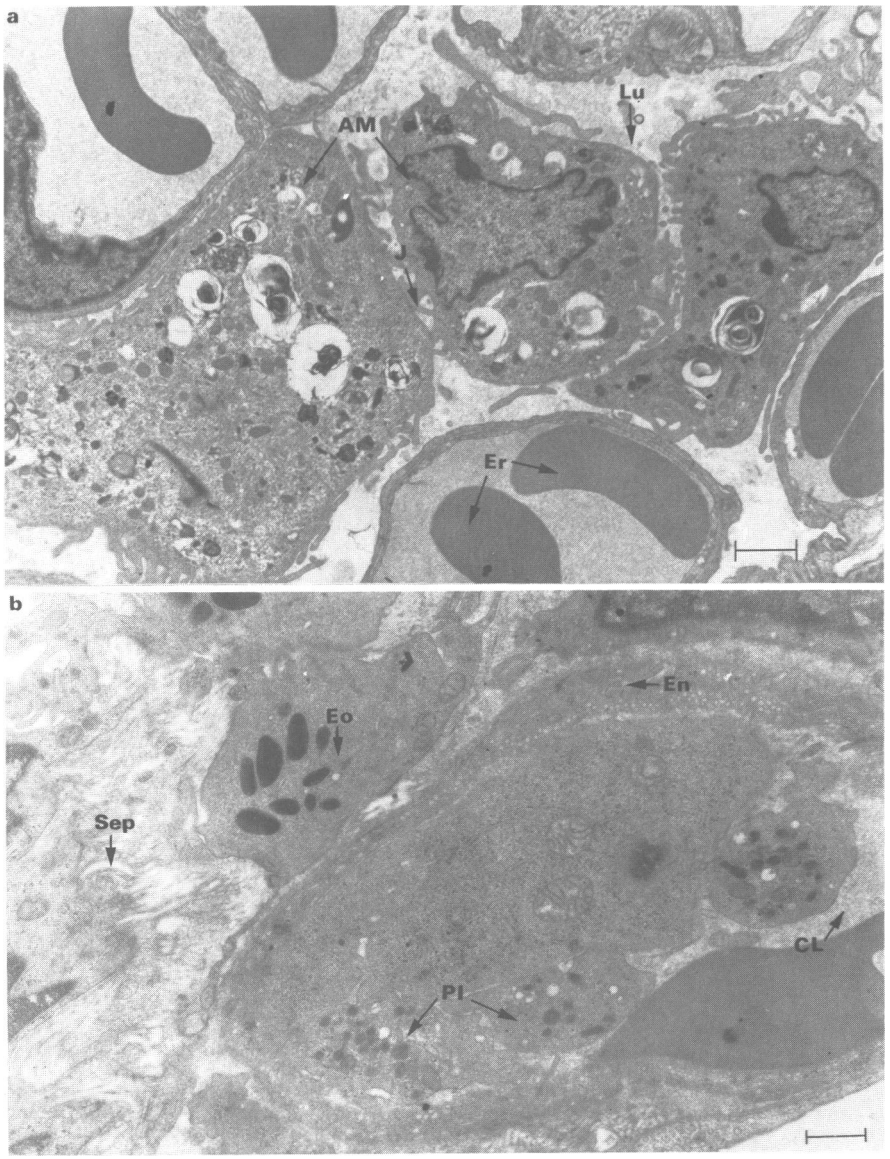
Three days after the i.v. administration of vinblastine or saline, the total number of leukocytes was $4200 \pm 523\,\mu l^{-1}$ in animals injected with saline vs $725 \pm 14\,\mu l^{-1}$ in animals injected with vinblastine ($P < 0.001$, $n = 4$ for each group). The percentage reduction of circulating leukocyte number was $82 \pm 3\%$. Furthermore, a significant loss of weight of the animals (-97 ± 17 g) was noted. In no instance was BC by i.v. (Figure 8) and an aerosol of FMLP (not shown) affected by vinblastine pretreatment; in fact, the response to $1\,\mu g\,kg^{-1}$ of i.v. FMLP was significantly enhanced. The BC induced by i.v. 5-HT $2\,\mu g$ ($18.9 \pm 2.9\,cmH_2O$) was not significantly modified in the vinblastine-treated animals; BC in control, untreated animals was $15.3 \pm 2.3\,cmH_2O$ in response to 5-HT.

Effect of FMLP on the isolated trachea

FMLP induced a dose-dependent contraction of the trachea. Indeed, the tensions recorded were 25 ± 5, 49 ± 8.5, 200 ± 33, 200 ± 60 and 245 ± 45 mg at 3, 10, 30, 100 and 300 nM FMLP, respectively. Mepyramine ($1\,\mu M$) given with indomethacin ($3\,\mu M$) failed to reduce these contractions (data not shown). In comparison, the standard bronchoconstrictor agonist acetylcholine, 0.1, 1 and $10\,\mu M$, added cumulatively at 3 min intervals, induced contractions of 56 ± 8, 105 ± 11 and 149 ± 18 mg (mean ± s.e.mean), respectively.

Discussion

In this study, we demonstrated that the administration of FMLP by aerosol induces dose-dependent


a
Lu
AM
Er
b
En
Eo
Sep
CL
Pl

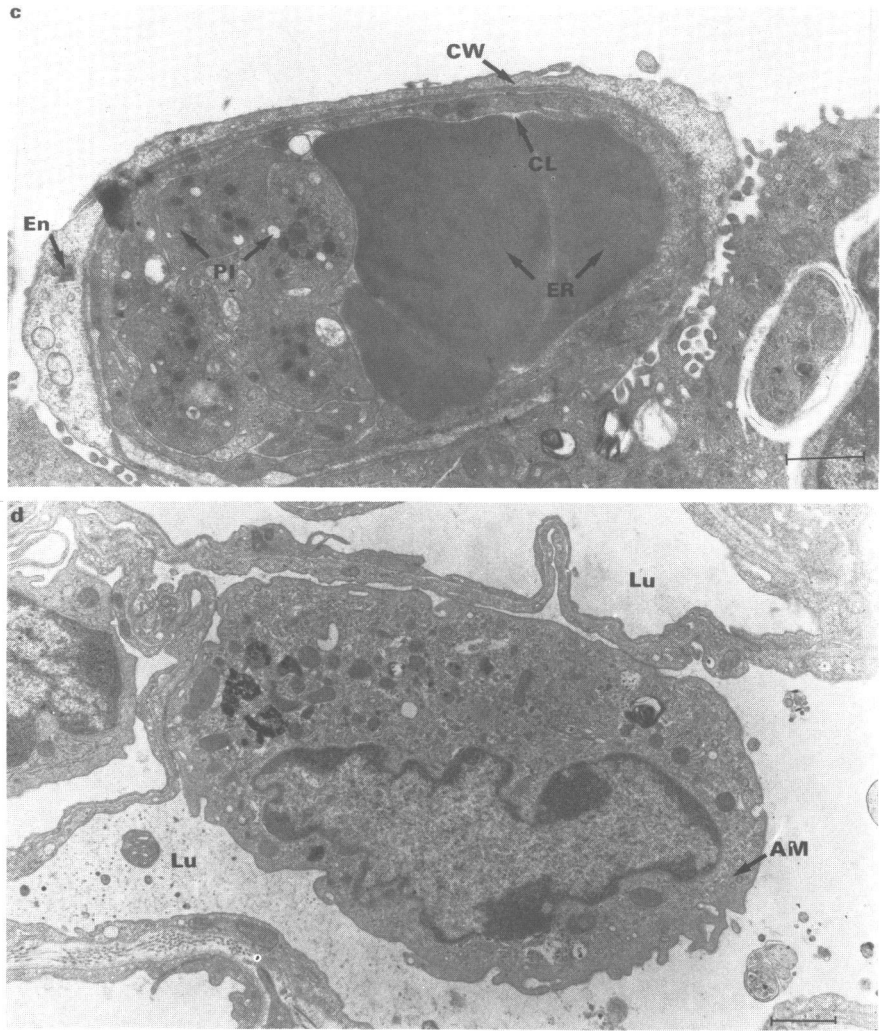


Figure 2 Electron micrographs of guinea-pig lung parenchyma 1 min after the administration of FMLP by aerosol ($100\,\mu g\,ml^{-1}$ for 2 min, $n = 4$). (a) Note the accumulation of alveolar macrophages (AM) in the alveolar lumen (Lu). These macrophages are activated and present numerous pseudopods and intercellular junctions (J), Er = erythrocyte; scale bar = $2\,\mu m$. (b) Micrograph showing accumulation of platelets (Pl) in the capillary lumen (CL), pinocytotic blebs in the endothelial cells (En) and eosinophils (Eo) containing their specific granules in the interstitial septum (Sep), scale bar = $0.5\,\mu m$. (c) A platelet microaggregate (Pl) is observed in the alveolar capillary lumen (CL); CW = capillary wall, Er = erythrocyte, En = endothelial cell; scale bar = $2\,\mu m$. (d) Electron micrograph represents a cross section after the inhalation of saline for 2 min ($n = 3$) and shows an inactivated alveolar macrophage (AM) in an alveolar septum; Lu = Alveolar lumen, scale bar = $2\,\mu m$.

BC and hypotension in the guinea-pig. Hypotension is probably due to the generation of vasoactive prostanoids, as indicated by its reduction by aspirin and indomethacin (not shown). BC is accompanied by the activation, accumulation and aggregation of alveolar macrophages in the alveolar spaces, and the presence of platelet microaggregates and of non-degranulated eosinophils. Stimulated alveolar macrophages secrete inflammatory mediators and thus participate in the pulmonary defence mechanisms (Rudent *et al.*, 1987; Nathan, 1987). Since guinea-pig eosinophils are not activated *in vitro* by FMLP (Ogawa *et al.*, 1981; Yamashita *et al.*, 1985; Coëffier, E., Joseph D. & Vargaftig, B. B., unpublished observations), the lung eosinophil infiltration we identified following the administration of FMLP by aerosol may be explained by the secretion of eosinophilotactic substances by the macrophages

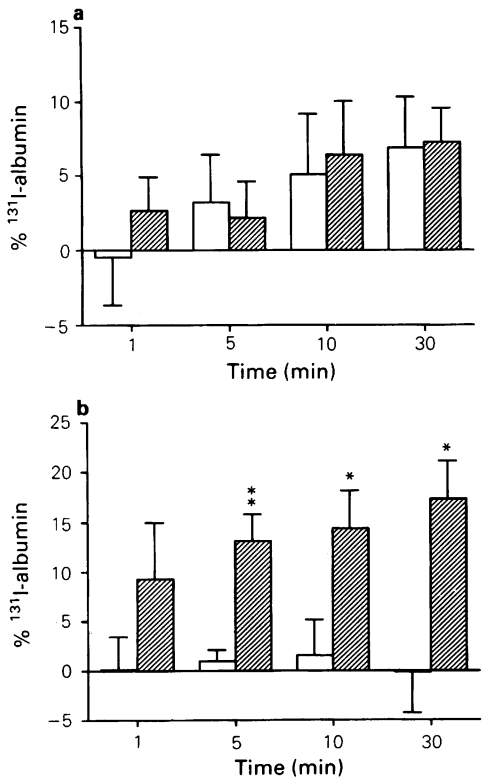


Figure 3 Values of extravascular ^{131}I-albumin, expressed as % of basal lung vascular content of radiolabelled albumin, 1, 5, 10 and 30 min after (a) administration of FMLP by aerosol ($100\,\mu g\,ml^{-1}$ for 2 min, hatched columns; $n = 5$) or (b) after an i.v. injection of FMLP ($10\,\mu g\,kg^{-1}$, hatched columns, $n = 5$). Open columns represent responses to saline. Vertical lines indicate s.e.mean. * $P < 0.05$, ** $P < 0.01$.

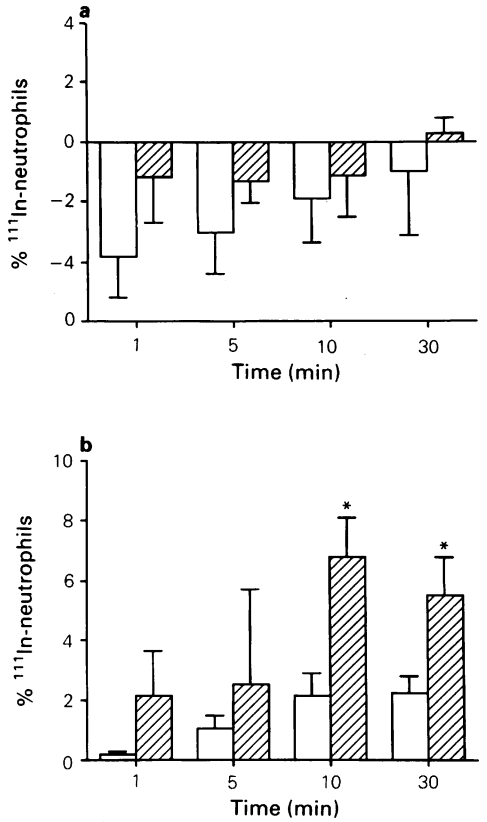


Figure 4 Values of ^{111}In-labelled neutrophils sequestered in the lungs, expressed as % of their basal blood content, 1, 5, 10 and 30 min after (a) administration of FMLP by aerosol ($100\,\mu g\,ml^{-1}$ for 2 min, hatched columns, $n = 5$) or (b) after its i.v. injection ($10\,\mu g\,kg^{-1}$, hatched columns, $n = 5$). Open columns represent responses to saline. Vertical lines indicate s.e.mean. * $P < 0.05$.

(Nathan, 1987). Hénocq & Vargaftig (1988) also found that eosinophils are elicited in FMLP-injected human skin; here again, substances released by macrophages may be involved.

Guinea-pig alveolar macrophages exposed to FMLP undergo extensive mobilisation of their endogenous arachidonic acid (Masliah *et al.*, 1988). Rat alveolar or guinea-pig peritoneal macrophages stimulated with calcium ionophore A23187 or zymosan (Chandler & Fulmer, 1987) or with synthetic peptides (Hartung *et al.*, 1983), respectively, release thromboxane A_2 (TxA_2). In addition, FMLP-induced contractions of the pulmonary parenchyma strips, which are accompanied by TxA_2 formation (Burka, 1984; Boukili *et al.*, 1986), are inhibited by aspirin and indomethacin and by the mixed cyclo-

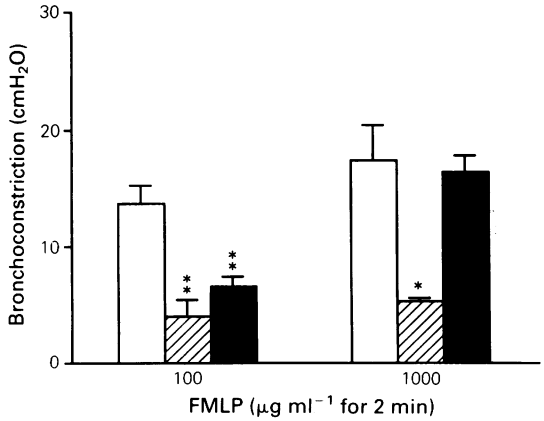


Figure 5 Effect of the i.v. administration of aspirin (hatched columns, 20 mg kg^{-1}, $n = 4$) and of indomethacin (solid columns, 10 mg kg^{-1}, $n = 4$) on the bronchoconstriction induced by an aerosol of FMLP (100 and 1000 μg ml^{-1} for 2 min). Open columns represent control responses to an aerosol of FMLP. Vertical lines indicate s.e.mean. * $P < 0.05$, ** $P < 0.01$.

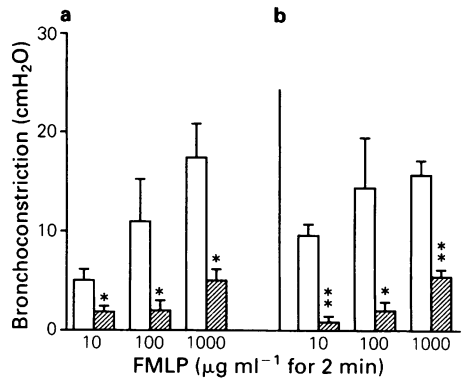


Figure 7 Effect of (a) aspirin (300 mg ml^{-1} by aerosol for 30 min, $n = 4$) and (b) indomethacin (100 mg ml^{-1} by aerosol for 20 min, $n = 4$) on the bronchoconstriction induced by an aerosol of FMLP (10, 100 and 1000 μg ml^{-1} for 2 min at 45 min intervals). Open columns represent control responses to an aerosol of FMLP. Vertical lines indicate s.e.mean. * $P < 0.05$, ** $P < 0.01$.

oxygenase/lipoxygenase inhibitor BW755C. The latter was more effective than pure cyclo-oxygenase inhibitors, since it suppressed the *in vitro* pulmonary parenchyma strip contractions, as well as the BC induced by i.v. (Boukili *et al.*, 1986) or an aerosol of FMLP suggesting the participation of lipoxygenase derivatives. In confirmation, exposure of lung parenchymal strips to FMLP is followed by the formation of leukotrienes (Shore *et al.*, 1987). There is thus agreement between *in vivo* and *in vitro* results with FMLP applied directly to lung tissues. By contrast, the suggested role of cyclo-oxygenase derivatives is inconsistent with the failure of indomethacin to reduce the contractile effects of FMLP on the isolated trachea. Nevertheless, in contrast to the parenchymal lung strips, the guinea-pig trachea does not synthesize TxA_2 (Grodzinska *et al.*, 1976). In fact, cyclo-oxygenase inhibition by indomethacin enhances histamine-induced contractions of the

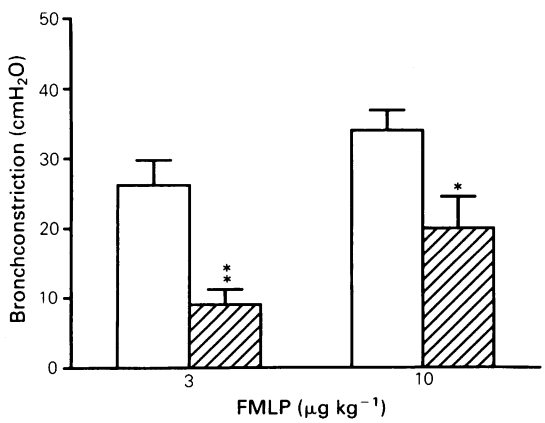


Figure 6 Effect of administration of indomethacin by aerosol (hatched columns, 100 mg ml^{-1} for 20 min, $n = 4$) on bronchoconstriction induced by i.v. FMLP (3 and 10 μg kg^{-1} at 45 min intervals). Open columns represent control responses to i.v. FMLP. Vertical lines indicate s.e.mean. * $P < 0.05$, ** $P < 0.01$.

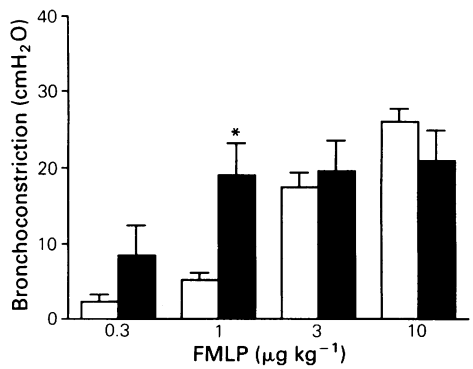


Figure 8 Effect of vinblastine pretreatment (solid columns, 0.75 mg kg^{-1} i.v., $n = 4$) of the bronchoconstriction induced by i.v. FMLP 0.3, 1, 3 and 10 μg kg^{-1}. Open columns represent control responses to i.v. FMLP. Vertical lines indicate s.e.mean. * $P < 0.05$.

guinea-pig trachea (Orehek *et al.*, 1975; Mitchell, 1982) and, accordingly, the responses of the isolated trachea do not reflect the events which occur in FMLP-induced BC *in vivo*. Finally, a role for histamine was ruled out by the failure of its potent antagonist mepyramine to suppress the *in vivo* and *in vitro* effects of FMLP.

Indomethacin administered i.v. may not reach the alveolar macrophage at the intervals we used, since it failed to block the BC induced by an aerosol of FMLP, whereas aspirin was effective. In addition, since an aerosol of indomethacin reduced the FMLP-induced BC, whatever its route of administration, it is clear that, contrary to our earlier contention (Boukili *et al.*, 1986), cyclo-oxygenase metabolites generated by the alveolar macrophages are involved in the BC induced by FMLP.

The repeated administration of FMLP by aerosol led to a reduced BC, as occurs when alveolar macrophages are exposed *in vitro* to FMLP (Daughaday *et al.*, 1985). This reduction is a true desensitization and was not due to interference with the access of the aerosol upon repeated administration, as a result of baseline changes or mucus secretion. Indeed, the intensities of BC were similar when guinea-pigs were first administered $1000\,\mu g\,ml^{-1}$ FMLP by aerosol or when FMLP was administered by inhalation at the same dose preceded by 10 and $100\,\mu g\,ml^{-1}$ at 45 min intervals. Under these conditions, it is likely that activation of the guinea-pig alveolar macrophages accounts, at least in part, for BC by FMLP and probably via the synthesis of TxA_2.

Neutrophil sequestration and albumin extravasation in lungs were only observed when FMLP was administered by the i.v. route (as previously shown by Bureau *et al.*, 1987, and confirmed by this study), but not by inhalation (Figures 3 and 4). This indicates that FMLP indeed activates neutrophils *in vivo* when given by the i.v. route, whereas it activates primarily the macrophages when given by aerosol (Figure 2a). Furthermore, an aerosol of FMLP did not induce leukopenia, as observed when it was injected i.v. (Boukili *et al.*, 1986). Nevertheless, BC by i.v. FMLP is not due to neutrophil activation or to their accumulation in the lungs, since the i.v. injections of FMLP still induced BC in leukocyte-depleted guinea-pigs (Figure 8). The finding that an aerosol of indomethacin inhibited the effects of i.v. FMLP (Figure 6) even though its i.v. administration was ineffective (Boukili *et al.*, 1986), reinforces the concept that the target cell involved in the FMLP-induced BC is not the circulating neutrophil.

In conclusion, our results suggest that the activation of the alveolar macrophage but not of the neutrophil, contributes to BC induced by FMLP administered by the i.v. and aerosol routes. BC is probably mediated by TxA_2 and/or by another cyclo-oxygenase metabolite of arachidonic acid. However, the marked effectiveness of BW755C and the surmountable inhibition by aspirin and indomethacin suggest the participation of lipoxygenase derivatives as well. Finally, since macrophage stimulation is followed by eosinophil and platelet recruitment, it is likely that other products secreted by the macrophages, explain the secondary recruitment of these inflammatory cells.

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Effect of acute and subchronic nicotine treatment on cortical acetylcholine release and on nicotinic receptors in rats and guinea-pigs

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1 The effect of acute and chronic (16 days) administration of nicotine on cortical acetylcholine (ACh) release, gross behaviour and brain nicotinic binding sites was investigated in rats and guinea-pigs.

2 The drug, injected either subcutaneously (0.45–0.90 mg kg^{-1}) or intracerebroventricularly (1, 3 and 5 μg) increased the cortical ACh release, in a dose-dependent manner, through mecamylamine-sensitive receptors for 1–2 h in both species.

3 Chronic treatment significantly increased basal ACh release in the rat and slightly lowered it in the guinea-pig, but the response to a challenging dose of nicotine was proportionally maintained in both species.

4 The number of nicotinic receptors was four times higher in the rat than in the guinea-pig and was not dependent on the radioligand used ([^{3}H]-nicotine or [^{3}H]-ACh, in the presence of atropine) to determine this. The nicotinic binding sites showed an apparent increase in chronically treated rats but no change in guinea-pigs.

5 Tolerance to the inhibitory effect of the drug, assessed with the T maze test, was found in the rat. No apparent change in gross behaviour was detected in the guinea-pig.

6 It is concluded that chronic nicotine treatment causes evident tolerance to its inhibitory effect on behaviour in the rat, but no adaptation to its excitatory properties on the cholinergic brain structures in rats and guinea-pigs.

Introduction

Nicotine is known to cause behavioural stimulation and EEG desynchronization in many animal species by interacting with specific CNS receptors, identified and distinguished in different subtypes with proper ligands (Domino, 1973; Morley *et al.*, 1979; Morley, 1981; Aceto & Martin, 1982; Hall, 1982; Larsson & Nordberg, 1985; Adem *et al.*, 1987; Wonnacott, 1987; Nordberg *et al.*, 1988a,b). The functional significance of these receptors still remains to be fully elucidated. Most of them are heteroreceptors present, for example, on noradrenergic, dopaminergic and 5-hydroxytryptaminergic cell bodies and axons (Schwartz *et al.*, 1984; Clarke & Pert, 1985; Härfstrand *et al.*, 1988). When stimulated, they increase the neuronal firing rate and transmitter release (Hall & Turner, 1972; Goodman, 1974; Giorguieff *et al.*, 1979; Yoshida *et al.*, 1980; Engberg & Swensson, 1980; Balfour, 1982; Westfall *et al.*, 1983; Clarke *et al.*, 1985; Egan & North, 1986; Rowell *et al.*, 1987; Mereu *et al.*, 1987). Mecamylamine and (sometimes) (+)-tubocurarine are effective blocking agents, suggesting the coexistence of ganglionic and neuromuscular junction subtypes in the same brain area (Morley, 1981; De La Garza *et al.*, 1987). Autoreceptors too seem to exist. This inference is based on binding studies carried out in human brains with spontaneous lesions of the basal cholinergic nuclei (Whitehouse *et al.*, 1986; Nordberg & Winblad, 1986) and on neurochemical investigations performed in animal brain synaptosomes (Rowell & Winkler, 1984; Moss & Wonnacott, 1985) and cortical slices (Beani *et al.*, 1985; Araujo *et al.*, 1987). The actual relevance of nicotine-induced facilitation of acetylcholine (ACh) release remains, however, to be firmly established in physiological, integrated models. In fact, *in vitro* studies might show the presence of nicotinic auto- and hetero-receptors which are devoid of any role in normal conditions. Also, no

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detailed data are available about the effects of nicotine on ACh release from the brain of the whole animal. Only preliminary observations have been carried out by Armitage *et al.* (1969) and Erickson *et al.* (1973). In addition, there are few results on the tolerance of the cholinergic systems to chronic nicotine.

The aim of the present study was to determine the effect of acute and chronic administration of nicotine on the cortical ACh release in two animal species, guinea-pigs and rats. Concomitantly, studies were performed to check for possible changes in behavioural responses and in the number of nicotinic binding sites following chronic treatment.

A preliminary account of this investigation has been recently published (Nordberg *et al.*, 1988c).

Methods

Wistar rats of either sex weighing 300–400 g and guinea-pigs of either sex weighing about 400 g were used. The animals were kept under a 12 h light/dark cycle and had free access to food and water.

Epidural cups

The epidural cup implantation on the left or right parietal cortex was performed according to the method described for the rabbit and guinea-pig as well as for the rat (Beani *et al.*, 1968; Beani & Bianchi, 1970; Casamenti *et al.*, 1980). In some animals an intraventricular cannula was also implanted into the ventricle on the opposite side (Beani & Bianchi, 1970).

Acute experiments

Two days after surgery rats and guinea-pigs underwent the experiment. Ringer solution containing 0.3 mM physostigmine (0.3 ml) was placed in the cup and renewed every 30 min. After 3–4 basal samples had been collected, (−)-nicotine was injected either subcutaneously (0.45–0.90 mg kg^{-1}, as base) or intracerebroventricularly (1, 3 or 5 μg base) and its effect was followed for at least 2 h. Changes in gross behaviour were evaluated by direct inspection of the animals. The amount of ACh present in the samples was determined either by bioassay on tetrodotoxin pretreated guinea-pig ileum (Beani *et al.*, 1978) or by an automatic luminometric assay method (Lundin *et al.*, 1984; Blomqvist *et al.*, 1987).

Chronic treatment

Rats and guinea-pigs were injected subcutaneously with nicotine 0.45 mg kg^{-1} twice daily for 16 days. Saline-treated animals were run in parallel. On the 15th day of treatment an epidural cup was implanted and the experiment on ACh release was carried out two days later. The same protocol as for the acute nicotine treatment experiments was followed.

Tolerance test

A T-maze consisting of a central space (24 × 24 × 28 cm) and two arms (27 × 8 × 8 cm) was used for testing of tolerance to the effect of nicotine in naïve rats and chronic nicotine-treated rats. On the morning of the 17th day each rat received a subcutaneous injection of nicotine (0.45 mg kg^{-1}) or saline. Five minutes later the rat was placed in the central space of the maze and the number of entrances (at least half of the body) into one of the arms was recorded for 5 min, according to the method described by Stollerman *et al.* (1973). The number of rears was also recorded during the same time period.

Nicotine receptor binding studies

Twelve hours after the last injection of nicotine the chronically-treated rats and guinea-pigs were killed by decapitation, their brains were taken out and placed on an ice-cold glass plate. The cerebral cortex was dissected and frozen at −70°C until binding experiments were performed. [^{3}H]-(−)-nicotine (7 nM; specific activity 80 Ci $mmol^{-1}$, Amersham, UK) was incubated with the prepared P2 fraction at 4°C for 40 min according to a method described previously (Larsson & Nordberg, 1985; Zhang *et al.*, 1987). [^{3}H]-ACh (10 nM, specific activity 81 Ci $mmol^{-1}$, Amersham, UK) binding was carried out with the P1 fractions at 4°C for 40 min in the presence of atropine 10^{-6} M (Adem *et al.*, 1987). The samples were filtered through Whatman GF/C glass filters presoaked in a 0.05% polyethylenimine solution. The filters were washed twice, dried and placed in scintillation tubes. Then 5 ml HP-B scintillation fluid was added and the radioactivity counted in a Nuclear Chicago scintillation counter. Specific binding for both ligands was calculated by subtracting the values for non-specific binding in the presence of unlabelled nicotine 10^{-3} M. All assays were performed in triplicate.

Measurement of nicotine and cotinine in plasma

Plasma nicotine and cotinine were analysed according to the method described by Curvall *et al.* (1983).

Drugs

Freshly prepared solutions of the following drugs were used: (−)-nicotine bitartrate, mecamylamine

Table 1 Effect of (−)-nicotine (0.45 mg kg^{-1}) on cortical release of acetylcholine (ACh) in naïve and chronically nicotine-treated guinea-pigs and rats

Experimental conditions	*No. of expts*	*ACh release* (ng cm^{-2} 30 min^{-1}) *Basal*	*30 min after (−)-nicotine*	*% increase*
Guinea-pigs				
Naïve	10	23.9 ± 3.1	36.0 ± 4.4*	150 ± 5.9
Nicotine-treated	10	19.0 ± 1.9	25.2 ± 2.5*	132 ± 5.7**
Rats				
Naïve	8	26.0 ± 6	40.0 ± 6*	153 ± 11
Nicotine-treated	8	44.0 ± 6**	62.0 ± 10*	140 ± 16

*Significantly different from pretreatment values, $P < 0.01$.
**Significantly different from naïve animals, $P < 0.05$.

HCl, physostigmine sulphate (Sigma), (+)-tubocurarine chloride (Wellcome).

Statistical methods

Statistical differences were checked by use of Student's *t* test for paired and unpaired data.

Results

The basal release of endogenous ACh from the cerebral cortex of naïve freely moving rats and guinea-pigs was similar (Table 1). No consistent differences in the values were detected when the assays were run in parallel with automatic luminometric assay and with bioassay on the guinea-pig ileum. The cortical ACh release remained at about the same level in control animals for some hours (see Beani & Bianchi, 1970). Therefore, the initial 2–3 samples before injecting nicotine were taken as internal standards in each experiment.

Acute nicotine treatment

Nicotine at doses as low as 0.10–0.20 mg kg^{-1} s.c. gave uncertain responses both in rats and guinea-pigs. However, when injected into rats at doses of 0.45 and 0.90 mg kg^{-1}, nicotine reduced spontaneous motor activity (Table 1) and increased cortical ACh outflow (Figure 1b). The maximal neurochemical effect was obtained in the first collection period (30 min) after injection and vanished within 120 min. Similar results on ACh release were obtained in the guinea-pig (Figure 1A) which, however, did not show any reduction in locomotor activity. In order to exclude peripheral effects, experiments were carried out in which nicotine was injected directly into the right or left ventricular space. As shown in Figure 2c and d the drug was able to increase, in a dose-dependent manner, the cortical ACh release in both animal species. Clearly the guinea-pig displayed a

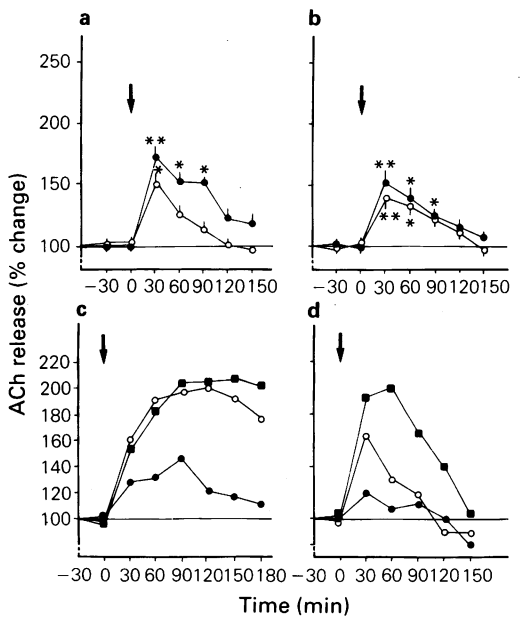


Figure 1 Effect of nicotine on the release of acetylcholine from the parietal cortex of naïve freely moving guinea-pigs (a and c) and rats (b and d). The drug was injected (arrow) at 0.45 (○) and 0.90 (●) mg kg^{-1} s.c. in (a) and (b) and at 1 (●), 3 (○) and 5 (■) μg in the lateral ventricles in (c) and (d). The values are expressed as percentages of the basal release (see Table 1) and are the mean (vertical lines show s.e.mean) of 5–10 experiments. Significantly different from the pre-injection values, $*P < 0.05$; $**P < 0.01$, Student's *t* test for paired data.

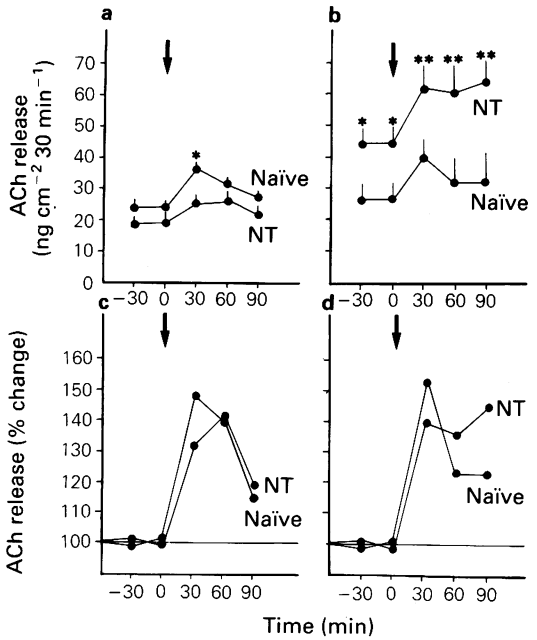


Figure 2 Effect of nicotine 0.45 mg kg^{-1} s.c. on the release of acetylcholine from the parietal cortex of sub-chronically treated (NT) guinea-pigs (a) and rats (b). The values are the mean (vertical lines show s.e.mean) of 5–10 experiments. For comparison, the response of naïve animals to the same dose is shown (see also Table 1). The data normalized with respect to the pre-injection release are presented in (c) (guinea-pigs) and (d) (rats). *$P < 0.05$; **$P < 0.01$.

greater and longer-lasting responsiveness compared with the rat. Again, the rat showed reduced locomotor activity and, after 5 μg nicotine, reduced muscle tone (mainly to the hind limbs). Mecamylamine (2 mg kg^{-1}, s.c.) injected one hour before nicotine (0.90 mg kg^{-1} s.c.) completely prevented the increase in ACh release both in guinea-pigs ($n = 4$) and in rats ($n = 4$).

Chronic nicotine treatment

The gross behaviour of chronically nicotine-treated animals and guinea-pigs (locomotor activity, responsiveness to tactile stimulation, interest for food and environment) did not differ from that of naïve animals. Also, no difference in body weight was found compared to saline-treated controls. When the rats were tested for behavioural effects of nicotine in a T-maze, partial tolerance in chronically treated rats was found (Table 2). In fact the number of entries and rears was drastically reduced by nicotine in naïve rats but was less evident in chronically treated rats.

The basal release of endogenous ACh was significantly higher in chronically treated rats in comparison with saline-treated controls (Figure 2b; Table 1), whereas a lower ACh release was found in chronically treated guinea-pigs (Figure 2b; Table 1). Nicotine (0.45 mg kg^{-1} s.c.) caused a similar, absolute increase of endogenous ACh release in naïve and chronically treated rats. In contrast, the drug evoked a lower response in chronically treated guinea-pigs. However, when the values were normalized with respect to the initial basal release a moderate, but not significant, reduction of the neurochemical response was evident (Figure 2c and d; Table 1).

Effect on nicotinic receptor binding

Unexpectedly, a consistent difference in the number of cortical high affinity nicotinic binding sites was found to exist between the two animal species, when the cerebral cortex was incubated with a certain concentration of the radioligand [^{3}H]-(−)-nicotine or [^{3}H]-ACh plus atropine. The number of binding sites for both ligands was four times higher in rat than in guinea-pig brain tissue. The reason for the higher values obtained with [^{3}H]-nicotine as compared to [^{3}H]-ACh have been reviewed and discussed previously (Wonnacott 1987; Nordberg *et al.*, 1988a). The difference between rat and guinea-pig might be due to differences in receptor affinity. After

Table 2 Effect of nicotine on the T-maze test performed on naïve and chronically treated rats

Groups	*No. of rats*	*No. of entries*	*No. of rears*
Controls	5	9.6 ± 1.2	17.2 ± 1.5
Naïve	5	2.1 ± 0.4**	0.6 ± 0.3*
Chronic treated	9	6.1 ± 0.8*	9.7 ± 2.1*

The chronically treated rats were injected with nicotine 0.45 mg kg^{-1} s.c. twice daily for 16 days. The drug was injected 5 min before the test (see Methods).
Significantly different from controls, *$P < 0.05$; **$P < 0.01$.

Table 3 Nicotinic receptors in the cerebral cortex following chronic treatment with nicotine (0.45 mg kg^{-1}, twice daily for 16 days)

Animal species	*Experimental conditions*	*Receptors* [^3H]-*ACh*	[^3H]-*nicotine*
Guinea-pig			
	Controls (6)	4.0 ± 0.45	7.1 ± 0.54
	Nicotine (7)	4.7 ± 0.52	8.3 ± 0.65
Rat			
	Controls (5)	16.9 ± 0.76	31.2 ± 1.4
	Nicotine (9)	20.5 ± 0.82*	40.0 ± 0.94*

The number of animals used is shown in parentheses.
Significantly different from control, *$P < 0.05$.

chronic nicotine treatment a significant increase in the number of nicotinic binding sites was detected only in the rats (Table 3).

Discussion

The main findings of this study are: (1) Nicotine, injected either s.c. or i.c.v. increases the cortical ACh release in a dose-dependent manner in both rats and guinea-pigs. (2) Chronic drug treatment significantly increased the basal ACh release in rats and slightly reduced it in guinea-pigs, but the response to nicotine was proportionally maintained in both species. (3) The number of nicotinic binding sites was found to be four times higher in rat brain than in guinea-pig brain (at a certain radioligand concentration). Also, only the receptors of the rat underwent apparent upregulation following chronic treatment. (4) Partial tolerance to nicotine developed, as judged from the cholinergic response and specifically from the T-maze test performed in the rat.

These four points require some comments. The increase of cortical ACh release found by Armitage *et al.* (1969) and by Erickson *et al.* (1973) was confirmed in the present study, which, in addition, demonstrated the central site of drug action. Obviously a peripheral afferent component (Hajos & Engeberg, 1988) cannot be excluded. The receptors involved in the cholinergic response are of the ganglionic type. This last finding is at variance with a previous study which showed that (+)-tubocurarine antagonised nicotine facilitation of ACh release in guinea-pig cortical slices (Beani *et al.*, 1985). Nicotine probably increases cortical ACh release in the whole animal by changing the firing rate of the subcortical cholinergic neurones (which have receptors of the ganglionic type), whereas at high concentrations, it is able to enhance transmitter release in cortical slices by acting upon receptors of the neuromuscular junction type. It has been suggested that this latter type of receptors might be present on the intracortical cholinergic nerve endings (Rowell & Winkler, 1984; Araujo *et al.*, 1987). However, the antagonism by tetrodotoxin of the effect of nicotine on unstimulated guinea-pig cortical slices (Beani *et al.*, 1985), together with some negative results on binding and biochemical studies on synaptosomes after lesioning of the subcortical cholinergic nuclei of the rat (Schwartz *et al.*, 1984; Meyer *et al.*, 1987), cast some doubts about such definite or exclusive localization. Further studies are needed to clarify if these supposed autoreceptors are also upstream (i.e. along the cholinergic axons), or in other nervous structures which in turn activate ACh release. Rats and guinea-pigs react differently to chronic nicotine treatment. When a complete washout of the plasma drug levels was achieved, the rat showed a higher basal ACh release whereas the guinea-pig displayed a lower transmitter outflow (Table 1). The reason for these opposite changes is not clear. Studies carried out in cortical slices demonstrate that the electrically-evoked ACh release is unaffected by chronic treatment (Nordberg, Romanelli, Sundwall, Bianchi & Beani, unpublished observations). Consequently, the repeated administration of nicotine seems to affect differently the average firing rate of the subcortical cholinergic neurones, but to leave the release process unchanged as such. In addition, the responsiveness of these neurones to nicotine is maintained.

After 16 days of nicotine administration the number of receptors, as measured with the two nicotinic radioligands, was apparently increased in the rat but not in the guinea-pig. This finding is in keeping with the different adaptative responses of these two animal species in terms of gross behaviour and ACh release. A shift of low affinity to high affinity binding sites due to desensitization (Changeux *et al.*, 1984) or the synthesis of new receptors might explain the increase found in the rat. Receptor competition experiments support the first hypothesis

(Romanelli *et al.*, 1988). The plasticity of the rat brain receptors is similar to that of the human brain. Recent studies carried out with positron emission tomography demonstrated an increased uptake of [^{11}C]-(−)-nicotine in the brain of smokers compared to non-smokers (Nybäck *et al.*, 1989). Similarly, an increased number of binding sites has been detected, with the standard techniques, in specimens of cerebral cortex of smokers compared to non-smokers (Benwell *et al.*, 1988). However, the reason why the guinea-pigs do not show nicotinic receptor adaptation remains to be explained. Interestingly, this species is provided with a smaller number of receptors (lower receptor affinity?) than the rat, but shows a greater responsiveness to nicotine. These points raise the question as to the actual significance of the nicotinic binding sites (and related changes) as assessed by standard techniques. Probably their total number includes physiological synaptic receptors, pharmacologically responsive extrasynaptic receptors, and, finally, extraneuronal (glial?) binding sites (Morley, 1981). Therefore, in order to bypass the limits of the binding technique, nicotine tolerance must be evaluated by measuring functional and/or biochemical responses to the drug in normal and chronically treated animals.

The experiments performed with the T-maze test have shown tolerance to the inhibitory behavioural effect of the drug in the rat. In contrast, the increase of ACh release (considered as an excitatory response) was not consistently modified by the chronic treatment, if the results were normalized with respect to the basal values. This means that moderate or no tolerance to the stimulating properties of this drug was evident (Ksir *et al.*, 1985). This fact may explain the persistent reward detected in animals and man submitted to self administration tests (Goldberg *et al.*, 1981; Henningfield *et al.*, 1983) and can justify the profile of nicotine as a drug of abuse.

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The cholinergic pharmacology of tetrahydroaminoacridine *in vivo* and *in vitro*

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1 The effect of tetrahydroaminoacridine (THA) on cholinergically mediated behaviour in the rat and mouse has been investigated. In addition the actions of this compound on cholinesterase activity and on muscarinic and nicotinic receptors has also been examined.

2 Administration of THA (5–20 mg kg^{-1}, i.p.) produced a dose-dependent increase in tremor, hypothermia and salivation in both rats and mice. A similar profile of activity was seen following physostigmine (0.1–0.6 mg kg^{-1}) administration.

3 THA was approximately fifty fold less potent than physostigmine in inducing behavioural change but its effects persisted for over twice as long as those of physostigmine. For example THA-induced hypothermia was still present at 4 h in the mouse and 8 h in the rat.

4 *In vitro* THA was a potent non-competitive inhibitor of rat brain cholinesterase (IC_{50}: 57 ± 6 nM) and bovine erythrocyte acetylcholinesterase (IC_{50}: 50 ± 10 nM) but was a more potent inhibitor of horse serum butyrylcholinesterase (IC_{50}: 7.2 ± 1.4 nM).

5 Radioligand binding studies indicated that THA binds non-selectively but with moderate potency to both M_1 (K_i: 600 nM) and M_2 (K_i: 880 nM) muscarinic receptors. THA also interacted with the allosteric site present on cardiac M_2 receptors.

6 It is concluded that THA is a reversible non-competitive inhibitor of cholinesterase with a long half life (compared with physostigmine). It also may antagonize muscarinic receptors at high doses. The long half life may account for its reported efficacy in the treatment of Alzheimer's disease.

Introduction

The central cholinergic deficits found in patients with clinically diagnosed Alzheimer type dementia (ATD) have been reported to correlate with the degree of cognitive impairment (Perry *et al.*, 1978; Francis *et al.*, 1985). Therefore many attempts have been made to reverse the cognitive deficits by increasing cholinergic activity (reviewed by Brinkman & Gershon, 1983; Kopelman, 1986). In general these attempts have met with little success. Directly acting agonists such as bethanechol or arecoline produce little or no improvement (Christie *et al.*, 1981; Penn *et al.*, 1988) although the short acting cholinesterase inhibitor physostigmine has been reported in some studies to induce small, transient improvements in cognitive function in ATD patients (Smith & Swash, 1979; Mohs *et al.*, 1985). Recently a more substantial improvement in a small group of ATD patients has been reported with another cholinesterase inhibitor, tetrahydroaminoacridine (THA) (Summers *et al.*, 1986).

The reason for the greater efficacy of THA in comparison with other cholinergic treatments is not clear. It has been reported that THA has direct actions on potassium channels in both nueronal and cardiac tissue (Osterrieder, 1987; Stevens & Cotman, 1987). THA has also been reported to have effects on monoamine uptake and release (Drukarch *et al.*, 1988; De Souza *et al.*, 1989). However, these effects occur at relatively high concentrations and little appears to be known about the direct actions of THA at muscarinic receptors or about its pharmacology, pharmacokinetics and duration of action, factors which may be important in accounting for the differences in therapeutic efficacy between THA and other esterase inhibitors such as physostigmine. In the present series of experiments we have characterized the cholinergic actions of THA *in vivo* and compared them to those produced by physostigmine,

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the behavioural actions of which are due primarily to its inhibition of cholinesterase. We have also examined the interactions of THA with cholinergic system enzymes and receptor binding sites *in vitro*. Some of this work has been reported in preliminary form to the British Pharmacological Society (Hunter *et al.*, 1988).

Methods

Animals and drug administration

Male C57BL/6J mice (20–25 g) or male Lister Hooded rats (250–300 g) supplied by Harlan Olac, Bicester, were used in these experiments. They were housed in groups of 6 under a 12 h light/12 h dark cycle with food and water available *ad libitum*. THA was dissolved in a solution containing 0.9% saline and 20% dimethylsulphoxide (DMSO). Scopolamine hydrobromide and physostigmine sulphate were dissolved in distilled water. Vehicle alone was administered as the control for each experiment and the dosage volumes were $10\,ml\,kg^{-1}$ for mice and $1\,ml\,kg^{-1}$ for rats. All weights were expressed as base.

Comparison of the acute behavioural effects of THA and physostigmine

Groups of animals (6 animals per group) were injected (i.p.) with vehicle or various doses of THA ($5–20\,mg\,kg^{-1}$) and observed 30 min later for effects on the cholinergic system. In the mouse these were tremor (scored 0–3), salivation (0–3) and rectal temperature. In the rat these were tremor (scored 0–3), salivation (weight in 10 mg units), analgesia as measured by tail flick latency and rectal temperature. Other behavioural effects in the rat such as chewing were also recorded. The effects of various doses of physostigmine ($0.1–0.6\,mg\,kg^{-1}$) were studied in the same way. To confirm that these effects were mediated via muscarinic receptors, scopolamine ($2.0\,mg\,kg^{-1}$, s.c.) was administered immediately before THA in a separate experiment and behavioural observations made at 30 min post-injection.

Comparison of the duration of action of THA and physostigmine

Two series of observations were made, one each in rats and mice. In each series, one experiment was carried out with THA ($20\,mg\,kg^{-1}$) and one with physostigmine ($0.4\,mg\,kg^{-1}$ in the mouse and $0.6\,mg\,kg^{-1}$ in the rat). In each experiment six groups of animals were injected with vehicle and six groups with drug, with 6–8 animals in each group. Tremor, salivation and rectal temperature were measured at 30 min and 1, 2, 3, 4, 8 and 24 h post-injection in the experiments with THA and at 15 min, 30 min and 1, 2, 3, and 4 h in the experiments with physostigmine.

Measurement of acetylcholinesterase activity

Rat striata were homogenized in 40 volumes of 20 mM sodium phosphate buffer pH 7.7. Acetylcholinesterase activity was assessed by the colourimetric method of Ellman *et al.* (1961) with acetylthiocholine as substrate. In some experiments bovine erythrocyte acetylcholinesterase was used as the enzyme source. Butyrylcholinesterase was assayed by a similar method with butyrylthiocholine iodide as substrate. Drugs were added immediately before assay and the reaction was initiated by the addition of enzyme.

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

Rat heart was minced, homogenized in 50 volumes of ice cold sodium phosphate (10 mM; pH 7.4) in an Ultra-Turrax rotating blade homogenizer and filtered through muslin to remove connective tissue (10,000 r.p.m.). Rat cerebral cortex was homogenized as above but it was not necessary to filter the homogenate through muslin. Homogenates were centrifuged at 15,000 g for 15 min at 4°C, and the membrane pellets frozen at −20°C before use. Pellets were resuspended in 50–80 volumes (original wet weight) of sodium phosphate (10 mM; pH 7.4) before assay.

For [^{3}H]-pirenzepine binding, incubations contained [^{3}H]-pirenzepine (1 nM), displacing agents and 160 µl of cortical membrane suspension in a total volume of 200 µl of buffer. Following incubation at 30°C for 1 h, bound ligand was separated by filtration over glass fibre filters using a Skatron cell harvester. For [^{3}H]-N-methyl scopolamine ([^{3}H]-NMS) binding 160 µl of heart membranes was incubated with [^{3}H]-NMS (1 nM) and displacing agents in a total volume of 200 µl for 90 min at 30°C. Bound ligand was separated by filtration over glass fibre filters.

For both ligands, non-specific binding was defined as that not displaced by atropine (10 µM). To study the dissociation of [^{3}H]-NMS from heart membrane binding sites, membranes were incubated with 1 nM [^{3}H]-NMS for 90 min at 30°C. Dissociation was initiated by the addition of 10 µM atropine sulphate plus drug under investigation and incubations were filtered at various times following addition of the atropine.

[^3H]-oxotremorine-M binding

Rat cortex was homogenized in 20 volumes of 20 mM Tris-HCl containing 3 mM $MgCl_2$ pH 7.4 in an Ultra-Turrax homogenizer. The homogenate was centrifuged at 15,000 *g* for 15 min, the resulting pellet was resuspended in Tris $MgCl_2$ buffer and incubated at room temperature for 15 min. The preparation was then centrifuged at 15,000 *g* for 15 min, resuspended in 40 volumes of Tris $MgCl_2$ buffer containing 0.1% dichlorodivinyl phosphate (DDVP), and used immediately.

Incubations contained 160 μl of membrane preparation, [^{3}H]-oxotremorine-M (1.5 nM) and displacing agents in a total volume of 200 μl. Following incubation at 25°C for 1 h, bound ligand was separated by filtration over glass fibre filters which had been presoaked in 0.05% polyethyleneimine. Non-specific binding was defined as that not displaced by atropine (10 μM).

[^3H]-nicotine binding

Rat brain minus cerebellum was homogenized in 50 μM Tris-HCl pH 7.4 in an Ultra-Turrax homogenizer. The homogenate was centrifuged at 15,000 *g* for 15 min at 4°C and the pellet washed once by rehomogenization and centrifugation. The final membrane pellet was resuspended in 50 mM Tris-HCl pH 7.4 containing 100 mM KCl_2, 40 mM $CaCl_2$ and 20 mM $MgCl_2$. Incubations contained 160 μl membrane preparation, 4 nM [^{3}H]-nicotine and displacing agents in a total volume of 200 μl. Following incubation at room temperature for 1 h, bound ligand was separated by filtration over glass fibre filters presoaked in polyethyleneimine (0.1%). Non-specific binding was defined as that not displaced by nicotine (10 μM).

Data analysis

Behavioural data were analysed by analysis of variance where applicable, followed by protected *t* tests. For non parametric data analysis was by Kruskal Wallis tests or chi squared tests. Inhibition of ligand binding was calculated from Hill plots and linear regression analysis.

Drugs and chemicals

[^{3}H]-pirenzepine (80–90 Ci mmol^{-1}), [^{3}H]-N-methylscopolamine (80–90 Ci mmol^{-1}), [^{3}H]-(−)-nicotine (80 Ci mmol^{-1}) and [^{3}H]-oxotremorine-M (80–90 Ci mmol^{-1}) were obtained from New England Nuclear, U.K. Dichlorodivinylphosphate (DDVP) was purchased from Greyhound Chemicals, U.K. Acetylcholinesterase (bovine erthrocyte, type XII-S), butyrylcholinesterase (horse serum, type XI), physostigmine and scopolamine hydrobromide were all obtained from Sigma, U.K. THA.HCl was purchased from Aldrich Chemical Company.

Results

Acute behavioural effects

THA and physostigmine both produced a dose-dependent increase in all the above measures of cholinergic function in both rat (Figure 1) and mouse (data not shown). In the rat and mouse doses of THA greater than 20 mg kg^{-1} were fatal. In the rat physostigmine and THA both produced a dose-dependent increase in tremor and salivation and a decrease in temperature (Figure 1). These compounds also produced analgesia, decreased rearing in an open field and increased frequency of chewing behaviour (Figure 1).

In the mouse THA produced a much larger decrease in temperature than physostigmine and maximal doses also produced higher levels of tremor and salivation (data not shown). In both species THA was approximately 50 fold less potent than physostigmine in producing cholinergic effects, which were blocked by scopolamine (2 mg kg^{-1}) given immediately before THA (10 mg kg^{-1}).

Duration of action of THA and physostigmine

In the rat the effect of physostigmine on tremor and salivation was maximal 30 min post-injection and had disappeared by 2 h and 3 h respectively (Figure 2), although at this time there was still a significant decrease in temperature, compared to controls. Although THA produced a maximal effect on tremor and salivation at 30 min post-injection, some salivation and tremor were still present at 4 h and 8 h respectively (Figure 2). The temperature decrease produced by THA was maximal at 4 h and there was still a significant decrease compared to controls at 8h (Figure 2).

In the mouse a similar pattern was observed except that the effects of physostigmine disappeared by 2 h, whereas THA-treated animals still showed a significant drop in temperature and some tremor. The duration of action of THA in the mouse was approximately twice as long as physostigmine (data not shown) but the effect of both drugs was considerably shorter in the mouse than in the rat.

Inhibition of acetylcholinesterase by THA

THA was found to be a potent inhibitor of both rat brain cholinesterase ($IC_{50} = 57 \pm 6$ nM) and bovine

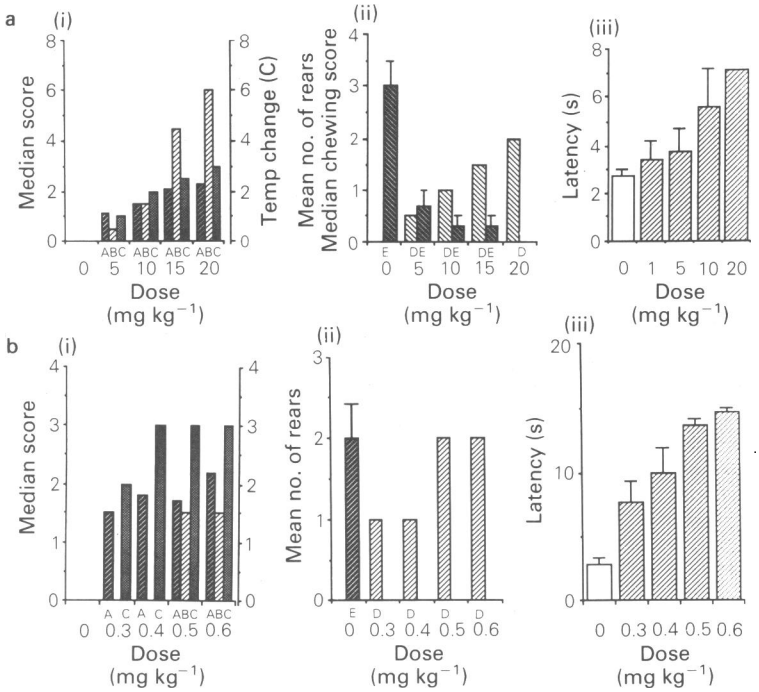


Figure 1 The effects of (a) tetrahydroaminoacridine (THA) and (b) physostigmine in the rat on (i) temperature, salivation and tremor; (ii) chewing and rearing; (iii) latency to tail flick. In (ai) and (bi), A = temperature change, B = salivation, C = tremor; in (aii) and (bii) D = chewing, E = rearing. Data for salivation, tremor and chewing are expressed as the median, all other data are shown as the mean with s.e.mean shown by vertical bars.

erythrocyte acetylcholinesterase ($IC_{50} = 50 \pm 10$ nM) with very similar potency. THA was eight fold more potent as an inhibitor of horse serum butyrylcholinesterase ($IC_{50} = 7.2 \pm 1.4$ nM). The kinetic analysis of the interaction of THA with rat brain acetylcholinesterase indicated that inhibition is apparently non-competitive (Figure 3) with a calculated K_i value of 80 nM. The inhibition of acetylcholinesterase by THA was unaltered after a 30 min preincubation period. In addition, incubation of tissue homogenate with 10^{-7} M THA (sufficient to inhibit > 50% activity) followed by a 10 fold dilution immediately before assay did not result in significant inhibition of cholinesterase.

Interaction of THA with muscarinic receptors

[^{3}H]-pirenzepine was used to label muscarinic M_1 receptors in cerebral cortex, and [^{3}H]-NMS to label M_2 receptors in heart. THA had significant activity

Table 1 Interaction of tetrahydroaminoacridine (THA) with muscarinic and nicotinic cholinoceptor binding

Receptor ligand	Nicotinic ([^{3}H]-nicotine)	M_1 ([^{3}H]-pirenzepine)	M_2 ([^{3}H]-NMS)	*Agonist binding* ([^{3}H]-oxo-M)
Tissue	*Whole brain*	*Cerebral cortex*	*Heart*	*Cerebral cortex*
THA	3400	600 ± 86	880 ± 180	770
Atropine	–	0.77 ± 0.15	1.01 ± 0.15	1.72
Carbachol	–	14500	–	13.1
Pilocarpine	–	4900	–	73.4
Nicotine	3.5	–	–	–

Values are apparent $K_i \times 10^{-9}$ M, expressed as the mean ± s.e.mean of at least 3 determinations, or the mean of 2 determinations. Apparent K_i values were calculated using the equation $K_i = IC_{50}/(1 + ([L]/(K_d))$ where [L] = ligand concentration and K_d = dissociation constant of the ligand.
[^{3}H]-NMS = [^{3}H]-N-methylscopolamine; [^{3}H]-oxo-M = [^{3}H]-oxotremorine-M

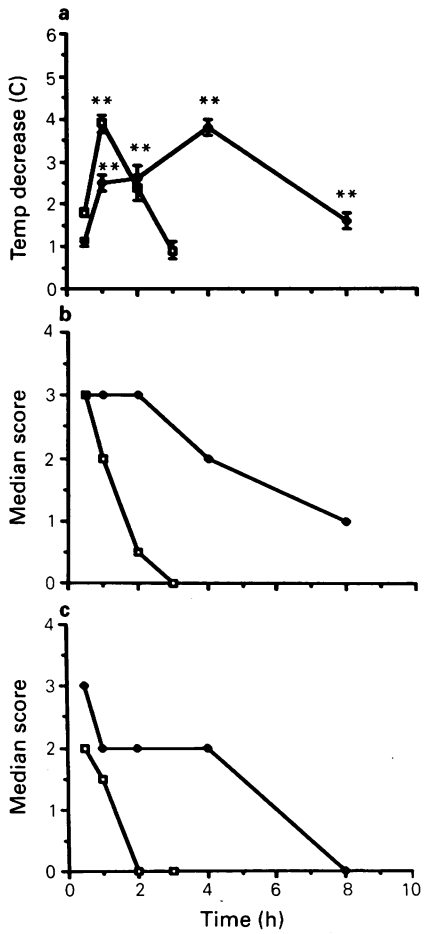


Figure 2 The duration of action of tetrahydroaminoacridine (THA 20 mg kg^{-1}, closed symbols), physostigmine (0.6 mg kg^{-1}, open symbols) in the rat: (a) shows the effects on temperature. By 24 h THA-treated animals were not significantly different from controls. Data are the mean with s.e.mean shown by vertical bars. In (b) and (c) are shown the duration of action on tremor and salivation respectively. Data are expressed as medians.
* $P < 0.01$, ** $P < 0.001$ compared to controls.

at both binding sites. It can be seen from Table 1 that THA did not distinguish between M_1 and M_2 receptors. THA was a weak inhibitor of [^{3}H]-oxotremorine-M binding, the ratio of K_i values in [^{3}H]-pirenzepine and [^{3}H]-oxotremorine-M binding was similar to that of atropine (Table 1) and considerably less than that of the agonist carbachol or the partial agonist pilocarpine (Table 1).

Kinetic experiments were performed *in vitro* on the dissociation of [^{3}H]-NMS from rat heart binding sites. In the presence of either gallamine

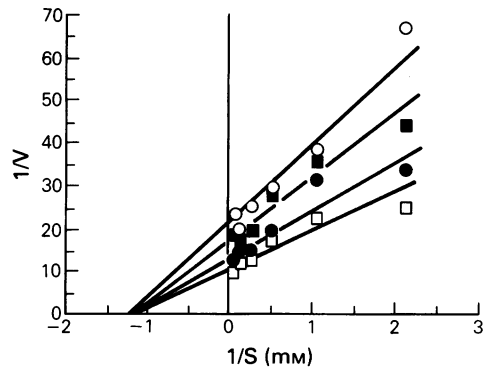


Figure 3 Kinetic analysis of the inhibition of brain acetylcholinesterase by tetrahydroaminoacridine (THA). The graph shows a double reciprocal plot of acetylcholinesterase activity in the presence of increasing concentrations of THA. Data are from a single experiment which was replicated three times: (□) control; (●) 2×10^{-8} M; (■) 5×10^{-8} M; (○) 10×10^{-8} M.

(3×10^{-4} M) or THA (3×10^{-4} M), the dissociation rate of [^{3}H]-NMS was markedly reduced (Figure 4a). Concentration-response curves for this effect (Figure 4b) suggested that THA was equipotent with gallamine, although neither compound produced a maximum effect.

Three hours following administration to rats of either gallamine (20 mg kg^{-1}) or THA (20 mg kg^{-1}) both drugs had produced a significant reduction in [^{3}H]-NMS binding to heart preparations (Figure 5a). In addition, the dissociation of [^{3}H]-NMS from heart preparations from gallamine- and THA-treated rats was significantly lower than tissue prepared from control rats (Figure 5b).

Discussion

In vivo THA had a similar profile of action in the rat and mouse with the effects on the cholinergic system being comparable with those produced by physostigmine in both species. These results are in agreement with previous studies in rodents which have indicated that physostigmine and THA produced similar improvements in memory in poorly trained or lesioned mice (Flood *et al.*, 1986; Davis *et al.*, 1987).

The main difference between the two compounds was in the duration of action and potency. Physostigmine was some 50 fold more potent than THA, but its duration of action was much shorter. The short duration of action of physostigmine behaviourally corresponds well with the time course of its inhibition of brain cholinesterase which is maximal between 5 and 20 min after injection (Hallak & Giacobini, 1986; 1987). Unfortunately due to the rapid

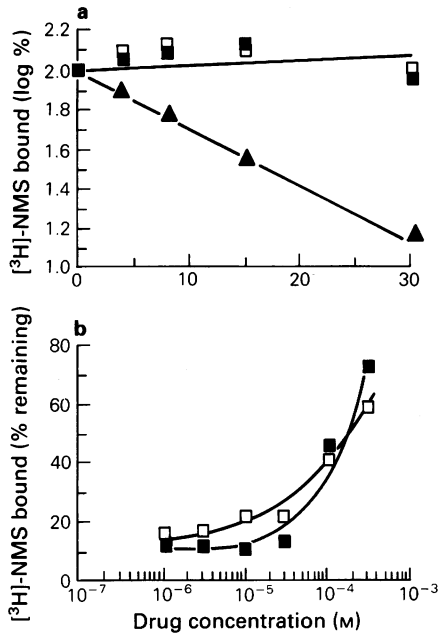


Figure 4 (a) Dissociation of bound [^{3}H]-N-methylscopolamine ([^{3}H]-NMS) from rat heart membranes. The graph shows a semilogarithmic plot of the dissociation of [^{3}H]-NMS under control conditions (▲) and in the presence of either gallamine (■, 3×10^{-4} M) or tetrahydroaminoacridine (THA) (□, 3×10^{-4} M). (b) Effects of increasing concentrations of gallamine (□) or THA (◆) on the dissociation of bound [^{3}H]-NMS from rat heart membranes. The dissociation of [^{3}H]-NMS is given as the percentage of bound [^{3}H]-NMS remaining 30 min after dissociation was initiated by the addition of 10 μM atropine.

reversibility of the inhibition of acetylcholinesterase by THA (see below), the degree of brain cholinesterase inhibition could not be measured directly in our study. Nevertheless it seems reasonable to assume that the duration of behavioural change reflects the duration of enzyme inhibition.

The biochemical actions of the two compounds also appear different. Although both THA and physostigmine are potent inhibitors of both central and peripheral cholinesterase they appear to bind to different sites on the enzyme. Kinetic analysis demonstrated that THA was a non-competitive inhibitor of rat brain cholinesterase. It is clear that THA is not an irreversible inhibitor of acetylcholinesterase, as the inhibition was not time-dependent and was rapidly reversible by dilution. The results indicate that THA does not bind to the catalytic ('esteratic') site and it has been suggested by others (Steinberg *et al.*, 1975) that THA might interact with a hydrophobic binding site adjacent to the catalytic site and exert its inhibitory effects either via an allosteric mechanism or via some steric effect. The present data would support an allosteric interaction as no change in the affinity of cholinesterase for acetylcholine was observed in the presence of THA. Physostigmine on the other hand, interacts with the catalytic site to produce a covalently modified enzyme (Aldridge, 1971).

In addition, THA, unlike physostigmine, bound with moderate potency to muscarinic receptors. THA does not distinguish between M_1 and M_2 receptor subtypes, since the calculated K_i values of THA for cortical M_1 and cardiac M_2 receptors were 600 nM and 880 nM respectively. Although these affinities are considerably less than those of atropine, they may still be relevant to the actions of THA at high doses. It has been estimated that about 80% inhibition of brain cholinesterase is required to induce a significant increase in central cholinergic function (Holmstedt *et al.*, 1967). At this level of cholinesterase inhibition, 30% of central M_1 receptors would be occupied by THA. It is possible therefore

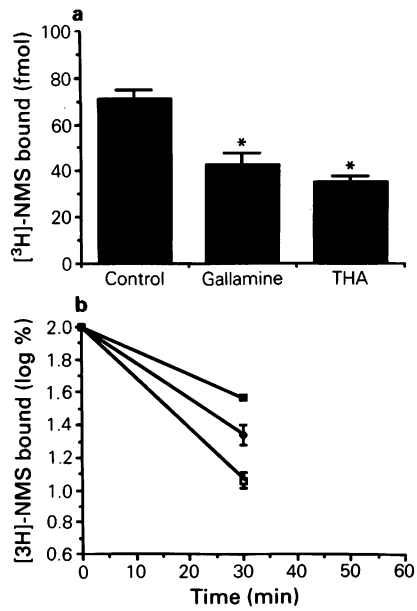


Figure 5 The interaction of tetrahydroaminoacridine (THA) and gallamine with heart [^{3}H]-N-methylsiopolamine ([^{3}H]-NMS) binding *ex vivo*. Animals were treated as described in the methods: (a) shows the amount of [^{3}H]-NMS binding in heart homogenates containing 3.2 mg of tissue, $P^* < 0.05$ compared to control; (b) shows the dissociation of [^{3}H]-NMS from heart membranes prepared from THA (■) or gallamine (◆)-treated animals; (□) control. Data are mean with s.e.mean shown by vertical bars; $n = 6$ per group.

that at high doses of THA some antagonism of its cholinergic effects may occur. However, at the maximum dose used in the present study this was not apparent.

The ligand binding data provide no information on the agonist/antagonist action of THA. However the ratio of THA potencies in [^{3}H]-pirenzepine binding versus [^{3}H]-oxotremorine-M binding was similar to that of the antagonist atropine, suggesting that THA may well be an antagonist (see Freedman *et al.*, 1988).

THA also interacted with the allosteric site present on rat heart M_2 receptors. The physiological significance of this interaction is unclear, although it has been suggested that the cardiac effects of the nicotinic agent, gallamine may be mediated by an interaction with this site (Stockton *et al.*, 1983). In the present study THA appeared to be at least as active as gallamine *in vivo* suggesting that cardiac muscarinic M_2 receptor antagonism could occur following administration of high doses of this drug.

In conclusion the present studies show that THA is a potent, reversible, non-competitive inhibitor of acetylcholinesterase *in vitro*, albeit some 50 fold weaker at producing effects on the cholinergic system than physostigmine *in vivo*. THA interacts with muscarinic receptors at high concentrations. It also has a much longer duration of action *in vivo* than physostigmine and this factor may contribute to the alleged enhanced effectiveness of THA over physostigmine in the treatment of dementia (Summers *et al.*, 1986).

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Mechanisms underlying the antiarrhythmic properties of β-adrenoceptor blockade against ischaemia-induced arrhythmias in acutely prepared rats

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1 The mechanism underlying the limited antiarrhythmic effects of β-adrenoceptor blocking agents against occlusion-induced arrhythmias in acutely prepared, pentobarbitone-anaesthetized rats has been investigated.

2 Atenolol, ICI 111,581 and propranolol were given at low, medium and high doses calculated to shift dose-response curves to exogenous agonists by factors of 10–30, 100–300 and 1000–3000, respectively.

3 Arrhythmias, blood pressure, heart rate, ECG changes and serum K^+ were measured.

4 Antiarrhythmic activity was seen with β-blocker treatment. This was minimal with atenolol (0.1, 1 and 10 mg kg^{-1}) and only statistically significant with the highest dose of ICI 111,581 (5 mg kg^{-1}), and propranolol (10 mg kg^{-1}).

5 Treatment with β-adrenoceptor blockers elevated serum potassium concentrations, as compared with saline controls, especially when measured at 30 min post-occlusion.

6 Only ICI 111,581 (5 mg kg^{-1}) and propranolol (1 and 10 mg kg^{-1}) prolonged P–R interval.

7 In order to evaluate possible mechanisms of antiarrhythmic action, attempts were made to correlate antiarrhythmic activity with β-blockade, serum potassium concentrations, and/or with changes in the P–R interval of the ECG.

8 Reductions in arrhythmias did not correlate well with presumed β-blockade. Better correlation was obtained with elevations of serum potassium concentration, and with prolongation of P–R interval (a presumed Class I antiarrhythmic action).

9 These results suggested that antiarrhythmic effects of adrenoceptor blocking agents in acutely-prepared anaesthetized rats, subjected to occlusion of a coronary artery, are unrelated to cardiac β-blockade. The limited antiarrhythmic effects which were observed could be attributed to elevations in serum potassium concentration (due to peripheral β-blockade) and/or possible Class I antiarrhythmic actions.

Introduction

The role the sympathetic nervous system plays in determining the duration and severity of occlusion-induced arrhythmias in the rat has been investigated in several studies. In our laboratory the problem has been examined by use of various approaches: CNS ablation (Curtis *et al.*, 1985b), tetrodotoxin-induced autonomic nervous system blockade (Abraham *et al.*, 1988) as well as β-adrenoceptor blockade in acutely (Au *et al.*, 1983) or chronically prepared animals, both conscious (Botting *et al.*, 1983) and anaesthetized (Au *et al.*, 1983). Findings in these studies for the most part refuted the hypothesis that the sympathetic nervous system plays anything more than a minor arrhythmogenic role during myocardial ischaemia in the rat. Beta-blockade with propranolol at doses sufficient to produce full β-blockade had only a minor effect on the severity and duration of arrhythmias (Au *et al.*, 1983; Botting *et al.*, 1983). However, other studies in conscious (Siegmund *et al.*, 1979; Szekeres, 1979), and acutely-prepared anaesthetized rats (Campbell & Parrat, 1983) have demonstrated antiarrhythmic activity with β-receptor antagonists such as propranolol.

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The strongest evidence for the antiarrhythmic activity of β-receptor blocking agents originates in acutely-prepared anaesthetized animals. We have previously suggested (Curtis *et al.*, 1985a; Curtis *et al.*, 1986) that antiarrhythmic effects of β-blockers in such preparations are secondary to a β-blockade-dependent elevation of serum potassium levels. Elevated levels of serum K^+ have been documented in acute surgery, and elevation of serum K^+ has been shown to be antiarrhythmic, both clinically and experimentally (Nordrehaug & von der Lippe, 1983; Solomon, 1984; Curtis *et al.*, 1986). Catecholamines depress serum K^+ via a β_2-adrenoceptor mediated action (Brown *et al.*, 1983). This would suggest that, if β-antagonists are antiarrhythmic in acutely prepared anaesthetized rats, it may be because of their ability to antagonize the catecholamine-mediated supression of surgically-induced elevations in serum K^+. In addition, pentobarbitone anaesthesia probably elevates sympathetic tone and may also impair the ability of the body to regulate serum potassium. If the above is correct, it would explain the apparent contradiction between results obtained with β-blockers in acutely-prepared anaesthetized rats and those obtained in conscious animals. In the present study we assessed the anti-arrhythmic properties of atenolol (a 'selective' β_1-adrenoceptor blocker), ICI 111,581 (β_2) and propranolol (non-selective) in acutely-prepared pentobarbitone-anaesthetized rats. Antagonists were administered at low, medium and high doses designed to ensure adequate β-blockade.

Methods

Male Sprague-Dawley rats (250–400 g) were used throughout. All experiments were performed in pentobarbitone anaesthetized (60 mg kg^{-1}, i.p.) and artificially ventilated rats (Palmer Small Animal Ventilator) at a stroke volume and rate which keeps blood gases in the normal range.

Groups studied, doses and route of administration

The occlusion study was performed in 12 groups of animals in 3 stages with 3 saline-treated groups. Each group contained 9 rats. The three blockers were each administered at three dose levels expected to shift dose-response curves for dobutamine, salbutamol or isoprenaline, as appropriate to the antagonist, by factors of 10–30, 100–300 and 1000–3000. A total of 1 ml of the appropriate concentration of drug was administered to the animal over a 5 min interval and then the rate of infusion was slowed to 0.5 ml h^{-1} so as to maintain a constant level of blockade.

Preliminary experiments were conducted to ascertain the appropriate dose (bolus and infusion) regimens required to achieve the above blockade. These preliminary studies were performed in rats anaesthetised with pentobarbitone (60 mg kg^{-1}, i.p.) and with a cannulated femoral vein and artery. Heart rate and blood pressure responses were obtained for salbutamol, isoprenaline and dobutamine before and after administration of the β-blocker under test. After these initial dose-range studies the minimal doses chosen were 0.1 mg kg^{-1} propranolol, 0.1 mg kg^{-1} atenolol and 0.05 mg kg^{-1} ICI 111,581. The shifts in partial dose-response (blood pressure and heart rate) curves to the above three agonists were used to estimate dose-ratios associated with the low doses. After this measurement, the dose of antagonist under study was increased by a factor of 10. Dose-ratios for falls in blood pressure to salbutamol and isoprenaline were 20 for the low dose of ICI 111,581 and propranolol. Ratios of approximately 100–200 were found with 1 mg kg^{-1} propranolol and 0.5 mg kg^{-1} ICI 111,581. When measured, ratios increased approximately a further 10 times with the highest doses of these two antagonists. Similar ratios were found for heart rate responses with propranolol, whereas no shift in heart rate occurred with ICI 111,581 until 5 mg kg^{-1} was given.

With isoprenaline and dobutamine as agonists, atenolol (0.1 mg kg^{-1}) gave a dose-ratio of 30 for heart rate while the ratio for blood pressure effects was less than 10. At 1 mg kg^{-1}, atenolol gave a dose-ratio of greater than 200 for heart rate and less than 30 for blood pressure. These data were considered in keeping with the limited selectivity of atenolol for the β_1-adrenoceptor and the greater selectivity of ICI 111,581 as a β_2-adrenoceptor blocker.

Experiments were conducted according to the Lambeth Conventions (Walker *et al.*, 1988). Animals were allocated to each of the 12 groups in a double-blind and randomized manner. Rats were excluded from the study if they met exclusion criteria, which included a fall in mean blood pressure below 60 mmHg before drug administration, and/or an occluded zone (ischaemic ventricular mass) below 25%, or above 45%, of the total ventricular mass; eleven rats were so excluded.

Coronary occlusion

Antiarrhythmic actions against occlusion-induced arrhythmias were studied in pentobarbitone-anaesthetized and acutely-prepared rats implanted with a left anterior descending coronary artery (LAD) occluder, ECG electrodes (approximate lead V_3) and arterial and venous cannulae. The methods for preparation of rats, as well as the technique of occlusion, were according to those described by

Clarke *et al.* (1980). Animals were allowed 30 min to stabilize after completion of surgery and then drug administration was initiated. Animals were occluded by tightening the implanted ligature at time 0, i.e., 10 min after beginning drug administration. The variables measured were blood pressure (BP), heart rate (HR) and ECG changes (V_3 chest lead, Johnston *et al.*, 1983). Body temperature was measured by a rectal probe (YSI model 73A) and maintained at 37.0 ± 0.5°C by means of a heating lamp. Arrhythmia scores (AS) were calculated according to the occurrence of ventricular premature beats (VPB) and the number and duration of ventricular tachycardia (VT) and ventricular fibrillation (VF) episodes diagnosed according to the Lambeth Conventions (Walker *et al.*, 1988). At the end of experiment, i.e., 30 min after LAD occlusion, rats were killed and the occluded zone (OZ = zone-at-risk) measured (Johnston *et al.*, 1981; 1983). This involved perfusing the heart by the Langendorff method with a Krebs-Henseleit solution containing cardiac green so that the under-perfused tissue was clearly demarcated.

Three blood samples were taken (−15 min = control; −1 min = drug; +30 min = post-occlusion) from the carotid artery cannula for measurement of serum K^+ concentration by potassium ion selective electrodes (Ionetics Potassium Analyzer, Ionetics, CA, U.S.A.). In view of the perturbing effects of concomitant tachyarrhythmias and difficulty of obtaining blood samples while monitoring for arrhythmias on ECG and blood pressure traces, the +30 min post-occlusion value was taken as being indicative of values during the period of peak arrhythmias (8–12 min post-occlusion). The validity of this assumption was tested in trial experiments when blood was taken during the peak period of arrhythmias in control and propranolol-treated animals and compared with values obtained at +30 min. For controls ($n = 7$) the K^+ values at the peak of arrhythmias were 97 ± 5% of those at +30 min, while for propranolol the mean was 95%. During these experiments it was consistently noted that serum potassium values were elevated after the termination of a tachyarrhythmia in which blood pressure fell for more than a few seconds. Electrically-induced tachyarrhythmias similarly elevated serum potassium. Thus we decided to use the +30 min values as being indicative of those expected at the peak period for arrhythmias.

Drugs

Atenolol and (±)-propranolol HCl were purchased from Sigma Chemical Company St. Louis, Mo U.S.A.; ICI 111,581 (erythro-(±)-1-(7-methylindan-4-yloxy)-3-isopropylaminobutan-2-ol-HCl) was a gift from the ICI Company, Macclesfield, UK.

Data analysis

Statistical analysis was performed by ANOVA and statistical significance determined by Duncan's multiple range test for means using UBC computer packages (Gregg & Osterlin, 1977).

Results

All groups had the same mean occluded zone size (range 32.3 ± 1.9 to 38.1 ± 1.4 $\bar{x}$ ± s.e.mean) and therefore were presumably subjected to the same basic arrhythmic insult although the arrhythmogenicity of this insult may be modified by drugs or concomitant pathology.

Prior to the administration of saline, or a β-adrenoceptor blocker (−15 min values), all groups had similar mean heart rates with values ranging from 363 ± 11 ($\bar{x}$ ± s.e.mean) to 406 ± 9 beats min^{-1}. Saline treatment did not lower heart rate, whereas β-blocker treatment did, especially at the higher doses. The falls in heart rate with treatment were statistically significant in 5 out of 9 of the treatment groups just before occlusion (−1 min values) when comparisons were made between pre-drug and post-drug periods, but all doses produced falls in heart rate. Changes reached significance for 5.0 mg kg^{-1} ICI 111,581 (382 ± 9 to 362 ± 11 beats min^{-1}), but much larger statistically significant falls were seen with 0.1 mg kg^{-1} and 10 mg kg^{-1} atenolol and 1 and 10 mg kg^{-1} propranolol. The appropriate pre- to post-drug falls in heart rate with atenolol were 410 ± 18 to 330 ± 9 for 0.1 mg kg^{-1}, and 383 ± 11 to 293 ± 5 for 10 mg kg^{-1}. The corresponding figures for 1 and 10 mg kg^{-1} propranolol were 400 ± 15 to 365 ± 9, and 378 ± 14 to 280 ± 18, respectively.

All treatments tended to lower blood pressure when compared with their own control, unlike the case with saline where no significant falls in blood pressure occurred in saline-treated animals between the −15 min (pre-drug) and −1 min (post-drug) periods. Atenolol statistically significantly lowered blood pressure only at the 10 mg kg^{-1} dose (from 104 ± 5 to 69 ± 6 mmHg). With ICI 111,581 no statistically significant falls occurred while significant falls occurred with propranolol at 0.1 and 10 mg kg^{-1}. The above analyses involved pre-drug vs. post-drug comparisons. When post-drug values for drug-treated groups were compared with the same values for saline-treated controls, statistical significance was obtained with 10 mg kg^{-1} atenolol, 5 mg kg^{-1} ICI 111,581 and 0.1 and 10 mg kg^{-1} propranolol.

Treatment effects on serum potassium concentrations are shown in Figure 1. To illustrate fully the extent of such effects, values are expressed as changes

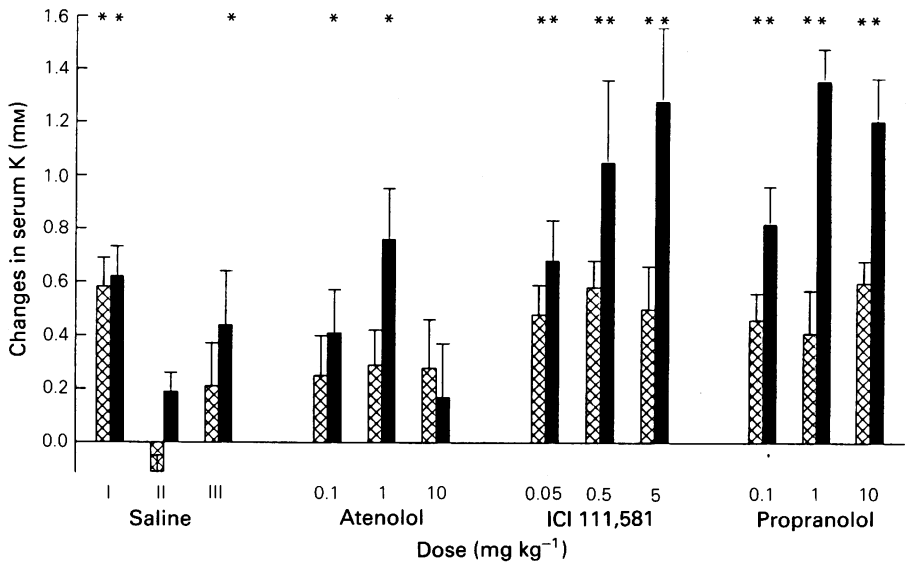


Figure 1 Effects of atenolol, ICI 111,581 and propranolol treatment on elevations in serum potassium concentrations in pentobarbitone anaesthetized rats. Drugs were administered as indicated in Methods and occlusion performed 10 min after beginning treatment. The values for the saline groups represents the control three groups. Results are presented as a change ($\bar{x}$ with s.e.mean shown by vertical bars of 9 rats per group) in serum potassium concentration from the initial pre-drug value, either 1 min before occlusion (hatched columns), or 30 min after occlusion (solid columns). $^*P < 0.05$ for significance of changes.

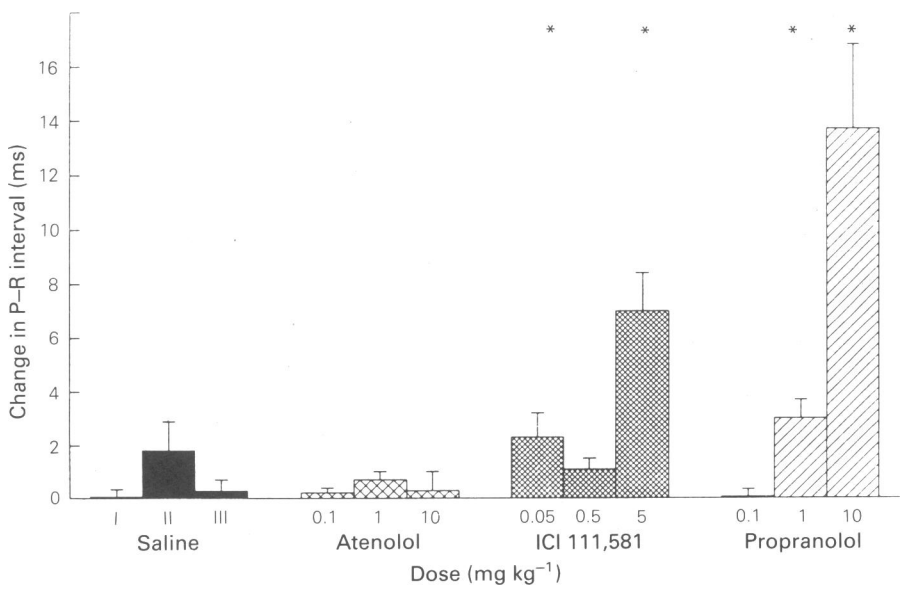


Figure 2 Effects of atenolol, ICI 111,581 and propranolol on P–R intervals in pentobarbitone anaesthetized rats. Drugs were administered as indicated in Methods. Each column is the mean with vertical bars showing s.e.mean; ($n = 9$) change in P–R interval 1 min before occlusion. $^*P < 0.05$ for difference.

from control. The control was the −15 min value which was obtained after surgery was complete, but just before starting treatment. Such control values varied between 3.4 ± 0.2 ($\bar{x} \pm$ s.e.mean) and 4.0 ± 0.2 mM. In all cases, with the exception of saline group II, there was an elevation at both −1 and +30 min and these elevations were generally statistically significant as a difference from their own control value. However, for saline groups I and III there were no statistically significant differences between the −1 and +30 values. On the other hand, in the β-blocker-treated groups, the elevation at +30 min was always statistically significant. In terms of the efficacy of the different β-blockers in raising serum potassium, both propranolol and ICI 111,581 may have been more efficacious than atenolol, especially prior to occlusion.

In terms of the ECG effects of β-blockade, the pattern of changes in acutely prepared animals were difficult to measure and analyse, compared with chronically prepared animals. As a result we only considered P–R intervals. Somewhat surprisingly, β-blockade (−15 min value versus −1 min value) did not cause statistically significant prolongation of the P–R interval, except in the case of the highest dose of ICI 111,581 and the two highest doses of propranolol (Figure 2). In the case of the latter drug, the prolongation appeared dose-related.

The effects of treatment on arrhythmias are summarized in Table 1. Statistically significant antiarrhythmic effects were only seen with the highest doses of ICI 111,581, and propranolol. With propranolol an increasing antiarrhythmic effect occurred with increasing doses. The above analysis was for VT and VF. The only statistically significant reduction in VPB occurred with the high dose of ICI 111,581. Since the arrhythmic history of an animal is adequately summarized by an arrhythmia score (Curtis & Walker, 1988) this is included in Table 1 as a summary.

In order to search for relationships between the various measured variables and arrhythmias, correlation analyses were performed. Figure 3 (a and b) shows two findings of significance. In Figure 3a the relationship between arrhythmia scores (as a percentage change from control values) and the +30 min serum potassium concentration (as $\log_e$ of elevation from control values) is shown. The linear correlation between the two variables was statistically significant as was the linear correlation between prolongation of P–R interval and the percentage reduction in arrhythmia score (Figure 3b).

Discussion

The three drugs in this study were chosen on the basis of their respective β-blocking profiles; atenolol for its partial β_1-adrenoceptor selectivity, ICI 111,581 for its β_2 selectivity, and propranolol for its lack of selectivity, as well as Class I antiarrhythmic activity. Atenolol has no reported Class I activity while if ICI 111,581 has, it has not been reported. The doses chosen were designed to span a full range

Table 1 Effects of atenolol, ICI 111,581 and propranolol on the occurrence of occlusion-induced arrhythmias in rats

Group	Dose ($mg\,kg^{-1}$)	Incidence (Number in groups having) VT	VF	VT and/or VF	log_{10} VPB	Arrhythmia score
Saline I		8	4	9	1.7 ± 0.2	4.1 ± 0.5
II		8	3	8	2.1 ± 0.1	3.4 ± 0.4
III		9	4	9	1.8 ± 0.2	3.7 ± 0.5
Atenolol	0.1	8	4	8	2.0 ± 0.1	3.6 ± 0.5
	1	8	4	8	1.8 ± 0.1	3.2 ± 0.5
	10	8	5	8	1.4 ± 0.3	3.3 ± 0.6
ICI 111,581	0.05	8	5	8	2.0 ± 0.2	3.7 ± 0.5
	0.5	9	4	9	1.8 ± 0.1	4.1 ± 0.4
	5	4	2	4	0.9 ± 0.3*	1.6 ± 0.7*
Propranolol	0.1	8	2	8	1.7 ± 0.1	3.3 ± 0.5
	1	6	1	6	1.5 ± 0.3	2.9 ± 0.6
	10	3*	2	3*	1.7 ± 0.2	1.1 ± 0.4*

Drugs were administered as a bolus followed by an i.v. infusion as described in Methods. Occlusion was performed 10 min after the beginning of treatment. Values are expressed as incidence (number of rats per group of 9 having VT, VF, VT and/or VF) or $\bar{x} \pm$ s.e.mean ($\log_{10}$ VPB) of 9 rats per group. Arrhythmia score (AS) is a normally distributed, linearly additive score which summarises an animal's arrhythmic history. *$P < 0.05$ vs. saline.

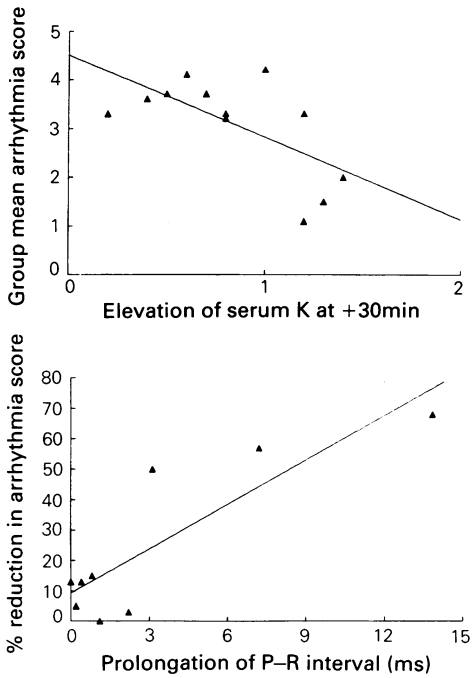


Figure 3 Correlation analyses between arrhythmia score as a percentage of control and elevated serum K^+ levels ($\log_e$ mM) (a); as well as the percentage reduction in arrhythmia score from control and P–R interval (b). In (a) the antiarrhythmic effect is shown as a decrease in arrhythmia scores with increasing elevations in serum potassium values (as $\log_e$ mM) compared with control values. The correlation coefficient r was 0.84 ($P < 0.001$). In (b) the decrease in arrhythmia scores from control values is plotted against the change from pre-drug values in P–R interval 1 min before occlusion. The correlation coefficient was 0.86.

of β-blockade. At the lowest doses, dose-ratios of 10–30 could be expected to reduce severely any normal activity of endogenous noradrenaline, or adrenaline, on β_1-, β_2 and/or β_1-plus β_2- adrenoceptors. The highest doses could be expected to block completely (99.9% blockade) even the highest level of endogenous noradrenaline likely to be found in ischaemic tissue.

With regard to their pharmacological actions, all three drugs lowered heart rate. This was so even with the selective β_2-antagonist. At the highest doses it can be presumed that the selectivity of both selective antagonists was minimal, especially with atenolol which has a limited selectivity (Tabrizchi, 1988). This is reflected in the fact that the highest dose of all three drugs produced similar elevations of serum potassium +30 min after occlusion. This potassium elevating action was presumably due to blockade of β_2-adrenoceptors the activation of which lowers serum potassium (Brown *et al.*, 1983). In this study it would not have been helpful to measure serum potassium concentrations at the peak time for the appearance of arrhythmias because of technical difficulties and confounding effects of recent arrhythmias. The decision was taken not to withdraw blood at this period for the major study. It appeared from the preliminary studies that +30 min values probably accurately reflect the levels at the time of peak arrhythmias assuming that no arrhythmias occurred to elevate values artificially. While making serum potassium determinations in many studies we have consistently found that short periods (s), even of relative ischaemia, elevate serum potassium. Thus, it is advisable to avoid sampling for serum potassium levels when blood pressure is liable to fall precipitously for varying periods as a result of the occurrence of arrhythmias.

In view of the possibility of Class I actions (blockade of sodium conductance) with high doses of propranolol, we attempted to measure Class I actions by monitoring the P–R interval of the ECG. This was done, despite the realization that β_1-adrenoceptor blockade could also be expected to prolong P–R. However, we have consistently found (Curtis *et al.*, 1985a,b; Curtis & Walker, 1986; Johnston *et al.*, 1983; Abraham *et al.*, 1988) that Class I antiarrhythmics are more effective than even calcium antagonists in prolonging the P–R interval of the rat. Interestingly, the presence of β-blockade, as judged by bradycardia, did not prolong the P–R interval, whereas high doses of propranolol and the highest dose of IC 111,581 did.

In view of the findings of bradycardia, elevated serum potassium, prolonged P–R interval, and limited antiarrhythmic effectiveness of β-blocker treatment, it was important to try and establish a causal relationships between these events. In the first place there was no clear correlation between the expected degree and efficacy of β_1-blockade (bradycardia) and antiarrhythmic effects. On the other hand, statistically significant reductions in arrhythmias correlated with prolongation of P–R interval (Figure 3b). Since large doses of ICI 111,581 and propranolol had to be given to cause P–R prolongation, we have identified prolongation as being indicative of sodium channel blockade. This is in agreement with previous observations that several β-blockers have actions on the Na^+ current in cardiac tissue (Morales-Aguilera & Vaughan Williams, 1965). Furthermore, atenolol, a drug with no reported sodium channel blocking actions, completely failed to prolong the P–R even at the highest doses. It is difficult to imagine that the highest dose of

atenolol was not producing complete cardiac β_1-adrenoceptor blockade.

The other correlation of interest was that between serum potassium and antiarrhythmic efficacy. According to Figure 3a, an e-fold rise in serum potassium could be expected to reduce arrhythmias by 40%. This can be compared with the value of 63% obtained by interpolation of data from previously reported studies (Figure 2, Curtis *et al.*, 1985a; Curtis *et al.*, 1986) and from an unpublished study in which 10 different groups of conscious rats, treated so as to produce hypo- or hyperkalaemia, were subjected to coronary occlusion. Extrapolation and calculation from the data in Figure 3a gave an arrhythmia score of 4.4 for a normal serum potassium which was the same score as that seen in untreated conscious rats (Johnston *et al.*, 1983). The lack of an absolutely unambiguous correlation in Figure 3a probably reflects the relative lack of precision in the measurement of arrhythmias in acutely-prepared anaesthetized rats.

From the above, we can tentatively draw the conclusion that any antiarrhythmic activity of β-blockers in acutely prepared pentobarbitone anaesthetized rats can be ascribed to mechanisms other than blockade of cardiac β_1-receptors. It is more likely that antiarrhythmic activity is due to a β-blockade-dependent elevation of serum potassium plus Class I antiarrhythmic actions. Parratt's group (Campbell & Parratt, 1983) have considered the latter possibility as being of secondary importance following an extensive series of investigations with a variety of β-blockers. Indeed, in conscious rats, enormous doses of propranolol (40 mg kg^{-1}, i.v. or more) have little antiarrhythmic activity (unpublished observations), despite being capable of inducing convulsions, presumably as a result of local anaesthetic actions in the brain.

It may be possible to reconcile the many apparent contradictions that are found in the literature concerning rats regarding the actions of β-blockers (see Curtis *et al.*, 1987) if the action of β-blockers on serum potassium are taken into consideration. In conscious rats serum potassium does not change with occlusion, and β-blockade or sympathectomy have no antiarrhythmic activity. Antiarrhythmic activity is only seen where recent surgical damage in the presence of presumed impairment of renovascular function results in a tendency for serum potassium to rise. Pentobarbitone anaesthesia in rats subjected to surgery is mostly associated with elevated heart rates and blood pressure (Au *et al.*, 1983) and, if this is taken as a sign of increased sympathetic activity, then the associated β-adrenoceptor activation can be expected to moderate any tendency for serum potassium to rise. Blockade of this β-adrenoceptor action can be expected to result in a further elevation of serum potassium. Finally, as speculation, it is interesting to wonder whether the ability of β-blockers to modulate serum potassium may be related to the ability of such drugs to reduce mortality in post-infarct patients.

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Ventricular fibrillation is reduced in hypothyroid rats with enhanced myocardial α-adrenoceptor responsiveness

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1 The severity of ventricular arrhythmias induced by coronary artery occlusion and reperfusion has been examined in control rats and animals made hypothyroid by pretreatment with 6-propylthiouracil (PTU). The maximal driving frequency and sensitivity of isolated left atria and papillary muscles to isoprenaline and to phenylephrine in the presence of propranolol, were also examined in tissues from control and hypothyroid animals.

2 Pretreatment with PTU resulted in a potentiation of responses to the α-adrenoceptor agonist phenylephrine in both left atria and papillary muscles, while responses to isoprenaline were depressed in left atria but unaltered in papillary muscles from hypothyroid animals.

3 In rats subject to coronary artery occlusion, PTU pretreatment reduced the incidence of ventricular fibrillation during acute myocardial ischaemia and abolished reperfusion-induced ventricular fibrillation. Mortality during myocardial ischaemia and reperfusion was also abolished. Diastolic blood pressure was similar in hypothyroid and control animals, but there was a small reduction in systolic blood pressure and a marked decrease in heart rate in PTU pretreated animals.

4 These results demonstrate that PTU-induced hypothyroidism represents a condition where cardiac α-adrenoceptor-mediated responses are enhanced but the severity of ischaemia- and reperfusion-induced arrhythmias is reduced.

Introduction

It has been suggested that the activation of α-adrenoceptors in the myocardium is a primary mediator of the ventricular arrhythmias which occur during myocardial ischaemia and reperfusion (Sheridan *et al.*, 1980). This hypothesis is based on the fact that in the anaesthetized cat, the α-adrenoceptor antagonists phentolamine and prazosin, were effective in preventing ventricular arrhythmias during myocardial ischaemia and reperfusion (Sheridan *et al.*, 1980).

In recent years the contribution of α-adrenoceptor stimulation to the genesis of ventricular arrhythmias has received much attention, but the evidence obtained is conflicting. Although α-adrenoceptor antagonists have been shown to prevent reperfusion-induced ventricular arrhythmias in the cat (Sheridan *et al.*, 1980), dog (Benfey *et al.*, 1984) guinea-pig (Sheridan & Culling, 1985) and rat (Williams *et al.*, 1987), it has been suggested that the antiarrhythmic properties of these compounds are due to additional membrane stabilizing effects (Dukes & Vaughan Williams, 1984; Bralet *et al.*, 1985; Daugherty *et al.*, 1986). The precise role of α-adrenoceptor stimulation in myocardial ischaemia and reperfusion is therefore unclear, particularly since α-adrenoceptor agonists have been shown to prolong the effective refractory period and promote spontaneous defibrillation in the rat (Sharma *et al.*, 1983).

Recently, this laboratory (Chess-Williams & Critchley, 1987a) and others (Ishac *et al.*, 1983) have demonstrated a potentiation of cardiac α-adrenoceptor-mediated responses in rats made hypothyroid with 6-propylthiouracil. The question that arises, is whether the increase in α-adrenoceptor responsiveness in these animals is associated with an increase in the incidence of ventricular arrhythmias during myocardial ischaemia and reperfusion. In the present study, this possibility has been investigated by examining the effects of 6-propylthiouracil treatment on cardiac α- and β-adrenoceptor-mediated

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responses and on the incidence and severity of ischaemia- and reperfusion-induced arrhythmias in anaesthetized rats.

Methods

Experiments were performed on male Wistar rats which had been bred in the departmental animal unit. Rats were housed in groups of 6 to 10, in a room maintained at 20°C on a 12 h light/dark cycle. Food (Labsure CRM diet) and water were available *ad libitum*. The treatment group of rats received 6-propylthiouracil (PTU) at a concentration of 0.4 mg ml^{-1} in drinking water. After 8 to 10 weeks of PTU treatment rats were then subject to either *in vitro* or *in vivo* experiments along with age-matched controls.

In vitro *experiments*

Animals were killed by a blow to the head and exsanguinated. Left atria and left ventricular papillary muscles were rapidly removed and set up in a Krebs-bicarbonate solution (composition in mM: NaCl 118.4, KCl 4.7, $NaHCO_3$ 25.0, glucose 11.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $CaCl_2$, 1.9) gassed with 5% CO_2 in O_2 at 37°C. Tissues were suspended under 0.8 g tension and paced at 1 Hz by square-wave pulses (5 ms duration; twice threshold voltage) delivered, via bipolar electrodes, from Grass S48 stimulators. Isometric developed tension was recorded via Lectromed UFI (57 g sensitivity range) force transducers, on a Lectromed MX2 polygraph.

Tissues were equilibrated for 30 min with several changes of bathing medium. In each tissue, maximal driving frequency (MDF) was determined at twice threshold voltage, by gradually increasing the pacing frequency until the tissue was unable to respond to each stimulus. After washing, a cumulative concentration-response curve to isoprenaline was obtained, followed after washout, by one to phenylephrine. Metanephrine (10 μM) and desipramine (1 μM) were added to the tissue bath 15 min before obtaining agonist responses; propranolol (1 μM) was added before constructing concentration-response curves to phenylephrine.

In vivo *experiments*

Rats were anaesthetized with sodium pentobarbitone 60 mg kg^{-1} i.p. and prepared for coronary artery occlusion as described previously (Coker & Ellis, 1987). Arterial blood pressure was measured from the left carotid artery and recorded along with a Lead I ECG. After performing a left thoracotomy the rats were ventilated with room air at a rate of 54 strokes min^{-1}, a stroke volume of 1.0 to 1.5 ml 100 g^{-1}, and a positive end expiratory pressure of 0.5 to 1.0 cmH_2O. An incision was made in the pericardium and the heart was exteriorised briefly while a fine silk ligature was placed around the left coronary artery close to its origin. If arrhythmias occurred within 10 min of this procedure the experiment was terminated. After the stabilization period the coronary artery was occluded and the arrhythmias that occurred during the first 25 min of myocardial ischaemia were quantified in accordance with the Lambeth Conventions (Walker *et al.*, 1988). In survivors, the total number of ventricular premature beats (VPBs) was counted and divided into those occurring as singles, bigeminy or salvos and those occurring as ventricular tachycardia (VT, defined as 4 or more consecutive VPBs). The incidence and duration of VT, and ventricular fibrillation (VF) were also recorded along with the mortality.

Separate groups of rats were used to study reperfusion-induced arrhythmias. For these experiments both ends of the ligature were threaded through a small polythene button which was placed in contact with the heart. Coronary artery occlusion was achieved by applying tension and clamping the ligature against the button with a small pair of rubber-sheathed Dieffenbach forceps. After 5 min of myocardial ischaemia the forceps were removed, thus releasing the tension and allowing reperfusion. The incidence of VT, VF and mortality was noted.

Prior to coronary artery occlusion an arterial blood sample (0.1 ml) was taken and Po_2, Pco_2 and pH were measured with a Corning 158 blood gas/pH analyser. If necessary, the stroke volume of the ventilation pump was adjusted to maintain Pco_2 within appropriate limits. The anaesthetized rats were maintained at a temperature of 37 to 38°C by means of a heated table.

Drugs

All drugs for *in vitro* experiments were obtained commercially from Sigma, and were made up fresh in Krebs-bicarbonate solution. All reagents were of Analar grade. PTU was also obtained from Sigma.

Statistical analysis

Increases in developed tension to isoprenaline and phenylephrine were plotted as a percentage of the maximum increase for each drug. Individual EC_{50} values were determined and geometric mean EC_{50} values with 95% confidence limits calculated. Student's unpaired *t* test was performed on logarithmic EC_{50} values to test for differences between control and PTU-pretreated rats. To compare maximum responses to phenylephrine in tissues from control and PTU-treated rats, the increases in devel-

oped tension to phenylephrine were plotted as a percentage of the maximum increase in developed tension induced by isoprenaline. This reduces the variability observed when responses are plotted simply as developed tension. Student's unpaired *t* test was then applied for a statistical analysis of the maximum phenylephrine responses in control and treated tissues. Differences in haemodynamics during *in vivo* experiments were also compared with an unpaired *t* test but a Mann-Whitney U test was used to compare ischaemia-induced VPBs since these data may not be distributed normally. A probability of $P < 0.05$ was considered to be significant. The incidence of events was compared with Fisher's exact test.

Results

In vitro *experiments*

Left atria: The resting tension of 0.58 ± 0.05 g developed by left atria from control animals was not significantly different from the 0.46 ± 0.14 g developed by atria from hypothyroid animals. However, the maximum driving frequency (MDF) for atria from PTU-pretreated rats (11.7 ± 0.7 Hz) was significantly lower ($P < 0.05$) than that obtained for control tissues (15.3 ± 0.9 Hz). The responses of these tissues to isoprenaline were also depressed with the EC_{50} values being significantly greater than in control tissues. The maximum developed tension to isoprenaline, however, was similar in control and hypothyroid animals (Table 1). In contrast, responses to phenylephrine obtained in the presence of propranolol, were enhanced in left atria from hypothyroid animals. Phenylephrine EC_{50} values were reduced in these animals and the maximum responses to phenylephrine relative to isoprenaline were significantly greater than in control tissues (Figure 1a; Table 1).

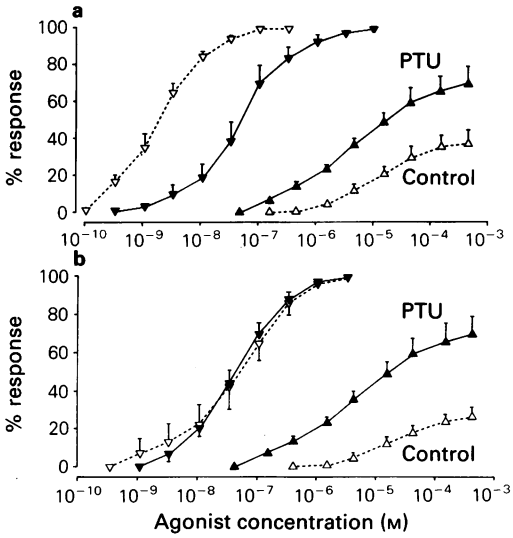


Figure 1 Mean ($n \geq 4$) concentration-response curves to isoprenaline (▽, ▼) and phenylephrine (△, ▲; in the presence of 1 μM propranolol) in (a) left atria and (b) papillary muscles from control (open symbols) and propylthiouracil (PTU)-pretreated (solid symbols) animals. Increases in developed tension are plotted as a percentage of the isoprenaline maximum.

Papillary muscles: As with left atria, the resting tensions developed by isolated papillary muscles were similar for control (0.63 ± 0.14 g) and treated tissues (0.53 ± 0.16 g). PTU-pretreatment reduced the maximum driving frequency of papillary muscles from 13.2 ± 0.7 Hz to 10.7 ± 0.5 Hz ($P < 0.05$). Responses of these tissues to phenylephrine were also enhanced, the EC_{50} values being reduced and the maximum response relative to isoprenaline being increased in papillary muscles from hypothyroid animals (Figure 1b; Table 1). However, in contrast

Table 1 Geometric mean EC_{50} values ($n \geq 4$) and maximum responses to isoprenaline and phenylephrine in isolated cardiac tissues from control and hypothyroid animals

	Control		*Hypothyroid*	
	Left atria	*Papillary muscle*	*Left atria*	*Papillary muscle*
Isoprenaline				
EC_{50} (nM)	1.9 (0.9–4.0)	43.9 (20.2–95.5)	51.0 (16.8–155.3)**	36.0 (5.7–228.7)
MDT (g)	1.00 ± 0.12	1.12 ± 0.25	0.76 ± 0.19	0.84 ± 0.14
Phenylephrine				
EC_{50} (μM)	12.6 (7.0–22.5)	21.6 (9.9–47.3)	6.4 (3.6–11.2)	3.5 (1.3–9.5)*
MDT (g)	0.57 ± 0.06	0.61 ± 0.07	0.67 ± 0.08	0.72 ± 0.16
MISO (%)	38.0 ± 7.3	27.0 ± 4.6	82.2 ± 8.1**	70.9 ± 9.6**

Maximum responses are expressed as the maximum developed tension (MDT) or the maximum increase in developed tension to phenylephrine as a percentage of the maximum increase in developed tension to isoprenaline (MISO).

*$P < 0.01$; **$P < 0.001$ compared with corresponding control, unpaired *t* test.

to that seen in left atria, papillary muscle responses to isoprenaline were not altered by PTU-pretreatment. Neither the EC_{50} nor the maximum tension developed to isoprenaline were significantly different in hypothyroid animals compared with controls (Figure 1b; Table 1).

In vivo *experiments*

Pretreatment of rats with PTU for 8 to 10 weeks reduced their weight gain. The weight of the control rats was 290 ± 9 g whereas the PTU-treated rats weighed 244 ± 9 g ($P < 0.05$). After anaesthesia and preparation for coronary artery occlusion, differences in heart rate and arterial blood pressure were observed between the two groups. Heart rate was 436 ± 12 beats min^{-1} in the controls ($n = 18$) compared with 256 ± 6 beats min^{-1} in the PTU group ($n = 20$), $P < 0.001$. Systolic but not diastolic blood pressure was also significantly ($P < 0.05$) reduced in the PTU-treated rats; $107 \pm 4/86 \pm 3$ mmHg compared with $126 \pm 6/91 \pm 6$ mmHg in controls. Arterial blood gas values before coronary artery occlusion were similar in both groups. The overall values were Po_2 87 ± 5 mmHg; Pco_2 30 ± 1 mmHg; pH 7.38 ± 0.02.

The severity of arrhythmias induced by acute myocardial ischaemia was reduced in the rats which had been pretreated with PTU. Although the incidence of VT was similar, the incidence of VF was reduced (but did not reach statistical significance at the commonly accepted 5% level) and none of the PTU-treated rats died as a result of sustained VF in contrast to the 50% mortality in the control group (Figure 2). The total number of VPBs that occurred in survivors tended to be greater in the PTU-treated rats but this was not statistically significant,

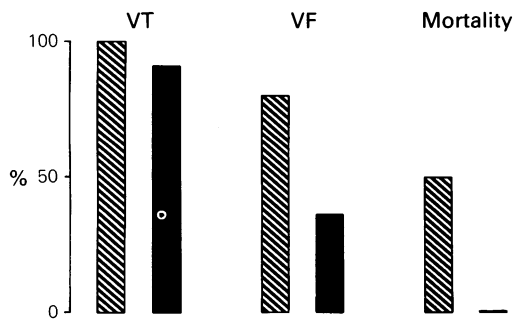


Figure 2 Incidence of ventricular tachycardia (VT), ventricular fibrillation (VF) and mortality during the first 25 min of myocardial ischaemia in control rats ($n = 10$, hatched columns) and propylthiouracil (PTU)-treated rats ($n = 11$; solid columns).
VF, $P = 0.11$; Mortality, $P = 0.025$; Fisher's exact test.

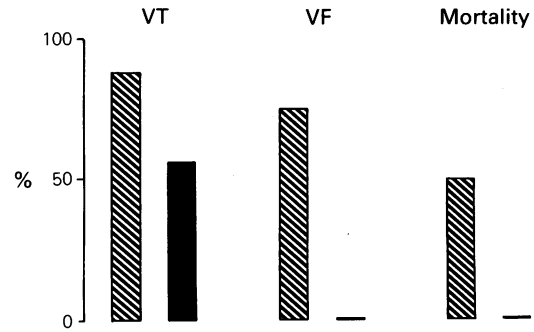


Figure 3 Incidence of ventricular tachycardia (VT), ventricular fibrillation (VF) and mortality induced by reperfusion after 5 min of myocardial ischaemia in control rats ($n = 8$, hatched columns) and propylthiouracil (PTU)-treated rats ($n = 9$, solid columns).
VF, $P = 0.005$; Mortality, $P = 0.059$; Fisher's exact test.

Table 2 The number of ventricular premature beats that occurred in rats which survived 25 min of permanent myocardial ischaemia

	n	*Ventricular premature beats* *Total*	*as VT*	*Others*
Control	5	746 ± 283	560 ± 245	186 ± 65
Propythiouracil	11	1521 ± 407	862 ± 377	659 ± 76*

*$P < 0.05$ compared with control, Mann-Whitney U-test.

although it should be noted that comparison had to be made between $n = 5$ in one group and $n = 11$ in the other because of the higher mortality in the controls. When these VPBs were divided into those occurring as VT and those occurring as singles, bigeminy or salvos (others) it can be seen that there was a significant increase in the less severe 'other' VPBs in the hypothyroid rats (Table 2).

The antiarrhythmic effect of PTU-induced hypothyroidism was more marked in the rats subject to reperfusion after 5 min of ischaemia. Although the reduction in the incidence of VT was not significant, none of the PTU-treated rats fibrillated. In contrast, the majority of the control rats succumbed to VF following reperfusion and in 50% of the group this was fatal (Figure 3).

Discussion

Propylthiouracil has been used widely to induce a hypothyroid state in experimental animals (Ciaraldi & Marinetti, 1977; Ishac *et al.*, 1983). In the present study PTU was administered in the drinking water

and we have shown previously that this method of administration results in changes in left atrial responsiveness identical to those found after thyroidectomy (Kunos, 1977; Chess-Williams & Critchley, 1987b). The marked fall in resting heart rate in the anaesthetized animals was also indicative of a hypothyroid state.

In rats subject to coronary artery occlusion, pretreatment with PTU resulted in a reduction in the incidence of ventricular fibrillation and mortality during ischaemia and reperfusion. This protective effect, occurred despite pretreatment being accompanied by an enhancement of cardiac α-adrenoceptor responsiveness. A similar increase in responsiveness to α-adrenoceptor agonists has been found in the ischaemic cat heart (Sheridan *et al.*, 1980) and since α-adrenoceptor antagonists exerted an antiarrhythmic effect in this species, it was proposed that α-adrenoceptor activation may contribute to the genesis of ventricular arrhythmias during ischaemia and reperfusion (Sheridan *et al.*, 1980). A protective effect of α-adrenoceptor blockade against reperfusion-induced arrhythmias has also been reported in the guinea-pig (Sheridan & Culling, 1985) and rat (Williams *et al.*, 1987). These previous studies suggest, therefore, that arrhythmogenesis may be expected to be greater in conditions where α-adrenoceptor responses are enhanced.

Since the cardiac adrenergic changes which occur in hypothyroid animals resemble those which occur during ischaemia, the present study examined the severity of ischaemia- and reperfusion-induced arrhythmias in hypothyroid rats. Not only are cardiac α-mediated responses enhanced in both conditions but the mechanism responsible appears to be similar with an increase in α-adrenoceptor number during ischaemia (Corr *et al.*, 1981; Allely & Brown, 1988) and hypothyroidism (Ciaraldi & Martinetti, 1977; Fox *et al.*, 1985). Of more physiological relevance is the finding that cardiac responses to noradrenaline may be similarly affected in the two disease states. Responses to noradrenaline or sympathetic nerve stimulation are mediated via β-adrenoceptors in the normal heart but during ischaemia (Sheridan *et al.*, 1980) and in hypothyroidism (Chess-Williams & Critchley, 1987b), α-adrenoceptors may be involved in the cardiac response. Thus the changes in the adrenergic system which occur in hypothyroidism resemble those which occur in myocardial ischaemia.

If α-adrenoceptor stimulation is involved in the genesis of ventricular arrhythmias, an increase in the severity of arrhythmias might therefore be predicted in hypothyroid animals. However, the opposite was found, with the incidence of ventricular fibrillation and mortality during ischaemia and reperfusion being reduced. This contrasts with the results of Williams *et al.* (1987) where no significant protective effect of PTU pretreatment could be demonstrated in rats subject to reperfusion-induced arrhythmias. However the method of PTU administration was different in the two studies. Williams *et al.* (1987) administered the drug as a single daily injection for 1 week which, considering its short half life (4 h) in the rat (Sitar & Thornhill 1972), may have failed to induce a hypothyroid state. This would explain the lack of a significant fall in heart rate found by these authors. In the present study, PTU was administered in the drinking water to ensure a constant dose of drug throughout the pretreatment period and resulted in a highly significant bradycardia in the treated animals. This fall in heart rate may have influenced the severity of arrhythmias seen in hypothyroid animals since it has been reported that heart rate correlates positively with the occurrence of ventricular tachycardia and fibrillation during ischaemia (Bolli *et al.*, 1986) and also the severity of arrhythmias induced by reperfusion (Coker & Parratt, 1985). Alternatively, the increase in α-adrenoceptor responsiveness itself may have exerted a protective effect, since α-adrenoceptor agonists have been shown to prolong the effective refractory period (Sharma *et al.*, 1983; Dukes & Vaughan Williams, 1984) and reduce ventricular automaticity (Rosen *et al.*, 1984), effects which would be expected to be antiarrhythmic. Indeed, Sharma *et al.* (1983) have found that α-adrenoceptor stimulation can promote spontaneous defibrillation in the rat and Williams *et al.* (1987) have shown that noradrenaline infusion can reduce reperfusion-induced ventricular fibrillation. These results appear to conflict with the reported protective role for α-adrenoceptor antagonists during ischaemia and reperfusion. However, in the rabbit (Dukes & Vaughan Williams, 1984) and the rat (Daugherty *et al.*, 1986; Bralet *et al.*, 1985) the protective effects of α-adrenoceptor blockers have been explained by the additional membrane stabilizing properties these compounds possess and may not be related to α_1-adrenoceptor antagonism. This conclusion is further supported by the demonstration that ischaemic conditions may enhance the local anaesthetic properties of these drugs (Vaughan Williams, 1978).

When considering the present results, it is important to note that the maximum frequency at which isolated left atria and papillary muscles could be paced was reduced in tissues from hypothyroid animals. This indicates a prolongation of the effective refractory period in the hypothyroid heart and agrees with the reported prolongation of the repolarization phase of the action potential in atria from hypothyroid rabbits (Freedberg *et al.*, 1970). Such an increase in effective refractory period would tend to reduce the severity of ventricular arrhythmias. It is

possible that the combined effects of reduced heart rate and increased effective refractory period may have overcome any proarrhythmic effect exerted by the elevated α-adrenoceptor responsiveness. Thus the role of cardiac α-adrenoceptors in the genesis of malignant arrhythmias remains uncertain, although we have been able to demonstrate a situation where α-adrenoceptor responsiveness is increased and the severity of ischaemia- and reperfusion-induced arrhythmias is reduced.

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Effects of yohimbine on submaxillary salivation in dogs

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1 The effects of yohimbine (0.5 mg kg^{-1} i.v.) on both resting and parasympathetic and sympathetic stimulation-induced submaxillary salivary responses were investigated in the anaesthetized dog.

2 Salivary secretion was increased significantly for a period of 45 min following an injection of yohimbine.

3 Sectioning of the chorda tympani (but not the cervical sympathetic) nerve abolished the yohimbine-induced increase in resting salivary secretion and potentiated that elicited by electrical stimulation of the chorda tympani nerve.

4 These results show that yohimbine increases submaxillary secretion by inhibition of presynaptic α_2-adrenoceptors located on the chorda tympani, which inhibit cholinergic transmission.

Introduction

Several experiments have suggested the involvement of postsynaptic α_2-adrenoceptors in the control of sympathetically and parasympathetically-mediated salivation in the rat (Bylund & Martinez, 1980; 1981; Martinez *et al.*, 1982; Elverdin *et al.*, 1984). In fact, a common side-effect of treatment with α_2-adrenoceptor agonists, especially clonidine, is reduced salivation and a dry mouth. Clonidine reduced submaxillary salivation provoked by either brainstem or peripheral nerve stimulation (Green *et al.*, 1979a); guanabenz, another α_2-adrenoceptor agonist antagonized the secretory responses to noradrenaline, methacholine, substance P (Kaniucki *et al.*, 1984; 1985) and to electrical stimulation of the chorda tympani (Terzic & Stojic, 1986). The decrease in salivation induced by guanabenz was virtually abolished by yohimbine, but not by the α_1-adrenoceptor antagonist prazosin (Terzic & Stojic, 1986). The effects of α_2-adrenoceptor antagonists on salivary secretion, in contrast, have been poorly investigated. Thus, the aim of the present study was to investigate the effects of yohimbine, an α_2-adrenoceptor antagonist (Goldberg & Robertson, 1983) on both resting salivation and that produced in response to parasympathetic and sympathetic nerve stimulation in the anaesthetized dog.

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Methods

Animals and general procedure

Dogs of either sex (7 to 15 kg) were anaesthetized with a mixture of chloralose (40 mg kg^{-1} i.v.) plus urethane (500 mg kg^{-1} i.v.). This combination of anaesthetics was chosen from information obtained in preliminary experiments (not shown) which indicated that under either chloralose or, urethane or sodium thiopentone anaesthesia, yohimbine remained ineffective. In view of the potential α_2-adrenoceptor antagonist properties of urethane (Armstrong *et al.*, 1982; Langer & Shepperson, 1982; Moore *et al.*, 1984), this drug was used at low subanaesthetic concentrations, devoid of α_2 activity. The trachea was intubated and the dog artificially respired. Adequate anaesthesia was maintained by injection of 1 mg kg^{-1} chloralose + 5 mg kg^{-1} urethane each hour. Femoral arterial blood pressure and heart rate were measured, respectively, with a Statham P23 Id pressure transducer connected to a Honeywell Bull recorder and a tachocardiometer triggered by an electrocardiogram (lead II) linked to a chart recorder. Body temperature and arterial pH were maintained constant around 38.5°C and 7.42 respectively during each experiment.

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Study of salivary secretion

In the anaesthetized dog, the skin and subcutaneous muscles were incised over approximately 8 cm, along a median path from the angle of the lower mandible to the mandibular arc. The spindle-shaped swelling of the digastric muscle was moved aside. This revealed, on either side of the median raphé, a thin muscle with transverse fibres: the mylo-hyoideus. It was raised on a probe in order to make an incision parallel to the median raphé and 1 cm to the exterior. Just below the mylo-hyoideus, on the deep cervical aponevrosis, near the angle of the mandible, the large lingual nerve was seen crossing the median line and the hypoglossal nerve perpendicularly. Just above the lingual nerve and perpendicular to it under the aponevrosis one can see the grey path of Wharton's duct with, to the inner side, the thinner duct of the sublingual nerve. Following the lingual nerve outwards one can see a thin externally concave nerve come off downwards and to the rear, the chorda tympani nerve, a branch of the facial nerve. This small nerve makes up the third side of a triangle formed in conjunction with the lingual nerve and Wharton's duct. Wharton's duct was catheterized by means of a polythene tube. Under these conditions, three experimental protocols were performed.

Experiment 1: Effect of yohimbine on spontaneous submaxillary salivation Yohimbine (0.5 mg kg^{-1} i.v., 3 ml, $n = 6$) and saline (0.9% NaCl 3 ml, in sham experiments, $n = 6$) were each injected after dissection had been completed, after at least 30 min in resting conditions and salivary secretion from both submaxillary ducts collected every 15 min thereafter for a period of 90 min.

Experiment 2: Effect of yohimbine on parasympathetic salivation A group of experiments (2a, $n = 6$) was performed in order to intestigate the effect of yohimbine on spontaneous salivation with and without the chorda tympani nerve being sectioned. The left chorda tympani nerve was then cut and the effects of yohimbine (0.5 mg kg^{-1} i.v.) on the spontaneous salivation from the intact (right) side compared with the values obtained on the sectioned (left) side.

A second group of experiments (2b) investigated the effect of yohimbine on electrically-induced parasympathetic salivation. After dissection, the left chorda tympani was cut, immersed in a pool of paraffin oil (38°C) and the peripheral end stimulated via bipolar stainless steel electrodes (15 s trains, supramaximal intensity, 0.1 ms, 10 Hz every 15 min, once before and six times after yohimbine (or saline) administration). These parameters of stimulation were chosen since they mimic the salivary secretion found with an intact nerve. Saliva was collected during these 15 s periods of stimulation and during the 15 min period following stimulation. The effects of saline (3 ml, i.v., $n = 6$) and yohimbine (0.5 mg kg^{-1} 3 ml, i.v., $n = 6$) injected at time 0 (i.e. at least 30 min after dissection) were compared on the output of saliva produced by stimulation of the chorda tympani nerve.

Experiment 3: Effect of yohimbine on sympathetic salivation In 6 other dogs, the left cervical sympathetic nerve was cut in the vagal sympathetic trunk and the effects of yohimbine (0.5 mg kg^{-1} i.v.) on the intact (right) side compared with the values obtained on the sectioned and stimulated side according to the protocol described in experiment 2b (15 s trains, supramaximal intensity, 0.1 ms, 10 Hz every 15 min, once before and six times after yohimbine or saline (3 ml) administration).

Drug administration

Yohimbine hydrochloride (Sigma), dissolved in 3 ml saline (0.9%) was injected via the saphenous vein. The dose (0.5 mg kg^{-1}) antagonized the depressor effects of 10 μg kg^{-1} i.v. clonidine on blood pressure, metabolism (plasma free fatty acids and glycerol) and submaxillary salivation (Taouis *et al.*, 1988). Controls received 3 ml saline i.v. as a sham injection.

Statistical analysis

Results are presented as mean values ± s.e.mean. Significance was estimated by use of Student's t test (after one-way analysis of variance). Student's t test for paired comparisons was used when intact and sectioned sides were compared. P values less than 0.05 were considered significant.

Results

Experiment 1: effect of yohimbine on spontaneous submaxillary salivation (Figure 1)

Compared with saline, yohimbine significantly increased salivary secretion during the 45 min following injection. After this time, due to the large individual variations, the increase was not significant. Yohimbine failed to modify blood pressure or heart rate (not shown) eliminating any possibility that variation in salivation arose from changes in blood pressure.

Experiment 2: effect of yohimbine on parasympathetic salivation

Experiment 2a After section of the chorda tympani nerve, the stimulating effect of yohimbine on sponta-

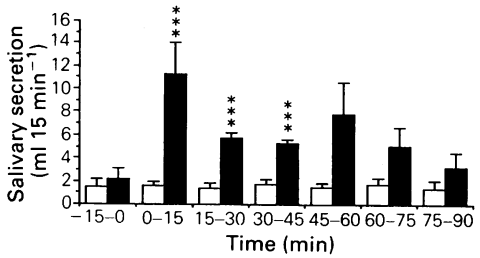


Figure 1 Comparison of the effects of yohimbine (0.5 mg kg^{-1} i.v. at time 0; solid columns) and i.v. saline (open columns) each 3 ml on the salivary secretion (ml 15 min^{-1}) in anaesthetized dogs (Experiment 1). Saliva was collected during the 15 min periods indicated from both submaxillary ducts. ***$P < 0.01$. Mean values are shown with vertical bars indicating s.e.mean; $n = 6$ in each case.

neous salivary secretion was abolished; yohimbine significantly increased salivary secretion from the intact innervated side but not from the sectioned side (Figure 2).

Experiment 2b Unilateral stimulation of the chorda tympani nerve every 15 min for 15 s elicited about 1 ml of saliva irrespective of the time of collection. Yohimbine significantly increased the magnitude of the salivary secretion, to about 2 ml every 15 min following yohimbine injection. After 45 min, the effect of yohimbine was not significant (Figure 3).

Experiment 3: effects of yohimbine on sympathetic salivation

Sectioning of the superior cervical sympathetic nerve failed to modify the spontaneous excito-secretory response to yohimbine (not shown) which was of similar magnitude in the intact (right) and the sectioned (left) sides. Moreover, yohimbine (0.5 mg kg^{-1} i.v.) failed to modify the excitatory response to stimulation of the sympathetic nerve (Figure 4).

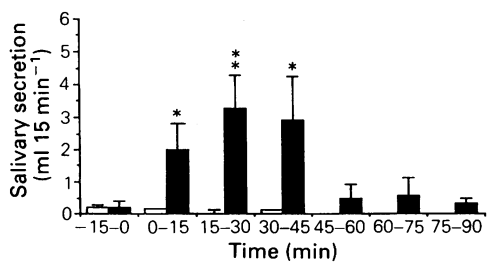


Figure 2 Effect of sectioning the chorda tympani nerve on the enhanced salivary response (ml 15 min^{-1}) produced by yohimbine (0.5 mg kg^{-1} i.v. at time 0) (Experiment 2a). The effects of yohimbine on the intact (right) side (solid columns) were compared with those on the sectioned (left) side (open columns). *$P < 0.05$; **$P < 0.02$. Mean values are shown with vertical bars indicating s.e.mean. $n = 6$.

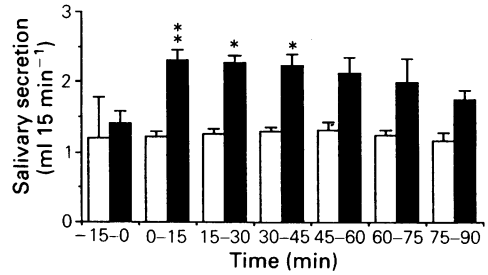


Figure 3 Comparison of the effects of yohimbine (solid columns) and i.v. saline (open columns) on the salivary secretion (ml 15 min^{-1}) elicited by peripheral electrical stimulation of the chorda tympani nerve (15 s trains, supramaximal intensity, 0.1 ms, 10 Hz every 15 min once before and six times after yohimbine (or saline) administration) (Experiment 2b). Stimulation was for 15 s at the beginning of each 15 min period before and after yohimbine (or saline) injection. *$P < 0.05$; **$P < 0.02$. Mean values are shown with vertical bars indicating s.e.mean. $n = 6$.

Discussion

Yohimbine induced a significant increase in the spontaneous salivary secretion in the submaxillary gland of the dog. The effect of yohimbine lasted for 45 min: this duration of action compares with a half-life in man of around 0.6 h (Owen *et al.*, 1987). This stimulating effect of yohimbine, an α_2-adrenoceptor

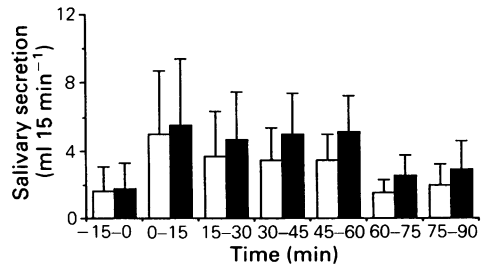


Figure 4 The effect of nerve section on the ability of yohimbine (0.5 mg kg^{-1} i.v. at time 0) to enhance the response of the dog submaxillary gland to stimulation of the superior cervical sympathetic nerve (15 s trains, supramaximal intensity, 0.1 ms, 10 Hz every 15 min once before and six times after yohimbine (or saline) administration) (Experiment 3). The effects of yohimbine on the intact (right) side (solid columns) were compared with those obtained from stimulation of the sectioned (left) side (open columns). Mean values are shown with vertical bars indicating s.e.mean. $n = 6$.

antagonist, in contrast with the inhibitory action of α_2-adrenoceptor agonists (for references see introduction) suggests the involvement of α_2-adrenoceptors in the control of submaxillary secretion.

This effect of yohimbine is dependent on the integrity of the parasympathetic innervation. Thus (1) it is suppressed by sectioning of the cholinergic chorda tympani nerve while (2) yohimbine itself potentiated the increase in salivary secretion in response to chorda tympani stimulation. In contrast, yohimbine did not modify the salivary volume obtained in response to sympathetic nerve stimulation while prior sectioning of the superior cervical sympathetic nerve did not change the magnitude of yohimbine-induced salivation. These results and those of Green *et al.* (1979a) suggest that α_2-adrenoceptors may regulate cholinergic (but not sympathetic) transmission and that yohimbine increases submaxillary secretion by inhibition of presynaptic α_2-adrenoceptors (located on the chorda tympani) which inhibit cholinergic transmission. The role of presynaptic α_2-adrenoceptors in the control of acetylcholine release has been well documented (Langer, 1977). In fact, an effect of yohimbine on postsynaptic inhibitory α_2-adrenoceptors located on the submaxillary gland can be ruled out since yohimbine failed to induce salivary secretion after section of the chorda tympani.

The effect of yohimbine could also have a central component which we cannot evaluate. The central effect is probably of importance since Green *et al.* (1979b) found that clonidine reduces submaxillary salivation evoked by brainstem stimulation in cats. However, the finding that yohimbine remained active during stimulation of the distal end of the chorda tympani (at a frequency which mimicked the salivary secretion found when the nerve was intact) suggests a peripheral action of yohimbine on the α_2-adrenoceptors located on the parasympathetic nerve endings.

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Baclofen and phaclofen modulate GABA release from slices of rat cerebral cortex and spinal cord but not from retina

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1 The effects of (−)-baclofen, muscimol and phaclofen on endogenous γ-aminobutyric acid (GABA) release from rat cortical slices, spinal cord slices and entire retinas were studied.

2 The spontaneous resting release of GABA from the three tissues was 3 to 6 pmol mg^{-1} wet wt $10\,min^{-1}$. Depolarization of cortical slices with KCl (50 mM) (high-K) produced an 8 fold increase in GABA release but high-K did not evoke an increased release of GABA from spinal slices or retinas.

3 When rats were injected with γ-vinyl-GABA (250 mg kg^{-1} i.p.) (GVG) 18 h before death, the tissue GABA stores were increased 3 to 6 fold and high-K then evoked striking Ca-dependent releases of GABA from all three tissues. Thus, in subsequent experiments, unless otherwise stated, the nervous tissues were taken from GVG-treated rats.

4 (−)-Baclofen (10 μM) significantly reduced the K-evoked release of GABA from cortical and spinal slices but retinal release was not affected, even at a concentration of (±)-baclofen of 1 mM. For cortical slices, the IC_{50} for baclofen was approximately 5.2 μM. The inhibitory effect of baclofen on GABA release from cortical slices also occurred in slices prepared from saline-injected rats, indicating that GVG treatment did not qualitatively affect the results.

5 The inhibitory effect of (−)-baclofen on the K-evoked release of GABA from cortical and spinal slices was antagonised by phaclofen (500 μM), confirming that baclofen was producing its effects by acting at the $GABA_B$-receptor.

6 Phaclofen (500 μM) increased the spontaneous resting release of GABA from cortical slices taken from GVG-treated rats but not from saline-injected rats. Phaclofen did not increase GABA release from spinal slices or retinas taken from GVG-treated rats.

7 Baclofen (10 μM) significantly reduced the K-evoked release from cortical slices of glutamate, aspartate, glycine and taurine.

8 Muscimol (10 μM) and δ-aminolaevulinic acid (10 μM) had no effect on either the resting or K-evoked release of GABA from cortical slices prepared from saline-injected or GVG-treated rats.

9 The results obtained with cortical and spinal slices are consistent with the presence of inhibitory $GABA_B$-autoreceptors. The phaclofen-induced increase in GABA release from cortical slices taken from GVG-treated rats, but not from saline-injected rats, implies that under conditions of high GABA release, considerable feedback inhibition is occurring via activation of the $GABA_B$ inhibitory autoreceptors. No evidence was found for $GABA_B$-autoreceptors on retinal GABAergic amacrine cells or for $GABA_A$-autoreceptors in cortical slices or spinal cord slices.

Introduction

γ-Aminobutyric acid (GABA) is the major inhibitory transmitter substance in the central nervous system and it is now generally accepted that it acts on two types of receptor (Bowery, 1988). The $GABA_A$-receptor, which is blocked by bicuculline and activated by muscimol, is linked to the chloride channel and is responsible for postsynaptic inhibition. The $GABA_B$-receptor is not blocked by bicuculline and is activated by baclofen. Until recently the absence of a specific $GABA_B$-antagonist has hindered our under-

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standing of the role of $GABA_B$-receptors. However, it is clear that many $GABA_B$-receptors are located on presynaptic nerve terminals and their activation inhibits transmitter release. Bowery *et al.* (1979) demonstrated that baclofen and GABA inhibited the release of [^{3}H]-noradrenaline from rat isolated atria. Subsequently, inhibitory presynaptic $GABA_B$- receptors have been found in many peripheral tissues (see Hill & Bowery, 1986, for references) and in central neurones utilizing several transmitters, including dopamine (Bowery *et al.*, 1980), 5-hydroxytryptamine (5-HT, Gray & Green, 1987) and cholecystokinin (Conzelmann *et al.*, 1986). However, in the case of GABAergic neurones themselves, the acceptance of presynaptic inhibitory autoreceptors has been slow and there is still no consensus in the literature on the classification of these presumed GABA autoreceptors. In several studies, muscimol was found to decrease the K-evoked release of [^{3}H]-GABA from brain slices or synaptosomes, suggesting the presence of $GABA_A$-autoreceptors on GABAergic nerve terminals (Mitchell & Martin, 1978; Brennan *et al.*, 1981; Kuriyama *et al.*, 1984). However, in similar experiments, others have found that baclofen, but not muscimol, decreased GABA release, suggesting the presence of $GABA_B$-autoreceptors (Anderson & Mitchell, 1985; Limberger *et al.*, 1986; Pittaluga *et al.*, 1987; Waldmeier *et al.*, 1988).

In the present study, we have attempted to clarify the status of GABA autoreceptors by examining the effects of $GABA_A$ and $GABA_B$ agonists and antagonists on the release of endogeneous GABA from three areas in the central nervous system. Our experiments provide support for the presence of $GABA_B$-autoreceptors in cerebral cortex and spinal cord but not in the retina.

Part of this work has been published previously in a preliminary form (Shah & Neal, 1989).

Methods

Preparation of tissues

Male Wistar rats (150–200 g) were killed by cervical dislocation. The eyes were enucleated and the retinas dissected in Krebs-bicarbonate medium at room temperature. The brains were removed and slices of cerebral cortex (0.2 mm thick) were prepared by means of a McIlwain tissue chopper as described previously (Cunningham & Neal, 1981). The spinal cord was removed following an extensive laminectomy and transverse (0.3 mm thick) slices were again prepared by a McIlwain chopper (Hopkin & Neal, 1971). In most experiments, tissues were taken from animals that had been treated with the irreversible GABA-T inhibitor, γ-vinyl-GABA (GVG) (250 mg kg^{-1} i.p.) 18 h before death.

Incubation of tissues

In order to measure the release of GABA from the tissues, slices of cortex (5–6 slices, weight 15 mg), spinal cord (5–6 slices, weight 15 mg), or two entire retinas (approx 20 mg) were placed in a small perspex chamber (volume 0.5 ml) containing Krebs-bicarbonate medium (250 μl) at room temperature and were gently agitated by a stream of 5% CO_2 in oxygen.

At 10 min intervals, the medium was drained off and replaced with fresh medium. The GABA and other amino acids in each of the resulting 10 min samples were measured by high performance liquid chromatography (h.p.l.c.) (Yazulla *et al.*, 1985). Potassium-evoked release of GABA was achieved by exposing the tissues for one 10 min period to KCl (50 mM). The effects of drugs on the K-evoked release of GABA was usually examined by exposing the tissue to the drug in the collection period immediately preceding the addition of KCl and during exposure to KCl.

High performance liquid chromatography

The samples were analysed on a Varian 5000 h.p.l.c. by use of precolumn derivatisation with *o*-phthalaldehyde (Turnell & Cooper, 1981).

Statistical analysis

Differences between the resting release of GABA and the K-evoked release of GABA were tested for significance by use of Student's *t* test.

Materials

Incubation medium Krebs Ringer bicarbonate of the following composition was used (mM): NaCl 118, KCl 4.8, $CaCl_2$ 2.4, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2, glucose 9.5. The medium was continuously gassed with 95% O_2 plus 5% CO_2.

Drugs γ-Vinyl-GABA (vigabatrin) was a gift from Merrell-Dow Pharmaceuticals Ltd. (−)-Baclofen was a gift from Professor N.G. Bowery. Phaclofen was obtained from Cambridge Research Biochemicals. Other chemicals (Analar Grade) were obtained from Sigma Chemical Company Ltd.

Results

Effect of high-K on GABA release

When cortical slices were exposed for 10 min to medium containing KCl (50 mM) (high-K) the release of GABA increased from approximately 3 to 26

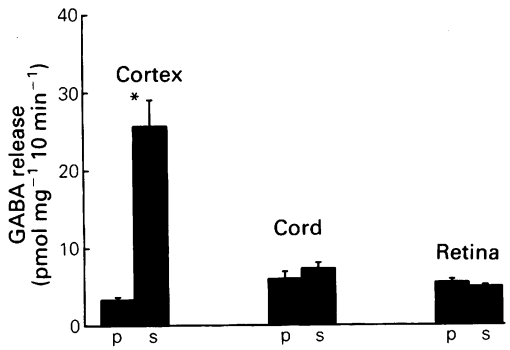


Figure 1 Effect of high-K on GABA release from rat cerebral cortex, spinal cord and retina. In this and subsequent figures, p represents release in sample preceding stimulation and s, release during exposure to KCl (50 mM). Each result is the mean of 8 experiments and bars show s.e.mean. High-K evoked a significant release of GABA only from cortical slices, s significantly different from p: $*P < 0.001$ (Student's t test).

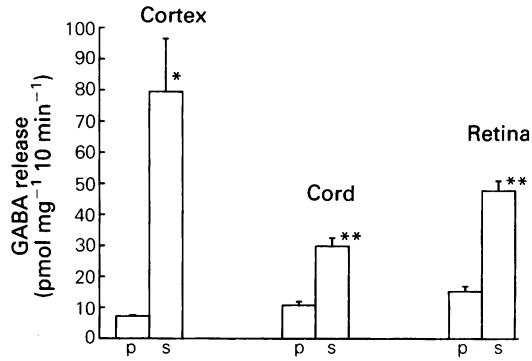


Figure 2 Effect of high-K on GABA release from nervous tissues taken from rats treated with γ-vinyl-GABA (GVG) 18 h before death. Note that GVG increases the resting release of GABA and enables a K-evoked release of GABA from cord and retina to be measured. Each result is the mean of 4–9 experiments and bars show s.e.mean; K-evoked release (s) significantly different from resting release (p): $*P < 0.001$, $**P < 0.002$.

Table 1 Effect of γ-vinyl-GABA (GVG) treatment on the GABA content of rat cerebral cortex, spinal cord and retina

Tissue	*GABA content* (μmol g^{-1} wet wt) *Saline injected*	*GVG injected*	*GVG/ saline*
Cerebral cortex	2.17 ± 0.29	5.85 ± 0.42	2.7
Spinal cord	0.89 ± 0.095	2.37 ± 0.16	2.7
Retina	2.77 ± 0.23	17.77 ± 1.05	6.4

Rats were injected (i.p.) with either normal saline or GVG (250 mg kg^{-1}) 18 h before death. Each result is the mean ± s.e. mean of 10 experiments. Values obtained from saline-injected animals were not significantly different from those obtained from uninjected animals (not shown).

pmol mg^{-1} wet wt 10 min^{-1} (Figure 1). However, exposure of spinal slices and retinas to high-K did not evoke any measureable increase in GABA release although the spontaneous resting release of GABA from these tissues was similar to that from cortex (Figure 1).

Effect of γ-vinyl-GABA on GABA release

In order to increase the storage levels of GABA, rats were treated with GVG (250 mg kg^{-1} i.p.) 18 h before death. The effect of this treatment on GABA levels in the cerebral cortex, spinal cord and retina is shown in Table 1. The GABA content of the retina was increased more than 6 fold, with the cortex and spinal cord showing less striking increases in GABA

Table 2 Ca-dependence of K-evoked GABA release

Tissue	*GABA release* (pmol mg^{-1} wet wt 10 min^{-1}) *Normal medium* *p*	*s*	*s/p*	*High-Mg/low-Ca medium* *p*	*s*	*s/p*
Cortical slices (S) (5)	2.7 ± 0.34	26.9 ± 3.04*	10	3.2 ± 0.52	2.4 ± 0.27	0.75
Cortical slices (GVG) (6)	7.6 ± 1.03	58.5 ± 2.88*	7.7	4.6 ± 0.63	4.6 ± 1.03	1.0
Spinal slices (GVG) (4)	7.6 ± 0.69	20.3 ± 2.35*	2.7	8.5 ± 1.04	7.1 ± 0.83	0.84
Retina (GVG) (6)	21.2 ± 2.42	52.6 ± 12.32†	2.5	4.6 ± 0.63	4.6 ± 1.04	1.0

(S) and (GVG) indicate rats were injected (i.p.) with normal saline or γ-vinyl-GABA 18 h before death, respectively. Each result is the mean ± s.e.mean of the number of experiments indicated in parentheses. p and s indicate resting release and K-evoked release, respectively. Significant evoked release $*P < 0.001$, $†P < 0.05$. High-Mg/low-Ca medium contained $MgCl_2$ (20 mM) and $CaCl_2$ (0.2 mM).

levels. The effect of GVG treatment on GABA release is illustrated in Figure 2. The spontaneous, resting release of GABA was increased from all three tissues but more importantly, high-K now evoked striking releases of GABA from the retina and spinal slices. In the case of cortical slices, not only was the resting release of GABA increased but the K-evoked release was almost doubled in slices from GVG-treated rats compared with controls (treated: 13 fold increase; untreated: 8 fold increase). GVG has been shown to cause the accumulation of GABA in glial cells (Neal *et al.*, 1989). However, release from glia seems unlikely in the present experiments because the K-evoked release from all three tissues was found to be highly Ca-dependent (Table 2). Since GVG treatment enabled us to measure a K-evoked, Ca-dependent GABA release from cord, retina and cortex, it was used in most of the subsequent experiments. Note that in the figures, solid columns indicate results obtained from untreated rats whilst open columns indicate results obtained from GVG-treated rats.

Effect of baclofen on GABA release from cortical slices

The effect of (−)-baclofen on GABA release in cortical slices from GVG-treated rats is shown in Figure 3. (−)-Baclofen (10 μM) did not affect the resting release of GABA but the K-evoked release was reduced by more than two thirds. It might be argued that the effects of baclofen could be abnormal because of the treatment of the animals with GVG. However, baclofen (10 μM) halved the K-evoked release of GABA in slices taken from animals not treated with GVG (Figure 3). Thus, it seems unlikely that GVG treatment fundamentally changes the mechanism of GABA release from nervous tissues. The EC_{50} of (−)-baclofen in inhibiting the K-evoked release of GABA was approximately 5.2 μM and 80% inhibition was produced by 20 μM baclofen.

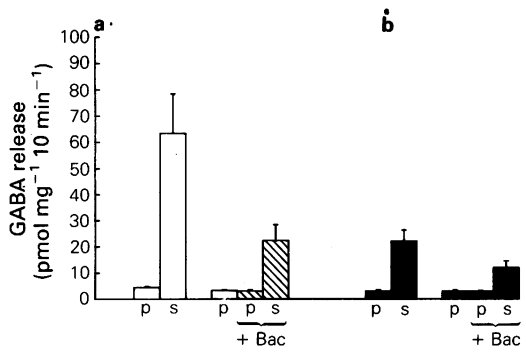


Figure 3 Effect of (−)-baclofen (Bac, 10 μM) on the K-evoked release of GABA from cortical slices prepared from brains of γ-vinyl-GABA (GVG)-treated rats (a) and saline-injected rats (b). In both cases baclofen significantly depressed the K-evoked GABA release ($P < 0.01$). Each result is the mean of 4–8 experiments; bars show s.e.mean.

Effect of phaclofen on GABA release from cortical slices

Exposure of cortical slices (from GVG treated rats) to the $GABA_B$ receptor antagonist phaclofen (Kerr *et al.*, 1987) increased the spontaneous resting release of GABA 4 fold and apparently blocked the depressant action of (−)-baclofen (10 μM) on the K-evoked release of GABA (Figure 4). However, phaclofen alone was found to increase significantly the K-evoked release of GABA (Figure 4). The effect of phaclofen on the resting and K-evoked release of GABA was not observed in cortical slices from saline injected rats (not illustrated).

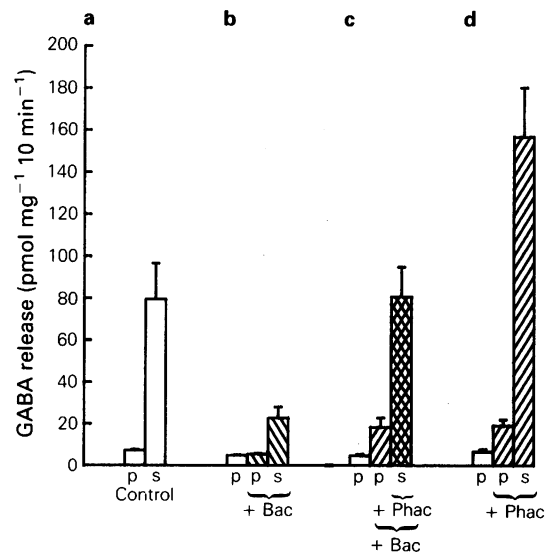


Figure 4 Effect of phaclofen (Phac, 500 μM) on GABA release from cortical slices (from γ-vinyl-GABA-treated rats). (c and d) Phaclofen significantly increased the resting release of GABA ($P < 0.01$) and reduced the effect of (−)-baclofen (Bac, 10 μM) on the K-evoked release of GABA (cross-hatched column in (c). The control response to high-K is shown on the left (a) and the control response to high-K in the presence of phaclofen is shown on the right (d). Each result is the mean of 4 experiments, bars show s.e.mean.

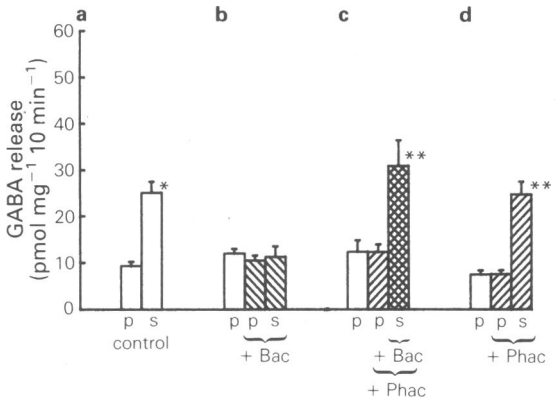


Figure 5 Effect of (−)-baclofen (Bac, 10 μM) (b) and phaclofen (Phac, 500 μM) (d) on the K-evoked release of GABA from spinal slices prepared from rats treated with γ-vinyl-GABA. In the presence of baclofen and phaclofen (c) the K-evoked release (s) was not significantly different from the control K-evoked release (a). Baclofen alone abolished the K-evoked release of GABA. Phaclofen alone did not increase either the resting or the K-evoked release of GABA (right). Each result is the mean of 5 experiments; bars show s.e.mean. K-evoked release (s) significantly different from resting release (p); $*P < 0.002$, $**P < 0.02$.

Effect of (−)-baclofen and phaclofen on GABA release from spinal slices

The effect of (−)-baclofen (10 μM) on GABA release in spinal slices from GVG-treated rats is illustrated in Figure 5. Baclofen completely inhibited the K-evoked release of GABA from spinal slices. This effect of baclofen was abolished by phaclofen, but unlike the effect of the antagonist on cortical slices, phaclofen did not affect either the resting or K-evoked release of GABA (Figure 5).

Effect of baclofen and phaclofen on GABA release from the retina

Neither (±)-baclofen (at concentrations up to 1 mM) nor phaclofen (500 μM) had any observable effect on the spontaneous resting release or the K-evoked release of GABA from retinas taken from GVG-treated rats (Figure 6).

Effect of baclofen on the K-evoked release of other amino acids from cortical slices

Exposure of cortical slices to high-K more than doubled the release of aspartate and glutamate. The release of glycine and taurine was also significantly increased by high-K but the evoked release of these amino acids was small (Figure 7). Baclofen (10 μM) abolished the K-evoked release of all four amino acids (Figure 7).

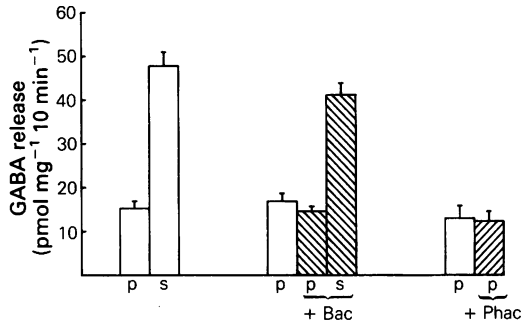


Figure 6 Effect of (±)-baclofen (Bac, 1 mM) and phaclofen (Phac, 500 μM) on GABA release in the retinas from γ-vinyl-GABA-treated rats. Neither the resting (p) nor the K-evoked (s) release of GABA was affected by the drugs. Each result is the mean of 4–9 experiments; vertical bars show s.e.mean.

Effect of muscimol and δ-aminolaevulinic acid on GABA release from cortical slices

Exposure of cortical slices from GVG-treated and saline-injected rats to the $GABA_A$ agonists muscimol and δ-aminolaevulinic acid did not have any effect on the resting or K-evoked release of GABA (Figure 8).

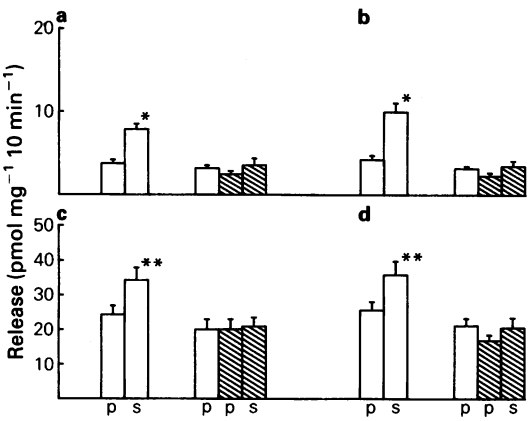


Figure 7 Effect of (−)-baclofen (10 μM hatched columns) on the release of (a) aspartate, (b) glutamate, (c) glycine and (d) taurine from cortical slices prepared from the brains of rats treated with γ-vinyl-GABA. Open columns: release in the absence of baclofen. Each result is the mean of 9 experiments; vertical bars show s.e.mean. K^+-evoked release (s) significantly different from resting release (p) $*P < 0.001$, $**P < 0.05$.

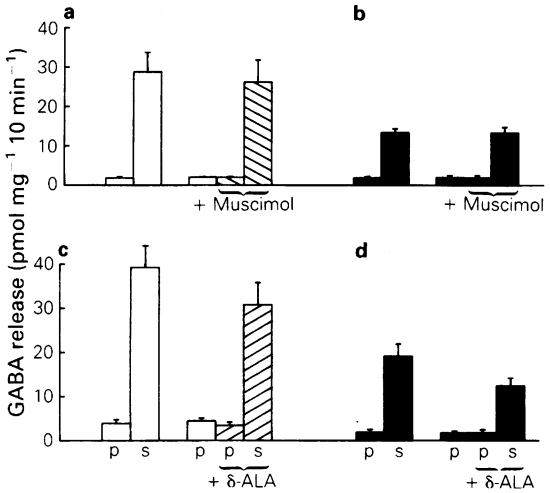


Figure 8 Effect of (a and b) muscimol (10 μM) and (c and d) δ-aminolaevulinic acid (δ-ALA) on the K-evoked release of GABA from cortical slices prepared from γ-vinyl-GABA treated rats (a and c) or saline-injected rats (b and d). Neither drug significantly reduced the K-evoked release of GABA. Each result is the mean of 4–6 experiments; vertical bars show s.e.mean.

Discussion

The present study clearly shows that the $GABA_B$ agonist (−)-baclofen inhibits the K-evoked release of GABA from cortical slices and spinal slices prepared from rats treated with the irreversible GABA-T inhibitor GVG. The possibility that treatment of the animals with GVG fundamentally alters the GABA release process seems unlikely because baclofen had similar effects on the K-evoked release of GABA from cortical slices prepared from saline-injected animals.

The effect of baclofen on the K-evoked release of GABA is consistent with the idea that GABAergic nerve endings in the cortex and spinal cord possess inhibitory $GABA_B$-autoreceptors. This suggestion is strengthened, at least in the spinal cord, by the finding that the inhibitory action of baclofen on GABA release was completely abolished by the specific $GABA_B$-receptor antagonist, phaclofen (Kerr *et al.*, 1987; Dutar & Nicoll, 1988). Under 'normal' conditions, phaclofen did not affect the spontaneous resting release of GABA from cortical slices, but when the resting release was increased by treatment of the animals with GVG, phaclofen caused a striking further increase in both the resting and K-evoked release of GABA. This implies that in the cerebral cortex under conditions where the relase of GABA is high, there is considerable feedback inhibition mediated via the presumed $GABA_B$-autoreceptors. This result may have implications for the use of GVG as an antiepileptic drug, because an additional anticonvulsant action might be obtained by the concurrent administration of GVG with a $GABA_B$-receptor antagonist. However, for reasons which are unknown, phaclofen did not increase the resting release of GABA from spinal slices taken from GVG-treated rats, suggesting that a combination of these drugs may have additive effects only in certain brain regions.

In the present experiments, we cannot rule out the possibility that baclofen is acting on a feedback loop. However, further support for the suggestion that baclofen acts at $GABA_B$-receptors located on GABAergic nerve terminals has recently been provided by Bonanno *et al.* (1988). They showed that the K-evoked release of [^{3}H]-GABA from rat cortical synaptosomes was inhibited by (−)-baclofen. This effect was antagonised by phaclofen but the conditions of their experiments were such that an effect on the spontaneous resting release of GABA was unlikely to be observed.

There have been several previous studies in which the effects of GABA agonists and antagonists on [^{3}H]-GABA release from brain slices or synaptosomes have been examined. Some demonstrated that muscimol inhibited [^{3}H]-GABA release, suggesting the presence of inhibitory $GABA_A$- autoreceptors (Mitchell & Martin, 1978; Brennan *et al.*, 1981; Kuriyama *et al.*, 1984). On the other hand, others found that muscimol was ineffective but that (−)-baclofen inhibited [^{3}H]-GABA release, a result consistent with the presence of $GABA_B$-autoreceptors (Anderson & Mitchell, 1985; Limberger *et al.*, 1986; Pittaluga *et al.*, 1987). We have found no evidence for the presence of inhibitory $GABA_A$-autoreceptors in rat cerebral cortex or spinal cord. Neither muscimol nor δ-aminolaevulinic acid, at concentrations of 10 μM, affected the release of endogeneous GABA. It might be argued that this concentration was too low, but 10 μM was the apparent IC_{50} for the inhibitory action of δ-aminolaevulinic acid on the K-evoked release of [^{3}H]-GABA from rat cortical synaptosomes (Brennan & Cantrill, 1979).

It is not clear why apparently similar experiments produce such different results. It may be that the use of [^{3}H]-GABA, often in conjunction with aminooxyacetic acid, resulted in the study of GABA release from inappropriate pools. It was for this reason that we chose to measure endogeneous GABA release. Perhaps lower concentrations of K would have revealed an inhibitory action of muscimol. However, this seems unlikely as our results agree closely with those of Waldmeier *et al.* (1988), who found that baclofen, but not muscimol, decreased the

electrically-evoked release of endogeneous GABA from slices of rat cerebral cortex, striatum and hippocampus. The suggestion that GABA autoreceptors are of the $GABA_B$-subtype has recently been greatly strengthened by electrophysiological studies, in which GABA-mediated monosynaptic inhibitory postsynaptic currents at synapses between embryonic rat hippocampal neurones in culture were measured. It was found that (−)-baclofen reduced inhibitory postsynaptic current amplitude but did not increase postsynaptic membrane conductance or block the postsynaptic response to GABA (Harrison, *et al.*, 1988).

There now seems to be a considerable amount of evidence from *in vitro* experiments that baclofen can decrease GABA release by an action on inhibitory $GABA_B$-autoreceptors. This has obvious implications for the mechanism of the antispastic action of baclofen, since it is difficult to see how a decrease in GABA release would produce this effect. It has been shown previously that baclofen can reduce the release of glutamate and/or aspartate from a variety of CNS preparations (Potashner, 1979; Johnson *et al.*, 1980; Collins *et al.*, 1982; Kato *et al.*, 1982; Zhu & Chuang, 1987) and in the present study we have confirmed that the K-evoked release of these excitatory amino acid transmitters from cortical slices is inhibited by baclofen. In addition, we also found that the K-evoked release of glycine and taurine was reduced. We have not examined the effects of baclofen on glutamate/aspartate release from other areas of the CNS, but it seems that the drug may have widespread inhibitory actions on the release of both the main excitatory and inhibitory transmitters in the brain. Presumably, the antispastic effect of the drug occurs because the decrease in excitatory transmitter (glutamate/aspartate) release outweighs the decrease in inhibitory transmitter (GABA/glycine) release. We are at present trying to test this idea by examining the effects of baclofen on central amino acid release by use of *in vivo* dialysis.

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Restricted usefulness of tetraethylammonium and 4-aminopyridine for the characterization of receptor-operated K^+-channels

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1 Recently, we suggested that the D_2-dopamine receptor involved in the inhibition of evoked [^{3}H]-acetylcholine release from rat striatum is coupled to K^+-channels.

2 In the present study, an attempt was made to elucidate further the role of these K^+-channels, using the K^+-channel blocking agents tetraethylammonium and 4-aminopyridine. With a superfusion method, the effects of both drugs on the D_2-dopamine receptor-mediated inhibition of the electrically evoked release of [^{3}H]-acetylcholine from rat striatal tissue slices was investigated.

3 Both tetraethylammonium (30 mM) and 4-aminopyridine (0.1 mM) significantly stimulated the electrically evoked release of [^{3}H]-acetylcholine and completely abolished the effect of the selective D_2-receptor agonist LY 171555 (1 μM) on evoked acetylcholine release. In addition, tetraethylammonium (0.03–30 mM) and 4-aminopyridine (0.003–1 mM) strongly increased the basal (non-evoked) release of radioactivity in a concentration-dependent manner. The results suggest that the effect of the drugs on the basal release of radioactivity and on the electrically evoked release of acetylcholine cannot exclusively be explained by their action on K^+-channels.

4 Furthermore, with the use of a receptor binding assay, data were obtained on the affinity of tetraethylammonium and 4-aminopyridine for D_2-receptors and various other neurotransmitter recognition sites. At concentrations in which both drugs are known to block K^+-channels, they were found to inhibit the specific binding of selective radioligands to their respective recognition sites.

5 It is concluded that due to their 'side-effects', both tetraethylammonium and 4-aminopyridine are of only limited value in the investigation of the alleged interaction between neurotransmitter receptors and K^+-channels.

Introduction

It is generally accepted that the release of acetylcholine (ACh) from striatal cholinergic interneurones is decreased by stimulation of D_2-dopamine receptors (for review, see Stoof & Kebabian, 1984). In a recent study, we have suggested that the D_2-receptor exerts its inhibitory control of depolarization (K^+)-induced [^{3}H]-ACh release from rat striatal tissue slices primarily via two mechanisms: (1) stimulation of potassium ion efflux by opening of K^+-channels and (2) inhibition of Ca^{2+} mobilization from intracellular storage sites (Drukarch *et al.*, 1989). In this study no attempt was made to characterize further the K^+-channels involved. Therefore, in the present study we investigated the effect of blockade of K^+-channels on the D_2-receptor-mediated inhibition of depolarization-induced [^{3}H]-ACh release from rat striatal tissue slices. For this purpose the well known K^+-channel blockers tetraethylammonium chloride (TEA) and 4-aminopyridine (4-AP) were used, which are usually supposed to block different types of K^+ conductances (for recent review, see Rudy, 1988). In electrophysiological studies, TEA and 4-AP have been shown to be effective as K^+-channel blockers only in high (mM range) concentrations (Rudy, 1988). In this range, however, TEA and 4-AP might interfere with ligand binding to various receptor recognition sites, thereby limiting their usefulness in investigating the alleged coupling of D_2-dopamine

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receptors to K^+-channels. Therefore, in addition, data are presented on the affinity of TEA and 4-AP for D_2-receptors and various other neurotransmitter receptor binding sites.

Methods

Release studies

Male Wistar rats (220–260 g) were killed by decapitation. The brains were removed and the striatum was dissected. The dissected tissue pieces were minced by passing them twice through a McIlwain tissue chopper (micrometer setting 300 μm). The tissue fragments obtained by this procedure were washed and incubated with 5 μCi [^{3}H]-choline in 2 ml of a Krebs-Ringer bicarbonate medium containing (in mM): NaCl 121, KCl 1.87, KH_2PO_4 1.17, $MgSO_4$ 1.17, $CaCl_2$ 1.22, $NaHCO_3$ 25 and D(+)-glucose 10. The medium was kept under a constant atmosphere of 95% O_2 and 5% CO_2. Following a 15 min incubation period the slices were washed and aliquots (approximately 5 mg of tissue) were transferred to a superfusion apparatus. Four to six chambers were used for each experimental condition. The chamber volume was 0.2 ml and the superfusion rate was 0.25 ml min^{-1}. After a 45 min presuperfusion period, four 15 min fractions were collected. Release of [^{3}H]-ACh was evoked electrically by exposing the tissue for 5 min to biphasic squarewave pulses (1 Hz, 24 mA, 4 ms) starting at the beginning of the collection of the third fraction (t = 75–80 min). Drugs were added to the medium 15 min before stimulation i.e. at the start of the collection of fraction 2 (t = 60 min). At the end of the experiments the remaining radioactivity was extracted from the tissue with 0.1 N HCl. For additional information concerning experimental conditions and procedures see Plantje *et al.* (1987). The amount of radioactivity (d.p.m.) in the collected fractions and tissue extracts was estimated by liquid scintillation counting (Kontron MR 300 automatic liquid scintillation system) in 12 ml of a scintillation cocktail (Packard Optifluor). In order to correct for differences in the amount of tissue present in each of the superfusion chambers the d.p.m. values were expressed as fractional rates (see Stoof & Kebabian, 1982). The effect of TEA and 4-AP on the basal (non-evoked) release of radioactivity was estimated, by dividing the amount of radioactivity obtained in the second fraction by the amount of radioactivity obtained in the first fraction. Subsequently, this ratio was expressed as percentage of control (control = ratio fraction 2/fraction 1 in the absence of drugs). The amount of radioactivity released only as a consequence of electrical stimulation was calculated by subtracting the mean of fractions 2 and 4 from fraction three and expressed as percentage of total tissue tritium content.

Radioligand receptor binding studies

Female Wistar rats (150 g) were killed by decapitation, the brains were removed and various brain regions (striatum, forebrain, total or frontal cortex or hippocampus) were dissected. Membrane fractions were prepared as previously described (Leysen *et al.*, 1986). The various receptors were labelled with the following radioligands: D_1-dopamine receptors in striatum: [^{3}H]-SCH 23390; D_2-dopamine receptors in striatum: [^{3}H]-haloperidol; muscarinic cholinoceptors in striatum: [^{3}H]-dexetimide; α_1-adrenoceptors in the forebrain: [^{3}H]-WB4101; α_2-adrenoceptors in the cortex: [^{3}H]-clonidine; β-adrenoceptors in the cortex: [^{3}H]-dihydro-alprenolol; 5-hydroxytryptamine$_{1A}$ (5-HT_{1A})-receptors in the hippocampus: [^{3}H]-8-OH-N,N-dipropyl-amino tetraline (8-OHDPAT); 5-HT_2-receptors in frontal cortex: [^{3}H]-ketanserin. For further details of the assay conditions see Leysen *et al.* (1988). Radioligand binding was estimated in the absence and presence of different concentrations of TEA or 4-AP. The data were corrected for non-specific binding and expressed as percentage of specific binding. The non-specific binding was determined in the presence of: piflutixol (1 μM, [^{3}H]-SCH 23390); (+)-butaclamol (2 μM, [^{3}H]-haloperidol); dexetimide (2 μM, [^{3}H]-dexetimide); noradrenaline (100 μM, [^{3}H]-WB 4101); noradrenaline (2 μM, [^{3}H]-clonidine); alprenolol (10 μM, [^{3}H]-dihydro-alprenolol); spiroxatrine (1 μM, [^{3}H]-8-OH-N,N-dipropyl-amino tetraline) and methysergide (1 μM, [^{3}H]-ketanserin).

Drugs and radioactive compounds

The radioactive compounds (specific activity: 10–85 Ci mmol^{-1}) were purchased from the following companies: [^{3}H]-ketanserin and [^{3}H]-haloperidol from Janssen Life Sciences Products; [^{3}H]-WB 4101 (N-[2-(2, 6-dimethoxyphenoxy)ethyl]-2, 3-dihydro-1, 4-benzodioxin-2-methanamine), [^{3}H]-dihydro-alprenolol, [^{3}H]-8-OH-N,N-dipropyl-amino tetraline, [^{3}H]-clonidine and [^{3}H]-dexetimide from New England Nuclear; [^{3}H]-SCH 23390 (7-chloro-2, 3, 4, 5-tetrahydro-3-methyl-5-phenyl-1H-3-benzazepine-7-ol) and [^{3}H]-choline from the Radiochemical Centre (Amersham). Tetraethylammonium chloride and 4-aminopyridine were obtained from Sigma and J.T. Baker respectively. LY 171555 (4, 4A, 5, 6, 7, 8, 8a, 9-octahydro-5-n-propyl-2H-pyrazolo-3,4-g-quinoline) was a generous gift from Eli Lilly. Additional unlabelled drugs were provided by the companies of origin.

Statistical analysis

Statistical analysis was performed by means of Student's two-tailed t test.

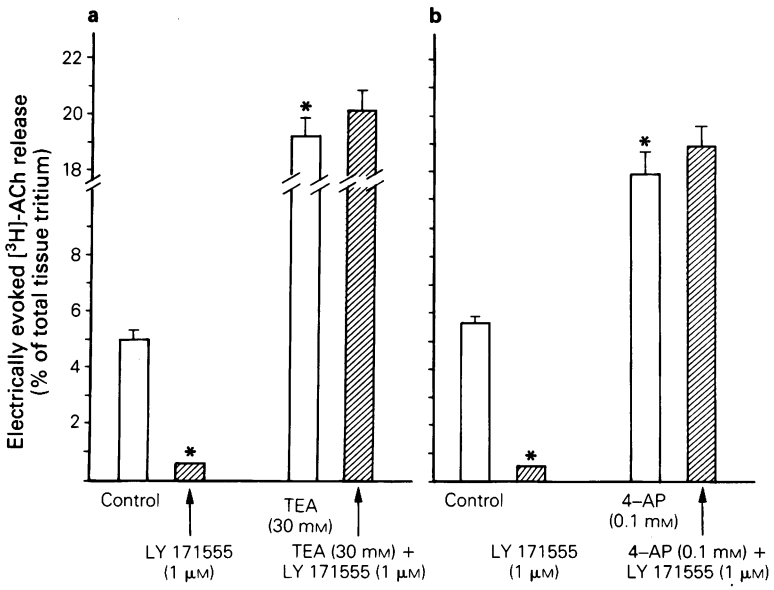


Figure 1 The effect of (a) tetraethylammonium (TEA) and (b) 4-aminopyridine (4-AP) on the D_2-dopamine receptor-mediated inhibition of electrically evoked [^{3}H]-acetylcholine ([^{3}H]-ACh) release from rat striatal tissue slices. Rat striatal tissue slices were incubated with [^{3}H]-choline and transferred to a superfusion apparatus. After a 45 min pre-superfusion period four 15 min fractions were collected. The release of [^{3}H]-ACh was stimulated electrically with biphasic squarewave pulses for 5 min, starting at the beginning of the collection of the third fraction. Drugs were added to the medium 15 min before the stimulation. The values represent the mean (bars show s.e.mean values, if large enough to be depicted) obtained from two experiments. $n = 8$ for experiments in which TEA was used; $n = 12$ for experiments in which 4-AP was used. *$P < 0.001$ vs respective control.

Results

Release studies

In contrast to our previous study (Drukarch *et al.*, 1989), electrical stimulation was used to evoke [^{3}H]-ACh release, since stimulation with a high extracellular K$^+$ concentration would interfere with the effect of blockade of K$^+$-channels by TEA and 4-AP, The selective D_2-receptor agonist LY 171555 (1 μM) inhibited the electrically evoked release of [^{3}H]-ACh from striatal tissue by approximately 90% (Figure 1). The addition of TEA (30 mM) or 4-AP (0.1 mM) to the medium led to a statistically significant stimulation of the electrically evoked release of [^{3}H]-ACh and completely abolished the effect of LY 171555 (1 μM) on evoked [^{3}H]-ACh release (Figure 1). Furthermore, under identical experimental conditions both TEA (0.03–30 mM) and 4-AP (0.003–1 mM) strongly enhanced the basal (non-evoked) release of radioactivity from the tissue slices in a concentration-dependent manner (Figure 2).

Radioligand receptor binding studies

In a concentration range of 0.01–10 mM both TEA and 4-AP inhibited the specific binding of the muscarinic receptor ligand [^{3}H]-dexetimide in a concentration-dependent manner, with an IC_{50} value of approximately 1 mM (Figure 3). In Table 1 it is shown that TEA at a concentration of 10 mM strongly inhibited the specific binding of both the selective α_1- and α_2-adrenoceptor radioligands. Furthermore, TEA at a concentration of 10 mM slightly interfered with the binding of the 5-HT_{1A}-, 5-HT_2- and D_2-dopamine receptor ligands (Table 1). At this concentration TEA did not interfere with binding of either the D_1-dopamine or β-adrenoceptor radioligands in the brain regions investigated (Table 1). Concentrations of TEA higher than 10 mM interfered with the assay and were therefore not used for receptor binding experiments. At a concentration of 0.3 mM 4-AP appeared to inhibit binding of the α_1- and α_2-adrenoceptor ligands (Table 1) and at 3 mM 4-AP considerably displaced selective ligands of 5-HT_{1A}- and 5-HT_2-receptors (Table 1). Also, 3 mM 4-AP showed some interaction with D_1-, D_2- and β-adrenoceptor ligand binding (Table 1).

Discussion

Recently, we have shown that high extracellular K$^+$ concentrations antagonise the D_2-dopamine

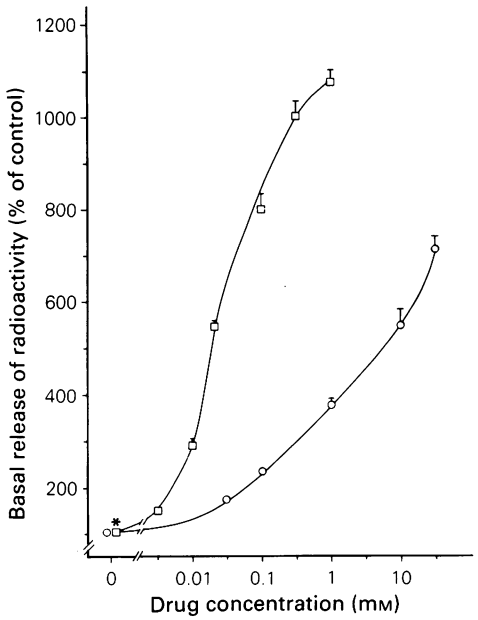


Figure 2 Enhancement of the basal release of radioactivity from striatal tissue slices incubated with [^{3}H]-choline by tetraethylammonium (TEA, ○) and 4-aminopyridine (4-AP, □). Rat striatal tissue slices were incubated in the presence of [^{3}H]-choline and transferred to a superfusion apparatus. After a 45 min pre-superfusion period four 15 min fractions were collected. Drugs were present from the start of the collection of the second fraction. The effect of TEA and 4-AP on the basal release of radioactivity was calculated by dividing the amount of radioactivity obtained in the second fraction by the amount of radioactivity obtained in the first fraction. This ratio was then expressed as percentage of control (control = ratio fraction 2/fraction 1 in the absence of drugs). The values represent the means ($n = 8$; $*n = 16$) obtained from two to four experiments; s.e.mean values are depicted by vertical lines only when they exceeded the size of the symbol. The ratio fraction 2/fraction 1 in the absence of drugs was 0.99 ± 0.03 (TEA group) and 0.87 ± 0.01 (4-AP group), respectively.

receptor-mediated inhibition of K^+-evoked [^{3}H]-ACh release from rat striatal tissue slices (Drukarch *et al.*, 1989). From this finding it was concluded that activation of this D_2-receptor stimulates (directly or indirectly) the opening of K^+-channels. Other investigators, using different methods, have also demonstrated the involvement of K^+-channels in signal transduction via D_2-dopamine receptors (Israel *et al.*, 1985; 1987; Ingram *et al.*, 1986; Memo *et al.*, 1987; Lacey *et al.*, 1987; 1988; Freedman & Weight, 1988). The present study was intended to obtain more direct evidence for the alleged association between inhibition of evoked ACh release from the striatum, by stimulation of the D_2-receptor, and opening of K^+-channels. The duration of an action potential is limited by outward directed K^+-currents. Both TEA and 4-AP are known to block outward K^+-currents and are therefore expected to increase the duration of action potentials. The influx of Ca^{2+} during an action potential is thought to be essential for the depolarization-evoked release of neurotransmitters from nerve terminals (Mulder, 1982). Thus, inhibition of K^+-currents by blockade of K^+-channels is assumed to facilitate evoked transmitter release, by allowing an increased entry of Ca^{2+} into the neurones to occur (for review, see Illes, 1986). Our data show that both TEA and 4-AP significantly enhanced the electrically-evoked release of [^{3}H]-ACh from rat striatum. More importantly, both drugs were able to antagonise completely the D_2-receptor-mediated inhibition of electrically-evoked ACh release induced by a supramaximal concentration of the selective agonist LY 171555. Superficially, these results seem to confirm the suggestion that the D_2-receptor mediates the decrease of evoked [^{3}H]-ACh release by opening of TEA- and 4-AP-sensitive K^+-channels. However, the results of our investigation further show that both drugs strongly enhanced the basal, non-evoked, release of radioactivity from the tissue slices. In our opinion, for several reasons it is highly unlikely that this effect of TEA and 4-AP on basal release can be exclusively accounted for by drug-induced blockade of K^+-channels. In the first place, TEA and 4-AP already stimulated basal release of radioactivity at concentrations shown to be ineffective for the blockade of neuronal K^+-currents in voltage-clamp experiments (Hermann & Gorman, 1981a,b). Moreover, in relatively low concentrations (μM range) 4-AP supposedly facilitates Ca^{2+} entry through opening of voltage-sensitive Ca^{2+}-channels (for review, see Illes, 1986). Furthermore, similar release experiments, using cortical tissue slices incubated in the presence of [^{3}H]-noradrenaline, did not show an effect of 4-AP on basal release of radioactivity in concentrations up to 0.1 mM (Schoffelmeer & Mulder, 1983). Only at higher concentrations did an increase occur. Recently, this has been confirmed in mouse vas deferens (Zimanyi *et al.*, 1988). To our knowledge, with respect to TEA, there have been no studies showing an enhancement of basal transmitter release by this drug. Therefore, it seems reasonable to conclude that various mechanisms are involved in the TEA- and 4-AP-induced stimulation of basal release of radioactivity from striatal tissue slices incubated with [^{3}H]-choline. Although the exact nature of these mechanisms remains to be elucidated, they, at least to some extent, appear to be specific for cholinergic neurones or to occur at lower drug concentra-

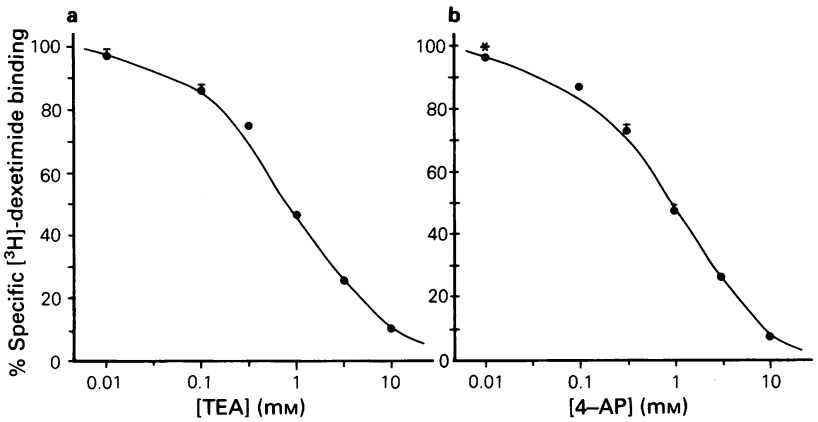


Figure 3 Concentration-dependent inhibition of the specific binding of [^{3}H]-dexetimide to rat striatal muscarinic receptor binding sites by (a) tetraethylammonium (TEA) and (b) 4-aminopyridine (4-AP). The values represent the mean and vertical lines show s.e.mean (if large enough to be depicted); $n = 4$, $*n = 2$.

tions in these neurones than in other types of neurone. Because these mechanisms can also be expected to contribute to the effects of TEA and 4-AP on the electrically-evoked release of ACh, both drugs are of limited use in providing specific evidence for the association between the D_2-receptor-mediated inhibition of evoked ACh release and the opening of K^+-channels.

In general, 4-AP and TEA have been shown to be active in blocking K^+-currents only at mM concentrations (for review, see Rudy, 1988). The results of our receptor binding experiments show that in this particular concentration range 4-AP and TEA almost completely inhibited the specific binding of the muscarinic receptor ligand [^{3}H]-dexetimide. If both agents act as antagonists on muscarinic autoreceptors, they would be expected to produce an increase in the release of ACh. However, under similar experimental conditions, the muscarinic receptor antagonist atropine only induced a marginal increase (approximately 15%) of the release (unpublished data). Although muscarinic receptor blocking effects of TEA and 4-AP might thus contribute to this increase, the dramatic effects observed seem to be mainly due to (an)other, at present unknown, mechanism(s) of action. Despite the observation that the effects of TEA, in concentrations up to 10 mM, seemed to be less marked than the effects of 4-AP on the binding of selective radioligands to various other receptor types, we would like to stress that in many electrophysiological studies TEA is used in concentrations much higher than 10 mM. The present findings indicate that TEA and 4-AP, at concentrations in which they block K^+-channels, also show considerable affinity for various neurotransmitter receptor binding sites. This again illustrates that caution seems warranted in interpreting the effects of TEA and 4-AP solely in terms of K^+-channel blockade. For instance, recently it has been shown that 4-AP, in a concentration of approximately 0.5 mM,

Table 1 The effect of tetraethylammonium (TEA) and 4-aminopyridine (4-AP) on the specific binding of selective radioligands to various neurotransmitter receptor recognition sites

		% *of specific binding*			
[^{3}*H*]-*ligand* (*region*)	*Control*	*TEA* (*1 mM*)	*TEA* (*10 mM*)	*4-AP* (*0.3 mM*)	*4-AP* (*3 mM*)
WB 4101 (Forebrain)	100 ± 0.4	92.9 ± 0.8*	54.4 ± 0.5**	56.6 ± 2.4**	6.6 ± 2.2**
Clonidine (Cortex)	100 ± 0.9	94.5 ± 5.5	59.8 ± 1.7**	36.9 ± 1.2**	4.6 ± 1.2**
Dihydro-alprenolol (Cortex)	100 ± 0.5	109.9 ± 6.9	93.8 ± 1.7	101.7 ± 3.4	76.4 ± 2.1**
SCH 23390 (Striatum)	100 ± 0.7	102.9 ± 1.3	97.0 ± 0.4	98.6 ± 0.6	90.7 ± 3.1
Haloperidol (Striatum)	100 ± 0.5	96.0 ± 7.1	86.9 ± 5.5	93.1 ± 5.3	72.2 ± 5.6*
8-OHDPAT (Hippocampus)	100 ± 0.5	98.6 ± 6.5	71.3 ± 6.3	90.5 ± 2.0	52.6 ± 2.5**
Ketanserin (Frontal cortex)	100 ± 0.4	95.9 ± 1.8	85.4 ± 2.5*	85.4 ± 2.7*	62.4 ± 1.7**

The values represent the mean ± s.e.mean ($n = 3$–4).
*$P < 0.01$, **$P < 0.001$ vs control.

not only inhibited the effect of α_2-adrenoceptor stimulants on evoked [^{3}H]-noradrenaline release from mouse vas deferens, but also completely prevented the action of a selective antagonist (Zimanyi *et al.*, 1988). On consideration of our results, these findings indicate that, besides blocking K^+-channels, 4-AP also interferes with the binding of ligands (agonist and antagonist) to α_2-adrenoceptors.

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Comparative effects of bepridil, its quaternary derivative CERM 11888 and verapamil on caffeine-induced contracture in ferret hearts

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1 The effects of bepridil, its quaternary derivative: CERM 11888 (methyl-pyrrolidinium bromide) (10^{-7}–10^{-5} M), and verapamil (10^{-7}–10^{-6} M) were compared on caffeine-induced contracture of isolated ventricular trabeculae of the ferret.

2 Bepridil diminished the amplitude of contracture in a concentration-dependent fashion, and this effect was significantly different from that of CERM 11888 which, like verapamil, only reduced the amplitude at the highest concentration used.

3 Bepridil (10^{-6} M) significantly shortened the time to peak tension and accelerated the relaxation phase of contracture. This latter effect was different from that of CERM 11888. Verapamil (10^{-6} M) also tended to accelerate the relaxation phase. At 10^{-5} M these actions of bepridil on the time to peak and relaxation tended to reverse.

4 At all concentrations bepridil and verapamil reduced the rate of repriming of contracture and this effect of bedpridil was significantly different from that of its quaternary derivative which only showed a significant effect at 10^{-5} M.

5 These results demonstrate a clear intracellular effect of bepridil in the ferret heart. Verapamil and CERM 11888 had only weak intracellular effects even at high concentrations.

6 Analysis of the results suggests that the main sites of action of bepridil in this model are the sarcoplasmic reticulum and one or two calcium compartments in the sarcolemma.

Introduction

The antianginal properties of bepridil seem to be due mainly to its inhibitory action on the slow inward calcium current, whereas an additional effect on the fast sodium current may be involved in the anti-arrhythmic properties of this compound (Marshall *et al.*, 1984; Alpert *et al.*, 1985). The voltage-clamp technique (Labrid *et al.*, 1979; Sassine *et al.*, 1984; Yatani *et al.*, 1986) has demonstrated the original electrophysiological profile of bepridil compared to that of first-generation calcium antagonists (Leboeuf *et al.*, 1984; Singh *et al.*, 1985a,b). Bepridil enters cells rapidly (Mras & Sperelakis, 1982; Cramb & Dow, 1983; Pang & Sperelakis, 1983; 1984) and this together with the fact that bepridil decreases the contraction force of isolated heart muscle at concentrations lower than those which inhibit the slow calcium current (i.e. pharmacomechanical uncoupling) suggests that it has intracellular sites of action (Vogel *et al.*, 1979; Brown *et al.*, 1985). Other studies have shown that therapeutic concentrations of bepridil have effects on both intracellular organelles and on calmodulin (Younes *et al.*, 1981; Younes & Schneider, 1984; Itoh *et al.*, 1984; 1986; Lynch *et al.*, 1985; Stoclet *et al.*, 1985). The quaternary derivative of bepridil (CERM 11888), although having effects similar to bepridil on the slow inward calcium current and contractile responses dependent upon extracellular calcium, has little effect on phenomena that are dependent upon intracellular calcium ions. These results suggest that unlike bepridil, the pharmacological effects of CERM 11888 are due to effects of the drug uniquely at the cell membrane level (Schaeffer *et al.*, 1986; Wahler *et al.*, 1986). The reference calcium antagonist, verapamil, is also known mainly for its inhibitory action on calcium movements at the level of the cell membrane but

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may also, to a much lesser extent than bepridil, enter the cell (Pang & Sperelakis, 1983; 1984).

The application of caffeine to isolated thin cardiac trabeculae, in the absence of stimulation, induces a phasic contracture which is independent of the slow inward calcium current (Chapman & Leoty, 1976; Konishi *et al.*, 1984; Chapman & Tunstall, 1988). Since the observed contracture of the myocardium results mainly from a movement of intracellular calcium, we thought it of interest to study the effects of verapamil and bepridil, at therapeutically relevant concentrations, on the contractile response to caffeine in order to elucidate any intracellular actions of the drugs. By measuring the effects of the quaternary derivative we were able to identify and rule out any possible influence of bepridil on background calcium conductance. The effects of compounds on intracellular movements of calcium have been discussed in terms of the model proposed by Chapman & Leoty (1976) and Chapman & Tunstall (1988). Results clearly show an intracellular action of bepridil upon intact ferret ventricular fibers.

Part of this work was presented at the Conference 'Biologie et Pathologie du coeur et des vaisseaux' at Vittel, 1987.

Methods

Ferrets weighing 0.7 to 1 kg were anaesthetized with a mixture of air and ether. Immediately after thoracotomy, the heart was removed and placed in a dissection tray containing a physiological solution of the following composition (in mM): NaCl 140, KCl 6, $MgCl_2$ 1, $CaCl_2$ 3, and Tris-HCl 8. Tris aminomethane ($\simeq$1 mM) was added to obtain a pH of 7.35 $\pm$ 0.05. Tris buffer was preferred to bicarbonate since it allowed the use of high divalent cation concentrations. The right ventricle was opened, and a free-running trabecula (50 to 250 μm in diameter and 1 to 4 mm in length) was removed and placed in a small perfusion chamber (2 × 1.5 × 1.5 mm), according to the method described by Chapman & Leoty (1976). Trabeculae with a larger diameter or small papillary muscles were not used because in these types of preparation caffeine induces either no contracture or contracture with very weak amplitude and no spontaneous relaxation. One end of the preparation was attached to a U-shaped platinum wire (100 μm in diameter), and the other end was snared by a platinum wire loop (30 μm in diameter) and connected to a small copper plate the position of which was estimated by means of a proximity detector (Kaman; KD 2300, 0.5 SU). The fibre was perfused continuously (20 ml min^{-1}) at ambient temperature (20–24°C). This temperature was selected since Chapman & Leoty (1976) showed that caffeine contractures are attenuated and of a duration of less than 1 s at temperatures higher than 30°C. A two-way perfusion switch permitted rapid transition from the physiological solution to the solution containing caffeine. At the end of the experiment, the fibre was weighed so that the force generated by the muscle could be expressed in terms of wet weight.

The contracture elicited by rapid perfusion with caffeine was transitory and the fibre relaxed completely even when caffeine perfusion was prolonged. After the muscle was placed in a caffeine-free medium and subsequently challenged again with caffeine, the contracture amplitude increased until the control value was attained. The greater the interval between the two caffeine exposures, the greater was the amplitude evoked (Figure 3).

In the control medium, several contractures were induced at 10-min intervals to check the stability of the response to caffeine. The repriming phenomenon was then studied. A test compound was continuously perfused and after 30 min a contracture was induced, which, in turn, was followed by measurement of repriming of the contracture. Finally, either the control medium or a higher concentration of a test compound was perfused. Signals were recorded on a Gould Recorder (BS 272). The parameters measured included amplitude of the caffeine-induced contracture, the time required for maximum contracture, the time constant of spontaneous relaxation, and the time constant of repriming of the contracture.

Drugs

Caffeine (Merck) was dissolved in the physiological solution to obtain a concentration of 4×10^{-2} M. Bepridil and CERM 11888 (RL-CERM) were dissolved in 10 ml of ethanol at a concentration of 10^{-2} M whilst verapamil (Biosedra), was dissolved at 10^{-2} M in 10 ml of distilled water. All solutions were then diluted in the physiological solution to obtain the desired concentration.

Statistical analysis

Student's *t* test for paired series was used to determine the significance of the effects of the various test compounds. Differences between the effects of similar concentrations of bepridil and CERM 11888 were analyzed using Student's *t* test for independent series.

Results

As shown in Figure 1, inclusion of caffeine (10^{-2} M) in the fluid perfusing isolated ferret heart trabeculae induced a contracture which relaxed spontaneously despite the continued presence of caffeine. The force

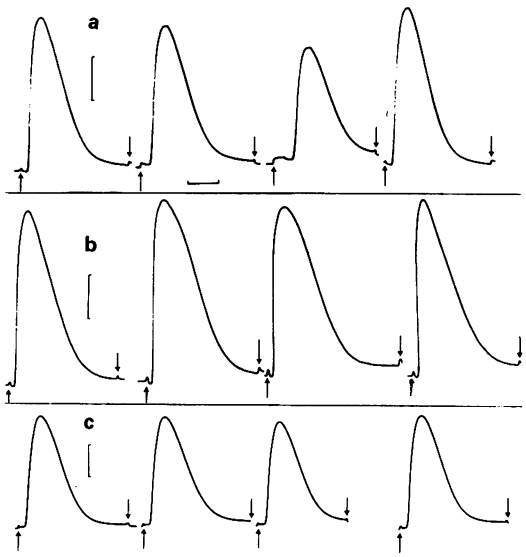


Figure 1 Effects of (a) bepridil, (b) CERM 11888, and (c) verapamil at 10^{-6} M on contractures induced by caffeine (10^{-2} M) in isolated ventricular fibres of the ferret heart: ↑ arrow indicates the start of caffeine perfusion and ↓ arrow indicates the end of caffeine perfusion. Responses from left to right: 1st response: caffeine (10^{-2} M) control response; 2nd response: caffeine (10^{-2} M) response after 30 min perfusion with antagonist; 3rd response: caffeine (10^{-2} M) response after 60 min perfusion with antagonist; 4th response: caffeine (10^{-2} M) response 30 min after removal of antagonist. Ordinates: contractile force developed. Scale: kN kg^{-1} Abscissae: time scale = 5 s (similar for a, b and c)

of contracture developed was dependent on the concentration of caffeine applied ($K_m = 3.71 \pm 0.4 \times 10^{-3}$ M). In these experiments, for the study of antagonist drugs, a concentration of 10^{-2} M caffeine was used since this induced submaximal contracture and facilitated determination of the repriming time constant (see Chapman & Leoty, 1976, for additional information).

Effects of drugs on the amplitude of the caffeine-induced contracture

Bepridil (10^{-6} M) reduced the amplitude of the caffeine contracture in a time-dependent manner, from control values of 3.5 kN kg^{-1} to 3.14 and 2.5 kN kg^{-1} following 30 and 60 min perfusion respectively with bepridil (Figure 1a). Similar experiments with CERM 11888 and verapamil showed that both compounds had little or no effect on the amplitude of caffeine contracture (Figure 1b and 1c). When expressed in percentage terms, results showed that CERM 11888 (10^{-5} M) and especially verapamil (10^{-6} M) did elicit small but significant reductions in the amplitude of the caffeine contracture. These reductions were however markedly inferior to those induced by bepridil (see Figure 2a).

Effects of drugs on the time to peak of the caffeine-induced contracture

Bepridil at 10^{-6} M significantly reduced the time to peak contracture but this effect was not concentration-dependent. Neither CERM 11888 nor verapamil had significant effects upon this parameter (Figure 2b).

Effects of drugs on the spontaneous relaxation of the caffeine-induced contracture

After 30 min perfusion with bepridil at the concentration of 10^{-6} M, the time constant of spontaneous

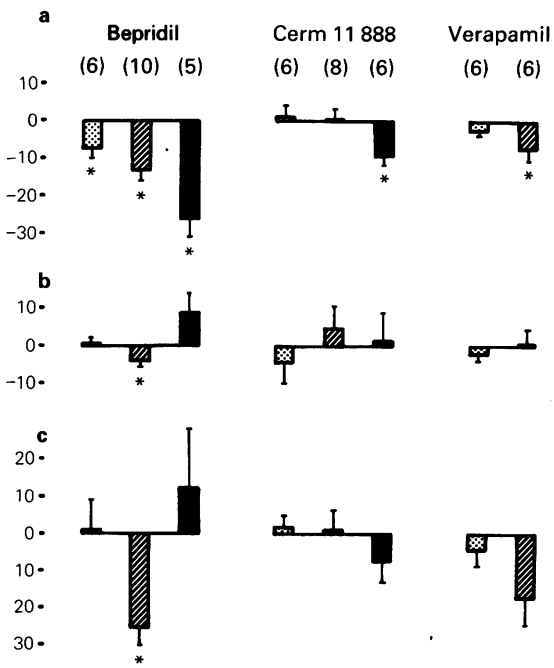


Figure 2 Changes in the contracture-induced by caffeine (10^{-2} M) following 30 min exposure of ferret heart trabeculae to bepridil, CERM 11888 and verapamil. Ordinates: Change in response, expressed as percentage change from control values (%Δ). (a) Amplitude of contracture; (b) time to peak contracture and (c) time constant of spontaneous relaxation. Stippled columns 10^{-7} M; hatched columns 10^{-6} M and solid columns 10^{-5} M of respective drugs. $^*P \leq 0.05$, Student's t test. The numbers in parentheses indicate the number of preparations obtained from different animals.

relaxation was significantly decreased from 2.63 ± 0.19 s (control) to 2.53 ± 0.16 s ($P < 0.05$, Figure 2c). This effect was significantly different from that induced by CERM 11888 which did not significantly modify this parameter even at the highest concentration studied. The effects of bepridil on the spontaneous relaxation were not however concentration-dependent (Figure 2c). Verapamil (10^{-6} M) also decreased the time constant of spontaneous relaxation from 5.42 ± 0.97 s to 4.18 ± 0.53 s; this change did not however, reach statistical significance ($P = 0.09$, Figure 2c).

Effects of drugs on the repriming phenomenon of the caffeine-induced contracture

The repriming phenomenon was markedly retarded by bepridil over the concentration-range 10^{-7}–10^{-5} M (Figure 3). Up to 10^{-6} M the effects of bepridil were concentration-dependent but increasing the concentration further to 10^{-5} M did not augment the effect (Figures 3 and 4). CERM 11888 (10^{-7}–10^{-5} M) had significantly less effect than bepridil upon the repriming of this caffeine-sensitive calcium store with small but statistically significant effects occurring only at 10^{-5} M (Figures 3 and 4). Verapamil (10^{-7} and 10^{-6} M) had greater effects on this parameter than did CERM 11888 but again these were much less than the changes induced by bepridil (Figure 3 and 4).

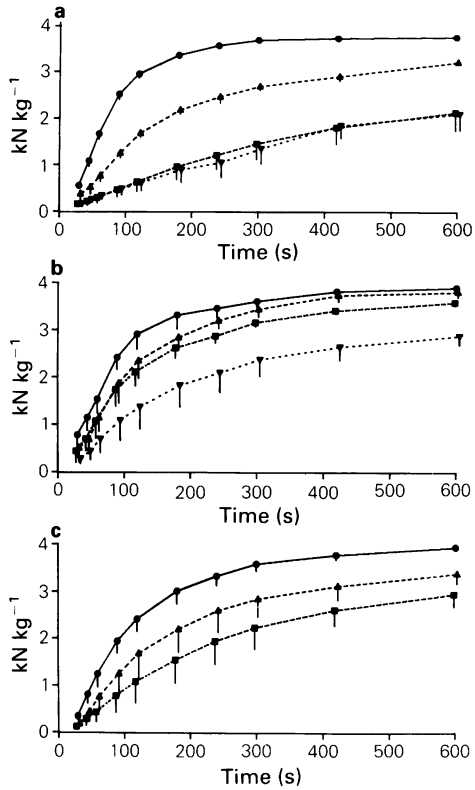


Figure 3 The time course of the effects of bepridil (a), CERM 11888 (b) and verapamil (c) at various concentrations on the repriming of contractures induced by caffeine (10^{-2} M): (●) Control; (▲) 10^{-7} M; (■) 10^{-6} M; (▼) 10^{-5} M. Ordinates: amplitude of contracture (kN kg^{-1}). Abscissae: interval (s) between two successive perfusions of caffeine. For the purpose of clarity, the values at 10, 15, and 20 s have been omitted from the figure.

Discussion

Under our experimental conditions, rapid perfusion of isolated quiescent trabeculae from the ferret heart with caffeine caused a contracture which relaxed spontaneously. Such findings are in general agreement with the results of Chapman & Leoty (1976) and Konishi *et al.* (1984).

Effects on the amplitude of the caffeine-induced contracture

Chapman & Leoty (1976) have shown that the amplitude of the caffeine-induced contracture, which is independent of the slow inward calcium current, is little affected by large changes in extracellular calcium ion concentration. Calcium release from the sarcoplasmic reticulum (SR) is therefore considered by the overwhelming majority of workers to be responsible for the contracture induced by caffeine (Chapman, 1983; Clusin, 1985; Bers, 1987; Chapman & Tunstall, 1988).

Bepridil induced a concentration- and time-dependent decrease in the amplitude of the caffeine contracture whilst its quaternary derivative CERM 11888 was without significant effects upon this parameter except at high concentrations. Since the quaternary derivative would be expected to penetrate into cells poorly, or at a rate much slower than that of bepridil, these findings implicate an intracellular effect of bepridil in the inhibition of the caffeine response in this preparation. With regard to the potential site(s) of action of bepridil within the cell, Solaro *et al.* (1986) have reported that bepridil significantly increased the submaximal force developed by chemically skinned pig heart trabeculae. Consequently, extrapolating from these results, the bepridil-induced reduction in the amplitude of caffeine contractures would appear not to be due to a direct effect of the drug on the myofibrils. Bepridil has been reported to modulate the sarcolemmal

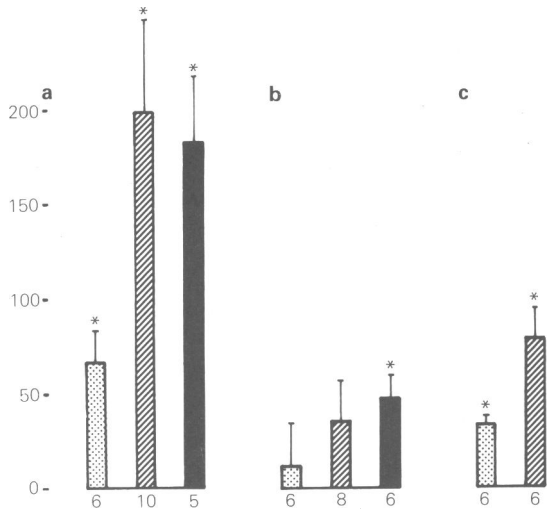


Figure 4 Percentage change in the time constant of repriming of caffeine-induced contracture after 30 min perfusion with bepridil (a), CERM 11888 (b) and verapamil (c). Ordinates: time course of repriming of the caffeine contracture expressed as % of time constant. Stippled columns 10^{-7} M; hatched columns 10^{-6} M; solid columns 10^{-5} M. The numbers beneath the columns indicate the number of preparations tested per concentration of test drug. $^{*}P < 0.05$, Student's t test.

calcium pump, as well as the sarcolemmal Na^{+}–Ca^{2+} exchange process (Lamers *et al.*, 1985; Van Amsterdam & Zaagsma, 1986; Kaczorowski *et al.*, 1988). Bepridil might therefore modulate calcium transport at the inner surface of the cell membrane. However whether such effects are responsible for the decrease in amplitude of caffeine contractures in the presence of bepridil must be questioned since these effects on Ca^{2+} transport are only observed following exposure to very high concentrations ($>10^{-5}$ M) of bepridil. We therefore suggest that most probably a direct inhibitory effect of bepridil on calcium release from the SR is responsible for the inhibitory effects of this compound on the amplitude of caffeine-induced contractures. Such an effect of bepridil could theoretically occur at the SR calcium channel labelled CH2 by Chapman & Tunstall (1988). Such a hypothesis is supported by studies showing that bepridil can compete with ryanodine for SR calcium binding sites (Wolleben & Sieg, 1987).

Bepridil has been shown to penetrate the cell membrane rapidly whilst verapamil, perhaps due to a lower lipophilicity, penetrates only slowly (Cramb & Dow, 1983; Pang & Sperelakis, 1983). The fact that verapamil (10^{-6} M) had relatively small effects upon the amplitude of the caffeine contracture is consistent with the fact that the effects of verapamil are predominantly manifested at the cell membrane level.

Effects on the time to peak of the caffeine-induced contracture

Bepridil (10^{-6} M) slightly but significantly decreased the time to peak contracture but increasing the concentration of bepridil to 10^{-5} M tended to increase rather than further decrease this parameter. This suggests that the time to peak contracture is a complex parameter probably modulated and controlled by several mechanisms. Two such mechanisms might involve a more rapid release of calcium from the SR in the presence of the drug and/or a greater sensitivity of the myofibrils to the released calcium. The latter effect has been reported for bepridil in the pig heart (Solaro *et al.*, 1986) and could explain the shortening in the time to peak tension observed with 10^{-6} M bepridil. It is not clear however why increasing the concentration of bepridil did not lead to increased effects on the time to peak response but it must be remembered that bepridil at high concentrations may also affect other cellular processes. Further studies are required in this respect.

Effects on spontaneous relaxation of the caffeine-induced contracture

As with the time to peak response, the spontaneous relaxation of the caffeine contracture is under the control of several processes and this complicates the interpretation of drug action. Bepridil had biphasic effects upon this parameter too, at 10^{-6} M accelerating, and at 10^{-5} M slowing spontaneous relaxation. The finding that the effects of 10^{-6} M bepridil were different from that of its quarternary derivative, CERM 11888, suggests that effects on external sarcolemmal sites are not responsible for the acceleration of spontaneous relaxation of the caffeine contracture observed in the presence of 10^{-6} M bepridil. It is known that the relaxing system in the ferret heart is heterogeneous (Chapman & Leoty, 1974; 1975; 1976). These authors have postulated that a four-compartment closed model could account for the effects of caffeine in this tissue. Three compartments represent components of the SR with the mitochondria as a side-branch reservoir and the fourth represented the sarcoplasm as a whole. In addition, caffeine also causes an increase in Ca^{2+} efflux (Rasmussen *et al.*, 1987; MacLeod & Bers, 1987), probably via sarcolemmal Na^{+}–Ca^{2+} exchange (see Clusin, 1985 for review). Bepridil has been reported to increase the Ca^{2+}–Mg^{2+}-ATPase activity of the SR and myofibrils (Younes *et al.*, 1981; Solaro *et al.*,

1986; Silver *et al.*, 1986). In addition, Lamers *et al.* (1985) reported that bepridil significantly increases the sarcolemmal calcium pump activity at high calcium concentrations (which is the situation for the initial phase of the spontaneous relaxation). Both effects could explain the faster relaxation of the caffeine-induced contracture in the presence of bepridil (10^{-6} M). At higher concentrations bepridil has been shown to slow down the sarcolemmal Na^+–Ca^{2+} exchange (Kaczorowski *et al.*, 1988) and SR Ca^{2+}–Mg^{2+}–ATPase activities (Younes *et al.*, 1981) which could account for the reverse effect observed with higher concentrations of bepridil (10^{-5} M). Verapamil has been reported to have no effect or to affect Ca^{2+}–ATPase activities only at very high concentrations (10^{-4} M), (Wang *et al.*, 1984; Movsesian *et al.*, 1985; Solaro *et al.*, 1986; Raess & Gersten, 1987) which is in good agreement with our results where we were unable to demonstrate a significant effect of verapamil on the spontaneous relaxation of caffeine-induced contracture.

Effects on the repriming of the caffeine-induced contracture

Bepridil and verapamil slowed the repriming of the intracellular calcium store involved in the caffeine-induced contracture whilst CERM 11888 was much less active in this respect. The effects of bepridil were markedly and significantly different from the quaternary derivative in this respect which suggests that an intracellular action is involved in the effects of bepridil upon repriming. Since some intracellular calcium is lost from the cell during relaxation (Rasmussen *et al.*, 1987; MacLeod & Bers, 1987), this calcium has to be recuperated by the cell to permit maximum responses to be obtained. Pang & Sperelakis (1981, 1982) have shown that bepridil and verapamil affect low-affinity calcium binding sites on cardiac sarcolemmal membranes. These binding sites may be required for the transport of calcium into the cell and thus could be indirectly involved in the repriming of the intracellular store essential for caffeine-induced contractures. This hypothesis could explain the dependence of the repriming on extracellular calcium ion concentration (Chapman & Leoty, 1976). Bepridil, unlike verapamil, also possesses potent calmodulin antagonist properties (Stoclet *et al.*, 1985; Itoh *et al.*, 1986) and could, in addition, antagonize calmodulin-dependent calcium uptake by the SR which might also explain the greater effects observed with bepridil over verapamil in this respect.

In conclusion these results have shown that a decrease in calcium release from the SR is probably responsible for the effects of bepridil on the amplitude of caffeine-induced contractures whereas its effects upon the relaxing system in this preparation are complex and the resultant of various processes. Such effects may involve an action upon Ca^{2+}–ATPase as well as effects upon plasma membrane sites. These results also suggest that at least one of the compartments described in the model of Chapman & Leoty (1976) must include a calcium pool in the sarcolemma as suggested by Hunter *et al.* (1982) and that the Na^+–Ca^{2+} exchange could be a good candidate. Additional studies are needed however to determine the number and precise location of the site(s) of action of bepridil in this respect. These actions of bepridil might be of benefit during ischaemia when intracellular calcium overload occurs and the maintainance of calcium homeostasis is of vital importance for cell survival. An intracellular effect of bepridil could also participate in the antianginal and antiarrhythmic effects of the drug (Lynch *et al.*, 1985) and contribute to the original electrophysiological profile of bepridil compared to other calcium antagonists which predominantly block only membranal slow Ca^{2+} channels at therapeutic doses (Singh *et al.*, 1985a,b; Schwartz *et al.*, 1985; Flaim & Cummings, 1986).

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The mechanism of amphetamine-induced inhibition of rat substantia nigra compacta neurones investigated with intracellular recording *in vitro*

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1 The effects of (+)-amphetamine (1–300 μM) applied by superfusion, on substantia nigra zona compacta neurones were studied in slices of the rat mesencephalon with intracellular recording techniques (current and voltage clamp mode).

2 Amphetamine inhibited the action potential discharge, hyperpolarized the cells and increased the membrane conductance. Under voltage clamp it produced an outward current.

3 The amphetamine outward current reversed at potentials negative to −100 mV (2.5 mM extracellular potassium) and was reduced by barium, (1 mM) suggesting an increased potassium conductance.

4 In slices treated with tetrodotoxin and calcium-free solution and added cobalt, amphetamine was still effective.

5 (−)-Sulpiride, (1 μM) a D_2 antagonist, blocked the effects of both amphetamine and dopamine.

6 In slices treated for more than 3 h with 30 μM α-methyl-DL-tyrosine, an agent known to block the synthesis of dopamine, the action of amphetamine was attenuated, while dopamine was still effective.

7 Responses to applied dopamine were potentiated by cocaine (10 μM) but unaffected by amphetamine (1–3 μM).

8 It is suggested that the inhibition of substantia nigra compacta neurones by amphetamine is mainly due to the release of endogenous dopamine.

Introduction

The dopamine-containing neurones of the mesencephalon are the main source of dopamine for the whole brain. This catecholamine is mainly released from the axon terminals of the dopaminergic neurones in the striatum and in the accumbens (Bjorklund & Lindvall, 1984).

Dopamine is also released from dendrites within the substantia nigra and ventral tegmental area (Geffen *et al.*, 1976; Cheramy *et al.*, 1981). Furthermore, the presence of dopamine autoreceptors (Carlsson, 1975) on the cellular dendrites, soma and terminals regulating the synthesis, the release of dopamine and the nerve impulse flow, is widely accepted. *In vivo* and *in vitro* electrophysiological studies have already shown that these cells respond to the administration of dopamine and dopamine agonists with an inhibition of firing (Aghajanian & Bunney, 1973; 1977; Pinnock, 1983). This effect is caused by a hyperpolarization due to an increase in a potassium conductance mediated by D_2 dopamine receptors (Lacey *et al.*, 1987; 1988a,b).

Amphetamine, inhibits presumed dopaminergic substantia nigra neurones recorded extracellularly in *in vivo* preparations (Bunney *et al.*, 1973a,b; Groves *et al.*, 1975; Aghajanian & Bunney, 1976; Browder *et al.*, 1981) and *in vitro* (Pinnock, 1983). Nedelgard *et al.* (1988) have recently shown that amphetamine causes a hyperpolarization.

Amphetamine stimulates the release of dopamine in the substantia nigra from dendrites (Paden *et al.*, 1976; Nieullon *et al.*, 1979) and in the striatum from dopamine cells' terminals (Chiueh *et al.*, 1975; Heikkila *et al.*, 1975a; Meyerhoff & Kant, 1978; Kamal *et al.*, 1981). Amphetamine is well known to produce

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increased locomotor activity, psychosis and worsening of schizophrenia (Beamish & Kiloh, 1960; Costa *et al.*, 1972; Sheel-Kruger, 1972; Snyder, 1974) that are related to an enhanced dopaminergic function (Jonsson, 1972; Snyder, 1974; Cole, 1978).

In the present study we employed intracellular electrophysiological recordings from a slice preparation of rat ventral mesencephalon, to determine the physiological and pharmacological mechanisms by which amphetamine affects the substantia nigra dopaminergic neurones. The effect of amphetamine was compared to that of dopamine and interaction between these substances was compared to the action of the catecholamine uptake blocker cocaine (Heikkila *et al.*, 1975b). Additionally, the dependence of the effect of amphetamine and dopamine upon newly-synthesized dopamine was examined by pretreating slices with the dopamine synthesis inhibitor α-methyl-DL-tyrosine (AMDLT) (Enna *et al.*, 1973).

Methods

Slices preparation

Albino Wistar rats (150–350 g) of either sex were killed by a blow to the chest under ether anaesthesia. The brain was quickly removed and slices of the mesencephalon (300–350 μm thick) which contained the substantia nigra were cut by an Oxford vibratome. A single slice was then transferred to a recording chamber and continuously superfused at a rate of 2.5 ml min^{-1}, with a solution maintained at 36°C and gassed with 95% O_2: 5% CO_2. The standard solution contained (mM): NaCl 126, KCl 2.5, NaH_2PO_4 1.2, $MgCl_2$ 1.2, $CaCl_2$ 2.4, glucose 10, $NaHCO_3$ 26. When $CoCl_2$ was added to the solution NaH_2PO_4 was omitted. The preparation of the slices has been described previously (Lacey *et al.*, 1987).

Intracellular recording techniques

Intracellular recording electrodes were filled with 2 M KCl and had a resistance of 30–100 MΩ. A single electrode voltage clamp amplifier was used (Axoclamp 2A) for both voltage and current recordings. Voltage clamp recordings were made with 2–3.5 Hz switching frequency, while the headstage signal was continuously monitored on a separate oscilloscope. Membrane potentials and currents were displayed on chart by a Hewlett Packard 7402A recorder. Steady-state current/voltage plots were obtained by hyperpolarizing neurones to between −110 and −120 mV and then depolarizing at a steady rate of 1 mV s^{-1}. Membrane current was plotted as a function of membrane potential with a Hewlett Packard 7035B X-Y recorder.

Drug application

Drugs were applied by superfusion by changing the control solution to one containing the drug at known concentration. Drug solutions entered the recording chamber no later than 20 s from turning a 3 way tap. Complete replacement of the medium in the chamber took 90 s.

The following drugs were used: (+)-amphetamine sulphate, dopamine hydrochloride, cocaine (Sigma), (−)-sulpiride (Ravizza), α-methyl-DL-tyrosine (Merck).

Numerical data were expressed as mean ± standard error of the mean (s.e.mean).

Results

Electrical properties of substantia nigra compacta neurones

Thirty-nine neurones of the substantia nigra zona compacta were recorded intracellulary for periods ranging from 0.2 to 5 h. Sixty-eight percent of these cells fired action potentials spontaneously mean 1.8 ± 0.2 spikes s^{-1}. The remaining cells were silent but action potentials could be evoked by depolarizing intracellular current injection. Action potential duration was longer than 1 ms. The membrane resistance showed a voltage- and time-dependent inward rectification on hyperpolarization. The peak resistance was 192 ± 20.8 MΩ, $n = 5$ (as measured within 200 ms at the onset of a hyperpolarizing pulse), the steady-state resistance was 140 ± 19.7 MΩ (as measured 600 ms after the onset of the current pulse). These properties correspond to those previously described for dopaminergic neurones (Lacey *et al.*, 1987; 1989).

Action of amphetamine

(+)-Amphetamine sulphate (1–300 μM) applied to the superfusate, depressed the spontaneous action potential discharge and hyperpolarized the membrane. Under voltage clamp at −50 to −60 mV holding potential it produced an outward current (Figure 1). Amphetamine also produced hyperpolarizations and outward currents in the neurones that were not spontaneously active. All thirty-nine cells, reported in this study, responded to the application of amphetamine. The amphetamine outward current depended on the concentration used (Figure 2). The concentration that caused the half maximal outward current (EC_{50}) was 13 μM (Figure 2b). These effects could be repeatedly observed on reapplication of amphetamine to single cells. They had an onset from the bath application of 1–3 min and took about

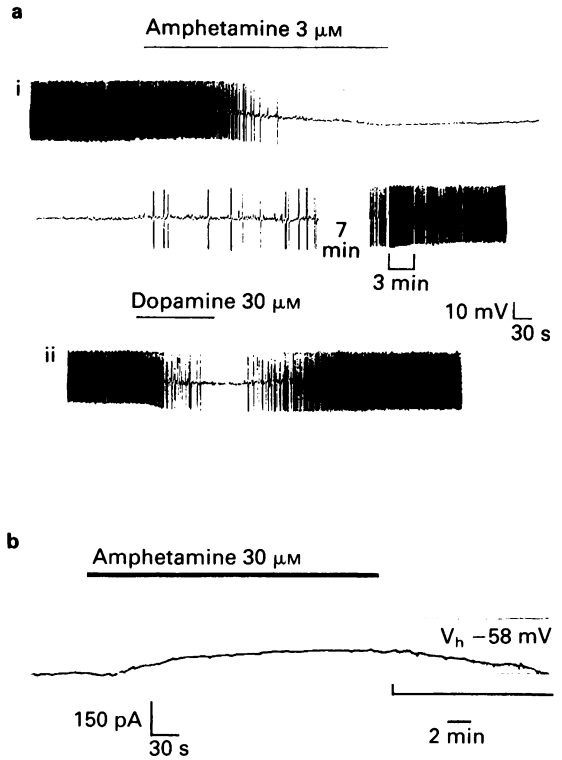


Figure 1 Effects of bath-applied amphetamine and dopamine on the firing rate and membrane potential and current (the action potential amplitude was not fully reproduced). (a) (i) Amphetamine (3 μM) applied for the period indicated by the bar, blocked the action potential discharge and hyperpolarized the membrane. The dotted line indicates an interruption in the recording. Note that during the recovery, the chart speed was also reduced for the time indicated by the bracket. (ii) Dopamine (30 μM) was later applied to the same cell. It hyperpolarized the membrane and blocked the firing activity. Note the different time course of the effects of the two drugs. (b) Amphetamine induced an outward current in another cell. Upper trace, voltage. Lower trace, current. Amphetamine 30 μM, applied in the superfusion solution for the period indicated by the bar, produced an outward current in the cell which was voltage clamped at −58 mV. The broken line indicates the original holding current of −50 pA. Note the different speed of the chart during the wash-out.

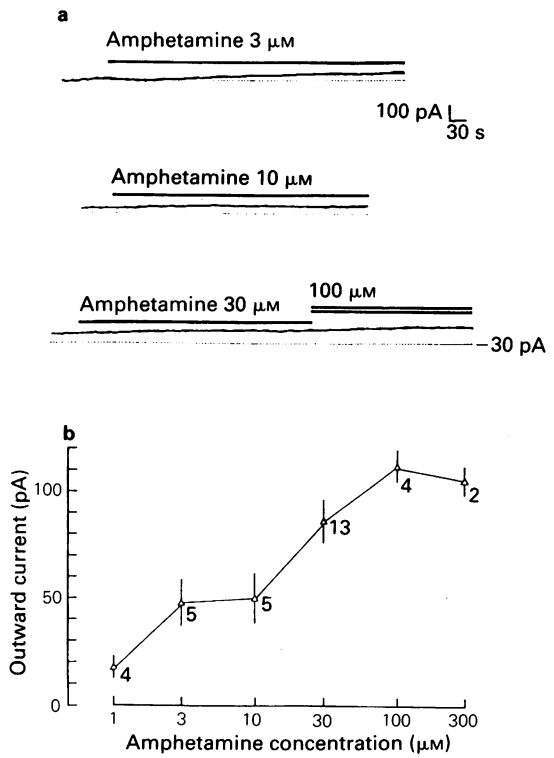


Figure 2 Effects of amphetamine on the membrane current as a function of the concentration applied. (a) Amphetamine was increased cumulatively to produce a concentration-dependent outward current in a single neurone. The three traces are consecutive. Original holding current −30 pA (dashed line). The membrane potential was clamped at −57 mV. (b) Concentration-response curve for the outward current caused by amphetamine. (Holding potential between −50, and −60 mV). Points show mean effect and vertical lines indicate s.e.mean for the numbers of neurones indicated.

2–6 min to reach a steady state. Recovery occurred within 8–80 min of wash-out. The higher the concentration applied the shorter the onset time to reach a steady state, but the longer the washout time required for full recovery. Full recovery was not observed when 100 or 300 μM amphetamine was used.

Comparison with the action of dopamine

The membrane effects induced by amphetamine resemble the ones produced by dopamine on substantia nigra cells (Lacey *et al.*, 1987; 1988a,b). Dopamine hyperpolarized the membrane (Figure 1a(ii)) and in voltage clamp produced an outward current (Figures 3 and 6). Previous studies have also shown that the actions of dopamine are mediated through the activation of D_2 type receptors (Pinnock, 1984; Lacey *et al.*, 1987; Suppes & Pinnock, 1987). The D_2 receptor antagonist (−)-sulpiride (1 μM) blocked the effects of both amphetamine and dopamine in 3 cells (Figure 3).

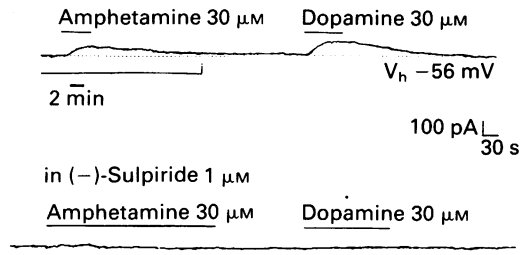


Figure 3 Amphetamine effects are blocked by (−)-sulpiride. Records are membrane currents from a single neurone voltage clamped at −56 mV. (a) Both amphetamine and dopamine produced an outward current (note the difference in time scale). (b) (−)-Sulpiride (1 μM) completely blocked the responses to amphetamine and dopamine.

In 3 neurones the membrane was hyperpolarized by current injection to values more negative than −100 mV and a steady state current voltage plot was obtained, by a ramp depolarization in the absence and presence of amphetamine (Figure 4). There was an increase in slope conductance throughout the voltage range during the action of amphetamine, and the membrane outward current became inward at potentials more negative than −100 mV (−104 ± 1.7 mV, $n = 3$) (Figure 4). Barium (1 mM), a potassium channel blocker, reduced the outward current produced by amphetamine by 42 ± 2.1% ($n = 3$) of control amplitude (Figure 4). The increase in membrane conductance induced by amphetamine was also seen as an increase in the current necessary to produce constant hyperpolarizing voltage steps (not shown).

Tetrodotoxin (TTX), which blocks fast sodium currents, at concentrations of 1–0.5 μM did not block the current induced by amphetamine. This was also observed in slices treated with zero-calcium and added cobalt (700 μM) (Figure 5d, 3 cells). The independence of the dopamine outward current on calcium entry has been already shown (Lacey *et al.*, 1987). In TTX, slow oscillations of the membrane replaced the fast action potentials (Figure 5b). These 'waves' were blocked in solution containing no calcium and added cobalt (Figure 5c).

Pretreatment with α-methyl-DL-tyrosine

α-Methyl-DL-tyrosine (AMDLT), an inhibitor of tyrosine hydroxylase, the rate limiting enzyme in catecholamine synthesis, depletes dopamine stores originating mainly from newly synthesized transmitter and thereby reduces amine release (Enna *et al.*, 1973). AMDLT (30 μM) applied in the bath for 3–6 h,

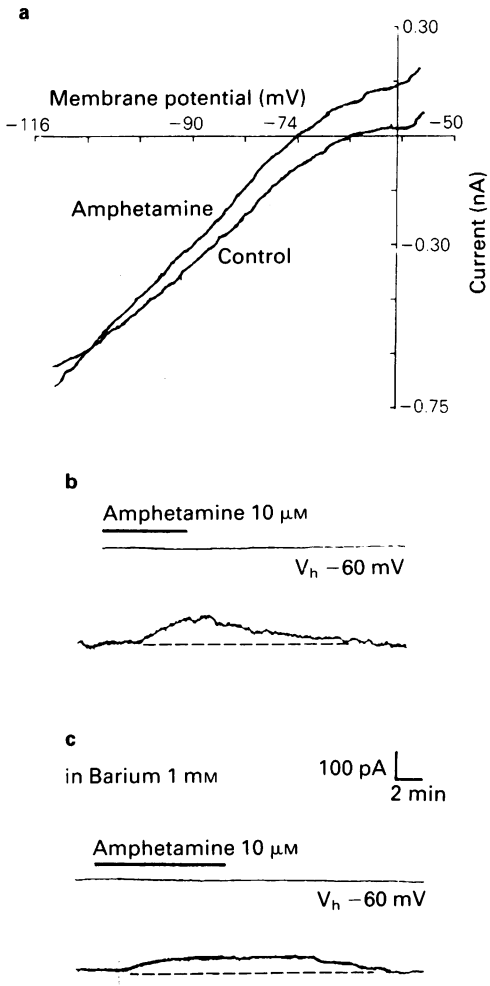


Figure 4 The amphetamine outward current reversed at values more negative than −100 mV and was reduced by barium (1 mM). (a) Current-voltage plots obtained by depolarizing the cell from −115 mV at 1 mV s^{-1} while measuring membrane current, in absence (control) and in presence of amphetamine (100 μM). Amphetamine current became inward negative to about −107 mV. (b) Amphetamine 10 μM, induces outward current in a nigral cell. (c) Barium (1 mM), reduced the outward current produced by the same concentration of amphetamine. Note, that in the presence of barium the time of amphetamine application was prolonged.

depressed the outward current produced by 30 μM amphetamine by 69% of control (26.5 ± 5.3 pA, $n = 4$ versus 87.5 ± 8.45 pA, $n = 13$, $P < 0.002$, Student's t test for independent samples). There was no change in the effects of dopamine on the same

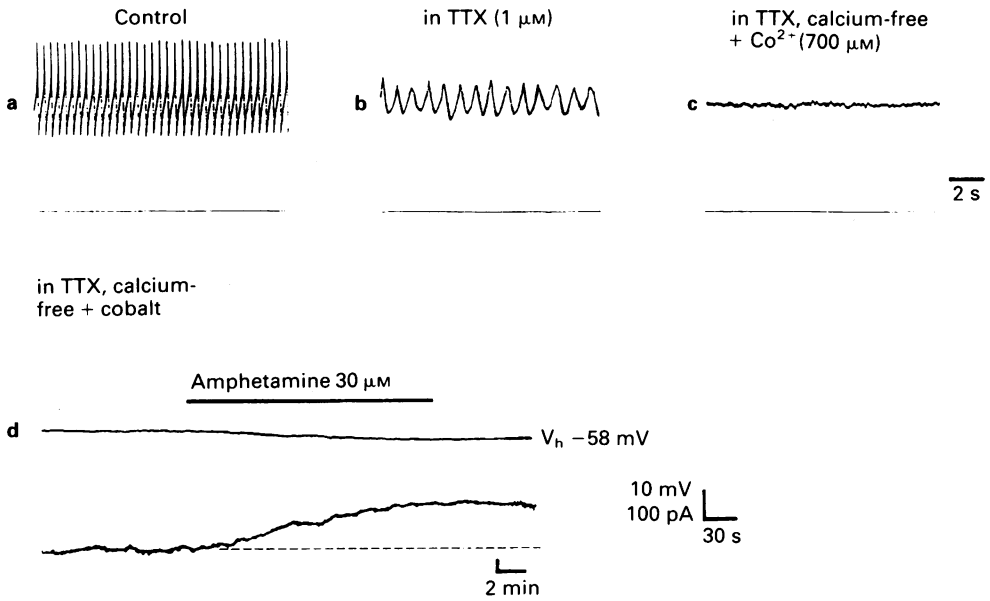


Figure 5 The amphetamine-induced outward current persists in tetrodotoxin (TTX) and calcium-free solution. (a, b,c,d) Upper records, voltage. Lower records, current. (a) Control, the nigral neurone is firing spontaneously at a regular rate (action potentials attenuated). In (b) in TTX (1 μM), fast action potentials were abolished, leaving slow 'waves' of membrane potential. In (c) the superfusion solution contained TTX, no calcium and cobalt (0.7 mM). The spontaneous 'waves' were blocked. In (d) in TTX, zero Ca^{2+} and Co^{2+}, under voltage clamp, amphetamine produced an outward current. Note that the time calibration in (a), (b), (c), is shown in (c) and that there was a change in the speed of the records in (d) at the point indicated.

cells. Dopamine 30 μM in AMDLT-treated slices produced an outward current of 86.6 ± 8.1 pA, $n = 3$, versus 95 ± 22 pA, $n = 6$ in normal solution, ($P = 0.682$). AMDLT did not clearly modify the membrane properties of the substantia nigra cells.

Interaction with dopamine

Cocaine 10 μM, an inhibitor of catecholamine uptake (Heikkila *et al.*, 1975b), caused a maintained membrane hyperpolarization or outward current (31.6 ± 4.3 pA, $n = 3$) and increased and prolonged dopamine responses (Figure 6a), (Lacey *et al.*, 1988a,b; Lacey, Mercuri & North, unpublished results).

Amphetamine has been reported to block catecholamine uptake processes (Glowinski & Axerold, 1965; Heikkila *et al.*, 1975a; Pelayo *et al.*, 1980). To evaluate whether the electrophysiological effects of amphetamine were attributable to dopamine uptake blockade, like those of cocaine, the action of amphetamine on the response to dopamine was examined. In the presence of amphetamine, the net membrane hyperpolarization and/or outward current had a higher peak than the one produced by dopamine prior to amphetamine application (Figure 6b,c). However, at high concentrations of either amphetamine or dopamine, the effect of amphetamine plus dopamine was less than the sum of the individual responses (Figure 6c). With low concentrations of amphetamine (1–3 μM) that produced hyperpolarizations or outward currents similar to the ones induced by cocaine (10 μM), the dopamine effects were not augmented (5 cells). On the contrary, in two cases these responses were slightly shortened (Figure 6b).

Discussion

Our results have shown that amphetamine inhibits the cells presumed to contain dopamine (Lacey *et al.*, 1989) in a concentration-dependent manner. Amphetamine hyperpolarized the membrane, caused an outward current and increased the membrane conductance.

In these respects and the following, amphetamine mimicked the effects of dopamine on these cells.

(a) (−)-Sulpiride antagonized both dopamine and amphetamine responses, which suggests a D_2 receptor involvement. The inhibitory action of dopamine

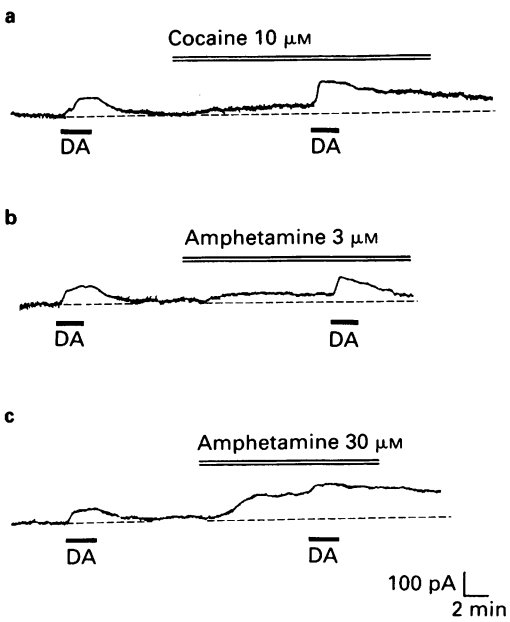


Figure 6 Unlike cocaine (10 μM), amphetamine does not potentiate the effect of dopamine. Membrane currents from the same nigral neurone during superfusion with dopamine (DA) 10 μM (bars), in absence and then in presence of cocaine (10 μM) and amphetamine (3, 30 μM). In (a) cocaine increased and prolonged the dopamine outward current: Holding potential −60 mV. In (c), amphetamine did little affect the dopamine response. In (b), the outward current produced by dopamine was almost abolished by the amphetamine outward current.

on these cells is attenuated by D_2 antagonists (Pinnock, 1984; Lacey *et al.*, 1987; Suppes & Pinnock, 1987).

(b) The amphetamine outward current reversed at potentials between −107 and −104 mV and was attenuated by barium suggesting, as shown for dopamine (Lacey *et al.*, 1987), an increase in potassium conductance. An amphetamine-induced, potassium conductance increase in substantia nigra zona compacta cells was proposed by others utilizing current-clamp intracellular recordings (Nedelgard *et al.*, 1988).

AMDLT, an agent known to block the synthesis of catecholamine, reduced the effect of amphetamine but did not affect the action of exogenous dopamine. This strongly supports the contention that the electrophysiological effect of amphetamine was mediated by the increased efflux of dopamine from dopaminergic neurones. A similar phenomenon accounts for the effect of amphetamine on rat neostriatal cells recorded intracellularly *in vitro* (Calabresi *et al.*, 1988).

In contrast, the amphetamine-induced efflux of dopamine is neither dependent on TTX-sensitive sodium channels nor on calcium entry into the cell, since the amphetamine-induced outward current was not blocked by solutions that contain TTX and/or zero calcium plus cobalt. These solutions block spontaneous electrical activity and synaptic release of transmitters. It might be argued that amphetamine releases dopamine independently from the persistance of synaptic chemical connectivity among neurones. It is worthwhile mentioning that the release of dopamine produced by amphetamine in the rat and rabbit striatum is also calcium-independent (Meyerhoff & Kant, 1978; Kamal *et al.*, 1981). These findings therefore provide insight into the mechanism of dopamine release by amphetamine and support the self inhibitory mechanism proposed by Groves *et al.* (1975).

The amphetamine outward current was dependent on the concentration applied and was maximal at 100 μM. This concentration of amphetamine is required to release dopamine in sufficient quantity to induce almost the same maximal effect produced by the superfusion of 100 μM dopamine on substantia nigra cells, (compare Figure 3b this paper with Figure 3 in Lacey *et al.*, 1987).

The prolonged time to peak of the amphetamine effect, relative to dopamine, could be explained by the processes of internalization of the drug in the soma and dendrites prior to the subsequent release of dopamine, (for a review see McMillan, 1983). The effects of applied amphetamine reverse less easily than those of applied dopamine on washout of the drug. The long period required to wash out could not only be caused by persistent outflow of dopamine from dendrites, but could also be due to the difficulty of fully reversing the effects produced by a prolonged application of a high concentration of dopamine on the cells (Lacey *et al.*, 1987). The observation that amphetamine, unlike cocaine, does not increase dopamine responses, argues that it does not block significantly the uptake of dopamine but induces the release of dopamine in the substantia nigra to produce the electrophysiological responses.

In conclusion, the ability of amphetamine to increase the efflux of dopamine from dopaminergic neurones is probably most relevant to the inhibitory action produced by the drug.

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Nicotinic excitation of rat ventral tegmental neurones *in vitro* studied by intracellular recording

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1 Intracellular recordings were made from presumed dopamine-containing neurones in the ventral tegmental area (VTA) in rat brain slices.

2 Nicotine (10–100 μM) and acetylcholine (ACh) depolarized the neurones. The depolarization caused by ACh was typically biphasic; both components were increased by neostigmine (0.1–10 μM), but only the slower component was blocked by scopolamine (1–10 μM).

3 The nicotinic action of ACh, studied in the presence of neostigmine and scopolamine, persisted in the presence of tetrodotoxin (1 μM) and cobalt (2–5 mM).

4 ACh or carbachol (30 μM) caused inward currents in neurones voltage-clamped near the resting potential. These currents reversed polarity at around −4 mV, were blocked by hexamethonium (1–100 μM) in a voltage-dependent manner, and showed desensitization with prolonged or repeated agonist applications.

5 Depolarizations caused by ACh and carbachol were reduced in slices pretreated with κ-bungarotoxin, but were not changed by α-bungarotoxin.

6 These responses to ACh and nicotine resemble those previously described on autonomic ganglion cells. The direct action on VTA neurones may contribute to the positive reinforcement associated with nicotine consumption.

Introduction

Acetylcholine (ACh) applied to presumed dopaminergic neurones during electrophysiological studies *in vivo* has been found to be either ineffective (Collingridge & Davies, 1981; Pinnock & Dray, 1982) or excitatory (Dray & Straughan, 1976; Lichtensteiger *et al.*, 1982; Scarnati *et al.*, 1986). Nicotine, applied systemically or by iontophoresis, excites neurones in both the substantia nigra (Lichtensteiger *et al.*, 1982; Clarke *et al.*, 1985) and ventral tegmental area (VTA) (Grenhoff *et al.*, 1986; Mereu *et al.*, 1987) of rats. Nicotine also increases dopamine concentrations in terminal regions of these cells, as do cocaine, amphetamine and morphine (DiChiara & Imperato, 1988). These findings are consistent with the idea that the excitation of VTA neurones by nicotine contributes to the reinforcement associated with tobacco consumption (Grenhoff *et al.*, 1986; Mereu *et al.*, 1987).

Although autoradiographic studies suggest that [^{3}H]-nicotine binding sites are on dopamine-containing neurones themselves (Clarke & Pert, 1985), electrophysiological experiments using extracellular recording *in vivo* are generally not able to resolve whether the nicotinic excitation is taking place on these cells or on other neurones afferent to them. Nor are those methods well suited to determine the type of nicotinic receptor involved. The purpose of the present experiments was to examine the actions of ACh and nicotine, by using intracellular recording from rat VTA neurones *in vitro*.

Methods

Intracellular recordings were made from cells in the VTA as previously described in detail by Lacey *et al.* (1987, 1989). In brief, coronal slices (300 μm) of rat ventral mesencephalon were maintained in a chamber completely submerged in a continuously

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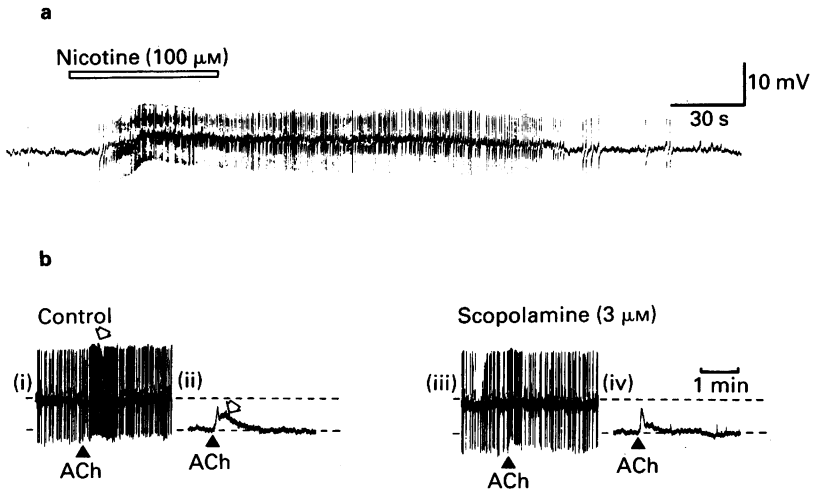


Figure 1 The effect of nicotine and acetylcholine (ACh) on ventral tegmental (VTA) neurones. (a) Nicotine (100 μM) depolarized the membrane and caused action potentials (full amplitude not shown). The open bar indicates the period during which the superfusing solution contained nicotine. Cell membrane was held at −57 mV (by passing −70 pA current) before the application of nicotine to prevent spontaneous action potentials; this current was constant throughout. (b) Nicotinic and muscarinic actions of ACh on a VTA neurone. ACh was applied from a blunt pipette (triangles) during spontaneous firing (no applied current, i and iii) and during passage of hyperpolarizing current to hold the membrane potential at −68 mV (ii and iv). (i) and (ii) Control. ACh caused a biphasic increase in firing rate (i) and, at −68 mV, a depolarization with early and late components (open arrows) (ii). (iii) and (iv) Muscarinic receptor antagonist scopolamine (3 μM) blocked the late but not the early component.

flowing (1.5 ml min^{-1}) solution containing (mM): NaCl 126, KCl 2.5, NaH_2PO_4 1.2, $MgCl_2$ 1.3, $CaCl_2$ 2.4 glucose 10 and $NaHCO_3$ 26, saturated with 95% O_2/5% CO_2 and heated to 36°C. NaH_2PO_4 was omitted when solutions contained cobalt chloride (2–5 mM). Intracellular recordings were made from VTA neurones by means of glass microelectrodes, of resistance 40–80 MΩ, filled with either KCl or CsCl (2 M). Signals were amplified with an Axoclamp 2A single electrode voltage-clamp amplifier and displayed on a Gould 2400 chart recorder. Most voltage-clamp recordings were made with electrodes coated with Sylgard (Dow-Corning) to reduce their capacitance; headstage voltage was continuously monitored.

With the exception of ACh, drugs were applied by changing the superfusion solution to one containing known concentrations of drug; new solutions entered the recording chamber within 30 s. The drugs used were: nicotine sulphate, carbamylcholine chloride (carbachol), (−)-scopolamine hydrochloride, hexamethonium chloride (Sigma), tetrodotoxin (TTX) and neostigmine methylsulphate (Calbiochem). ACh (Sigma) was applied by ejecting a few nl of a 100 mM solution from the tip of a blunt pipette positioned beneath the surface of the superfusing solution and just above the tissue slice; a pressure of 50–70 kPa for 50–500 ms was typically used.

Experiments with α-bungarotoxin and κ-bungarotoxin were carried out as follows. Slices were kept for 2 h at 36°C in 3–6 ml of continuously gassed superfusion medium, sometimes with added α- (1 μM) or κ- (0.5 μM) bungarotoxin. They were then transferred to the recording chamber and intracellular recordings obtained. Lyophilized purified bungarotoxins were a gift from Dr V.A. Chiappinelli, St. Louis, U.S.A.

Numerical data are expressed as mean ± s.e.mean.

Results

Effects of nicotine and acetylcholine

Fifty-nine cells were studied; all had properties similar to those of principal dopamine-containing neurones in the adjacent substantia nigra zona compacta, including hyperpolarization by dopamine (Lacey *et al.*, 1987; 1989). Nicotine (10–100 μM) depolarized the neurones and increased their firing rate, or caused an inward current in cells held at the resting potential ($n = 5$) (Figures 1a and 2c).

ACh caused a depolarization and an increase in cell firing rate in 27 of 35 cells. This depolarization often comprised a transient phase lasting for a few seconds, followed by a slower component lasting

tens of seconds. The slower component was reduced or abolished by scopolamine (1–10 μM; Figure 1b), indicating that a late part of the response to ACh was mediated by a muscarinic receptor. The effect of ACh was enhanced by the acetylcholinesterase inhibitor neostigmine (100 nM–10 μM); in the presence of neostigmine, ACh depolarized all 26 cells tested. Neostigmine itself had no effect on membrane potential or membrane current ($n = 28$). All subsequent experiments with ACh and carbachol were carried out in the presence of scopolamine and neostigmine.

Responses to ACh or carbachol persisted in the presence of tetrodotoxin (1 μM; 10 cells; Figures 2c and 3) or cobalt (2–5 mM; 4 cells; Figure 3a). Either treatment blocked spontaneous action potentials and action potentials evoked by the agonists: therefore, we expected that they would block release of transmitter resulting from the excitation of any adjacent neurones, making it likely that the nicotinic effect was due to a direct action of the agonists on the impaled neurone.

ACh (in the presence of scopolamine) was also applied to seven neurones in the substantia nigra zona compacta; six were depolarized.

Desensitization

The nicotinic response desensitized markedly: the response amplitude declined during 1–2 min superfusion with nicotine and carbachol (Figures 1a, 2b and c). The desensitization was also apparent when ACh was applied; reproducible responses were obtained only when the interval between applications of ACh was at least 30 s, and sometimes 2 min or more (Figure 2a). The depolarizations (or inward currents) caused by carbachol (30 μM) and nicotine (100 μM) showed cross-desensitization with ACh (Figure 2b and c).

Reversal potential

The depolarization or inward current caused by carbachol or ACh became larger with membrane hyperpolarization (11 cells) (Figure 3). The response was studied at potentials less negative than the resting potential, after reducing outward potassium currents with intracellular caesium (2 M in the electrode, 2 cells) or with extracellular tetraethylammonium (30 mM) plus barium (1 mM; 2 cells). TTX (1 μM) and cobalt (2–5 mM) were also present to block voltage-activated inward currents. Under these conditions the effect of ACh or carbachol reversed polarity at -3.7 ± 2.4 mV ($n = 4$).

Effects of antagonists

The response to ACh was reduced by hexamethonium (1–100 μM). The block by hexamethonium was greater at more negative potentials (6 cells; Figure 3b) and reversed on washing for 10–15 min. (+)-Tubocurarine (10 μM; 4 cells) also reduced the response by about 50%.

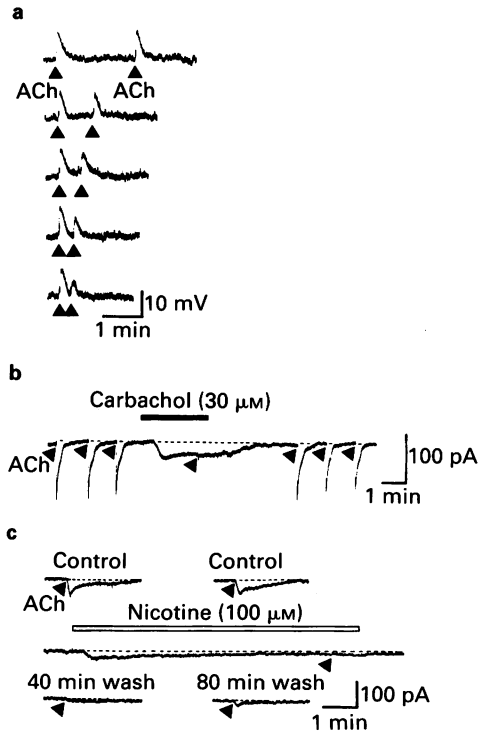


Figure 2 Desensitization of nicotinic responses. (a) Depolarizations caused by acetylcholine (ACh) became smaller when the interval between applications was reduced to less than 1 min. Membrane potential held at -72 mV by constant current injection. ACh was applied from a blunt pipette (triangles). Traces are continuous from top to bottom. Carbachol (30 μM, solid bar) caused an inward current that partially desensitized during the application. Responses to ACh were completely occluded by carbachol. (c) A similar experiment on a different neurone. Currents in response to ACh were occluded by nicotine (100 μM, open bar), which itself caused an inward current that desensitized. The response to ACh recovered only partially 80 min after washing out nicotine. Tetrodotoxin (TTX, 1 μM) was present in (c); scopolamine (3 μM) and neostigmine (100 nM–3 μM) were present in (a), (b) and (c).

We tested the effectiveness of ACh and carbachol (in the presence of scopolamine (3 μM) and neostigmine (100 nM)) to depolarize a further 37 VTA neurones in tissues pretreated with either α- or κ-bungarotoxin (or controls). Neurones were held at -70 ± 0.7 mV ($n = 37$) by passage of constant hyperpolarizing current. The maximum depolarization elicited by ACh was 6.7 ± 1.3 mV (9 cells)

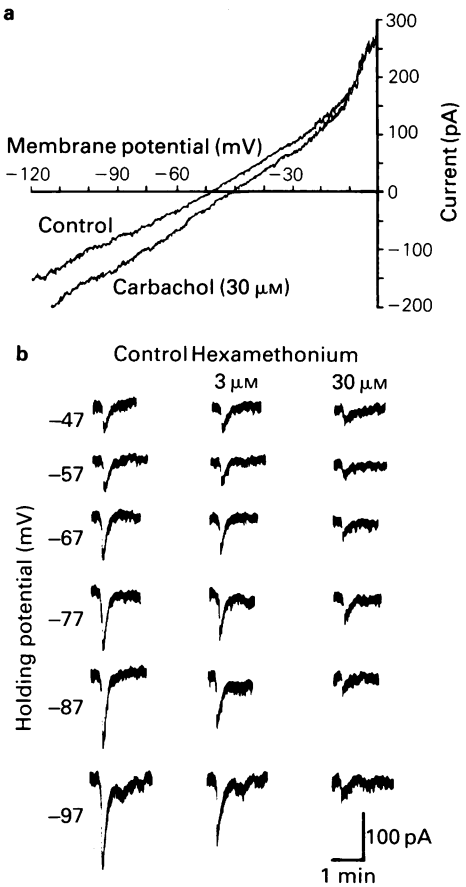


Figure 3 Voltage-dependence of the nicotinic current, and the inhibitory effect of hexamethonium. (a) Current/voltage relation before and after carbachol (30 μM). Membrane current recorded in response to slow ramp voltage command from −120 to 0 mV. Rectification was reduced by adding tetrodotoxin (TTX, 1 μM), cobalt (2 mM), barium (1 mM), caesium (2 mM) and tetraethylammonium (30 mM). Scopolamine (3 μM) was also present. The inward current caused by carbachol became zero at about −10 mV. (b) Membrane currents evoked by acetylcholine (ACh) in a single cell at holding potentials indicated before (left column) and after (middle and right columns) adding hexamethonium (3 and 30 μM). The effect of hexamethonium on the response to ACh is greater at more negative membrane potentials. Scopolamine (3 μM), neostigmine (100 nM) and TTX (1 μM) were present.

and by carbachol (30 μM) was 3.8 ± 0.5 mV (12 cells) in six slices incubated in control medium. In three slices preincubated with α-bungarotoxin (1 μM), ACh depolarized eight cells by 5.3 ± 1.1 mV and carbachol depolarized nine cells by 3.8 ± 0.7 mV. In four slices pretreated with κ-bungarotoxin (0.5 μM), the ACh depolarization was only 2.5 ± 0.6 mV (n = 14) and the depolarization caused by carbachol was 1.4 ± 0.3 mV (n = 14). κ-Bungarotoxin reduced the nicotinic depolarizations by more than half ($P < 0.01$, Dunnett's t test modification) whereas α-bungarotoxin pretreatment had no effect ($P > 0.1$)

Discussion

The action of acetylcholine on VTA neurones resembles that at nicotinic receptors on other cells. Thus the value of the reversal potential (−4 mV, Figure 3a) is close to that estimated for the reversal of current flow through nicotinic receptor channels in many tissues (Takeuchi & Takeuchi, 1960; Nishi & Koketsu, 1960; Dennis *et al.*, 1971; Egan & North, 1986). Desensitization is also characteristic of nicotinic responses (Katz & Thesleff, 1957). Block by hexamethonium is typical of nicotinic actions in ganglia (Paton & Zaimis, 1949) and brain (Egan & North, 1986; McCormick & Prince, 1987): its voltage-dependence (Figure 3b) has previously been shown in ganglion cells (Rang, 1982). The finding of voltage-dependent block by hexamethonium, along with the insensitivity of the response to TTX and cobalt, indicate that the response was due to direct activation of nicotinic receptors on VTA neurones. The cross-desensitization with nicotine and carbachol (Figure 2b and c) suggests that all three agents act upon the same receptors on these cells.

κ-Bungarotoxin blocks nicotinic receptors on ganglion cells, but not at the neuromuscular junction, in contradistinction to α-bungarotoxin (Chiappinelli, 1983; Loring *et al.*, 1985; Loring & Zigmond, 1988). It also blocks the nicotinic receptor expressed in the membrane of oocytes injected with mRNA directing the synthesis of α_3 and α_4 agonist-binding nicotinic receptor subunits (Boulter *et al.*, 1987), but not α_2 (Wada *et al.*, 1988). *In situ* hybridization experiments have demonstrated the presence of mRNA encoding the synthesis of α_3 and α_4 (but not α_2: Wada *et al.*, 1988) subunits in the VTA (Goldman *et al.*, 1986; 1987). Thus it may be that the nicotinic depolarization is mediated by receptors comprising either α_3 or α_4 subunits (or both). However, the failure to block completely the response with κ-bungarotoxin must necessarily temper this conclusion. It is possible that a mixed population of κ-bungarotoxin-sensitive and -insensitive receptors is present, or that in our experiments the binding of κ-bungarotoxin may have been incomplete at the time during which recordings were made.

The gratifying constituent of tobacco is nicotine, withdrawal from which can result in craving and physical distress (Johnston, 1949); it is therefore considered a drug of dependence (U.S. Surgeon

General's Report, 1988). Like other drugs of dependence, nicotine is rewarding; it is self-administered by animals (Goldberg *et al.*, 1981; Cox *et al.*, 1984) and by man (Henningfield *et al.*, 1983). Increased activity in the mesolimbic dopamine system, which arises predominantly from the ventral tegmental area (VTA), has been associated with a variety of rewarding behaviours including intracranial self-stimulation and self-administration of cocaine, amphetamine and heroin (Bozarth, 1983; Wise, 1984; Fibiger & Phillips, 1986). It appears likely that tobacco ingestion will activate nicotinic receptors of several different molecular species in the central and peripheral nervous systems. Thus, in view of the evidence implicating the dopamine system arising from the VTA in behavioural reinforcement and the rewarding properties of addictive drugs, further characterization of the nicotinic receptor type(s) present on these neurones may have a significant impact in the search for novel therapeutic strategies to reduce the consumption of tobacco products.

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Intracellular platelet-activating factor regulates eicosanoid generation in guinea-pig resident peritoneal macrophages

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1 The role of intracellular platelet-activating factor (Paf) in arachidonic acid (AA) mobilization from guinea-pig peritoneal macrophages has been investigated by use of the potent and selective Paf receptor antagonists, WEB 2086 and CV 6209.

2 Adherent macrophages contained cell-associated Paf which was increased by exposure to formyl-methionyl-leucyl-phenylalanine (fMLP), endotoxin and the ionophore, A23187. However, only endotoxin and A23187 caused release of detectable amounts of Paf into the extracellular medium.

3 Exogenous Paf and each of the above stimuli mobilized previously incorporated [^{14}C]-AA and increased the generation of prostacyclin (PGI_2) in resident macrophages.

4 WEB 2086 (10–100 μM) and CV 6209 (0.1–10 μM) reduced both basal and stimulated PGI_2 generation and WEB 2086 inhibited the mobilization of [^{14}C]-AA. In addition, WEB 2086 (10 μM) inhibited fMLP- and Paf-induced superoxide anion generation. Responses to A23187 were not inhibited by either antagonist.

5 Activation of macrophages by fMLP caused a short burst of intracellular Paf generation but none was detected in the supernatants. The time-course of PGI_2 synthesis followed closely that of Paf.

6 These data suggest that intracellular Paf generation is important for subsequent AA mobilization and may have a wider role in signal transduction processes.

Introduction

Evidence for an important role for platelet-activating factor (Paf) in a number of models of allergy, shock and inflammation has accumulated rapidly following the availability of potent and specific receptor antagonists (see Braquet *et al.*, 1987 for a review). These include endotoxin shock, pulmonary anaphylaxis, cardiac anaphylaxis, thrombosis and allergic and non-allergic inflammation. The participation of cells capable of synthesizing and releasing Paf is common to these disorders and includes macrophages (Camussi *et al.*, 1981; Roubin *et al.*, 1986), polymorphonuclear leukocytes (PMNLs, Camussi *et al.*, 1981; Mueller *et al.*, 1983; Jouvin-Marche *et al.*, 1984) and platelets (Chap *et al.*, 1981). However, macrophages and PMNLs also retain a high proportion of the synthesized Paf within the cell and the function of this intracellular Paf has not been established (Harris *et al.*, 1985; Henson, 1987).

We have recently shown that guinea-pig resident peritoneal macrophages possess a Paf receptor linked to prostacyclin generation which has lower apparent affinity for the Paf antagonists BN 52021, L-652,731 and WEB 2086 (Stewart & Dusting, 1988) than do the Paf receptors mediating platelet and PMNL aggregation. It is likely that the Paf receptors on macrophages are a distinct subclass. However, it is also possible that the low apparent affinities were due to decreased access of the antagonists to a receptor located intracellularly. The possibility of an intracellular site of action is also

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supported by our recent observation that Paf receptor antagonists inhibit bradykinin-induced prostacyclin (PGI_2) generation by cultured endothelial cells (Stewart *et al.*, 1989).

In this study we have investigated the role of exogenous and endogenous Paf in stimulating the metabolism of arachidonic acid in guinea-pig peritoneal macrophages using the potent, specific and structurally distinct Paf antagonists, WEB 2086 (Casals-Stenzel *et al.*, 1987) and CV 6209 (Terashita *et al.*, 1987).

Methods

Macrophage cultures

Macrophages were obtained from healthy male guinea-pigs (500–900 g) by peritoneal lavage (Stewart & Dusting, 1988). Following isolation by centrifugation (1000*g*, 4°C, 10 min), cells were resuspended in RPMI 1640 (20% foetal calf serum, FCS, 15 mM HEPES, pH 7.40, penicillin 100 u ml^{-1}, streptomycin, 100 $\mu g\,ml^{-1}$) at a concentration of 1–2 × 10^6 cells ml^{-1}, dispensed (0.5 ml) into Linbro 24 well plastic culture plates (6 well plates were used for the experiments described in Table 2) and allowed to adhere for 2 h. In experiments involving the release of [^{14}C]-arachidonic acid ([^{14}C]-AA), the macrophage adherence was carried out in RPMI 1640 (20% FCS) containing 0.2 $\mu Ci\,ml^{-1}$ [^{14}C]-AA (58 mCi $mmol^{-1}$) and indomethacin 2.8 μM. At the end of the adherence period the non-adherent cells were removed and the remaining cells (80–95%) were washed once with RPMI 1640 (containing 0.25% bovine serum albumin, BSA in place of FCS) and pre-incubated for 15 min in RPMI 1640 (0.25% BSA) containing either the vehicle (culture medium), WEB 2086 (10–100 μM) or CV 6209 (0.1–10 μM). The cells were exposed to a range of known stimuli for arachidonic acid metabolism for 60 min periods (corresponding to the maximum release for all stimuli) or as indicated. At the conclusion of the incubation period, the supernatants were harvested and cells were subjected to ethanol extraction as described below.

Radio-immunoassay of 6-oxo-$PGF_{1\alpha}$

PGI_2 generation by adherent macrophages was assessed by radio-immunoassay (RIA) of its chemically-stable breakdown product, 6-oxo-$PGF_{1\alpha}$ according to the method of Salmon (1978). None of the agents used in this study cross-reacted with the 6-oxo-$PGF_{1\alpha}$ antiserum or altered the sensitivity of the assay which was carried out in RPMI 1640 (0.25% BSA). One hundred microlitres of the 0.5 ml of the supernatant was used immediately in the RIA of 6-oxo-$PGF_{1\alpha}$, the remainder was stored at −20°C for bioassay of Paf.

Paf bioassay

Paf was bioassayed on rabbit washed platelets (Vargas *et al.*, 1982) suspended in Tyrode solution containing indomethacin 2.8 μM (Parente & Flower, 1985). Criteria for identification included: ability to induce an indomethacin-resistant platelet aggregation; inhibition of bioactivity by the selective Paf receptor antagonist WEB 2086 (1 μM); co-elution of pooled and re-extracted samples with authentic [^{3}H]-Paf on thin layer chromatography (t.l.c.), mobile phase 65:35:6 chloroform:methanol:water. Supernatants (up to 50 μl) were assayed without prior extraction whereas cells were extracted into 80% ethanol. Following removal of the precipitate by centrifugation, the extract was evaporated to dryness under reduced pressure and reconstituted in 250 μl of Tyrode solution (0.25% BSA). The recovery from this extraction was 94 ± 3%, $n = 53$. The limit of detection of the assay was 6 fmol. The maximum volume used was 50 μl. However, the effective detection limit varied with the cell number used. Thus, in experiments reported in Table 2, 6 well plates were used (containing 3–4 × 10^6 cells per well) giving a limit of detection of 0.01 pmol per 10^6 cells. In time-course experiments, 24 well plates were used and the limit of detection was 0.03 pmol per 10^6 cells.

Samples from different cultures were pooled, subjected to a Bligh-Dyer extraction and chromatographed on t.l.c. (Whatman LK 5D) as described above. One centimetre segments of the scraped silica were extracted into Tyrode solution (BSA 0.25%) and bioassayed on washed rabbit platelets.

Reverse-phase h.p.l.c. of [^{14}C]-arachidonic acid metabolites

At the end of the 60 min incubation period, supernatants from macrophages pre-labelled with [^{14}C]-AA were harvested and immediately frozen and stored at −20°C until chromatography. The supernatant pH was adjusted to 5.6, and brought to 65% methanol, centrifuged to remove the precipitate and filtered twice (0.22 μm millipore filter) before injection onto the rp h.p.l.c. column. Prior to sample preparation, approximately 4000 d.p.m. of [^{3}H]-leukotrienes B_4 and C_4 and [^{3}H]-5-hydroxy-eicosatetraenoic acid (5-HETE) were added to indicate the respective retention times. The h.p.l.c. system consisted of a radial compression module with a novapak C_{18} column and a mobile phase of methanol:H_2O:acetic acid, 65:35:0.1, pH 5.6, 1 ml min^{-1} for 17 min followed by a 1 min linear gra-

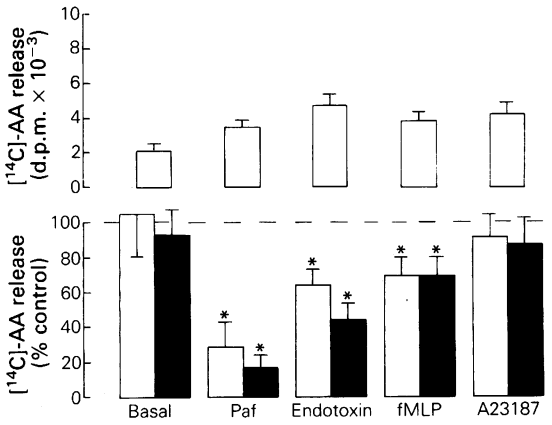


Figure 1 The effects of WEB 2086 (10–100 μM) on basal release of pre-incorporated [^{14}C]-arachidonic acid ([^{14}C]-AA) and that stimulated by Paf (0.1 μM) endotoxin (100 μg ml^{-1}), fMLP (1 μM) and A23187 (10 μM). The upper panel indicates the absolute release of [^{14}C]-AA in non-pretreated macrophages during a 60 min incubation period. The lower panel represents the release of [^{14}C]-AA in the presence of WEB 2086 expressed as a percentage of that in non-pretreated (control) macrophages (n = 11): WEB 2086 10 μM open columns; 100 μM closed columns. * $P < 0.05$, paired Student's t test, compared to untreated cells.

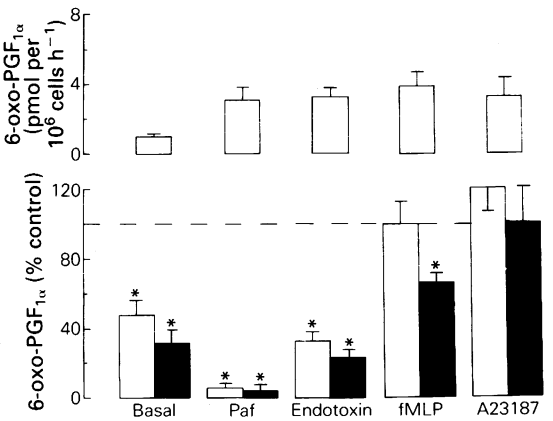


Figure 2 The effects of WEB 2086 (10–100 μM) on the generation of 6-oxo-PGF$_{1\alpha}$ in unstimulated (basal) macrophages and in those activated by Paf (0.1 μM), endotoxin (100 μg ml^{-1}), fMLP (1 μM) and A23187 (10 μM). The upper panel indicates the absolute generation of 6-oxo-PGF$_{1\alpha}$ in non-pretreated macrophages during a 60 min period. The lower panel represents the generation of 6-oxo-PGF$_{1\alpha}$ in WEB 2086-pretreated macrophages and the data are expressed as a percentage of the generation by the corresponding non-pretreated macrophages: WEB 2086 10 μM open columns; 100 μM closed columns. Data are presented as the means with s.e.mean shown by vertical bar (n = 8). * $P < 0.05$, paired Student's t test.

dient to methanol:acetic acid, 100:0.1, for 20 min. One ml fractions were collected, added to 4 ml Emulsifier-Safe Scintillant (Packard) and counted in a Packard 300C liquid scintillation spectrometer with software for dual counting of ^{3}H/^{14}C.

Superoxide anion generation

Stimulus-induced generation of superoxide anion by adherent macrophages was assessed by cytochrome C reduction according to the method of Johnston *et al.* (1978). Briefly, macrophages were incubated in 1 ml of Krebs-Ringer solution (composition, mM: NaCl 121, KCl 5, $CaCl_2$ 1.3, $MgSO_4$ 1.2, NaH_2PO_4 3.1, Na_2HPO_4 12.5, dextrose 11, pH 7.3) containing cytochrome C (80 μM) and were pretreated with WEB 2086 (10 μM) for 15 min. The cells were stimulated for a period of 90 min after which the supernatants were harvested and the absorbance read at 550 nm. The amount of cytochrome C reduced was calculated by the change in absorbance at 550 nM using the differential molar extinction coefficient, 21×10^3 M^{-1} cm^{-1}. The potential free radical scavenging activity of WEB 2086 was examined with a hypoxanthine (100 μM)/xanthine oxidase (25 mu ml^{-1}) superoxide anion generating system in the absence of cells.

Materials

All reagents and solvents used in this study were of analytical or higher grade. RPMI 1640 and FCS were obtained from CSL (Australia) and Flow Laboratories, respectively. Chemicals were obtained from the following sources: A23187; BSA, grade 5, essentially fatty acid-free; cytochrome C (horse heart type III); dextran, clinical grade C; endotoxin E. Coli Serotype 011 B4; formyl-methiony-leucyl-phenylalanine (fMLP); hypoxanthine; indomethacin; superoxide dismutase (Sigma Chemical Co.); hexadecyl platelet-activating factor (Paf, Bachem); prostacyclin (PGI_2, Na salt, Wellcome); 6-oxo-PGF$_{1\alpha}$ (Upjohn); 6-oxo-PGF$_{1\alpha}$ antiserum (Dr J. Salmon, Wellcome Research Laboratories); 6-keto [5,8,9,11, 12,14,15 (n)-^{3}H] prostaglandin F$_{1\alpha}$, 170 Ci mmol^{-1}; [1-^{14}C]-arachidonic acid, 58.3–59.6 mCi mmol^{-1} (Amersham); [hexadecyl-1′,2′-^{3}H(N)]-2-acetyl-sn-glyceryl-3-phosphoryl-choline, 59.5 Ci mmol^{-1}; [14,15-^{3}H(N)]-leukotriene B_4, 32 Ci mmol^{-1}; [14,15-^{3}H(N)]-leukotriene C_4, 39 Ci mmol^{-1}; [^{3}H]-5-hydroxyeicosatetraenoic acid, 150 Ci mmol^{-1}; (New England Nuclear); HEPES (N-2-hydroxy ethylpiperazine-N-2-ethane sulphonic acid, (BDH chemicals); heparin (Fisons Pty. Ltd.), CV 6209 (2-[N-acetyl-N-(2-methoxy-3-octadecyl-carbamoyloxy-propoxycarbonyl)aminomethyl] - 1 - ethylpyridinium chloride) (Takeda Chemical Industries Ltd.); WEB 2086 (3-(4-(2-chlorophenyl)-9-methyl-6H-

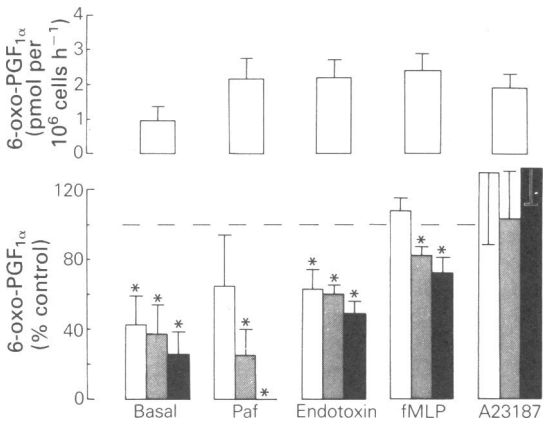


Figure 3 The effects of CV 6209 (0.1–10 μM) on the generation of 6-oxo-$PGF_{1\alpha}$ in unstimulated (basal) macrophages and in those activated by Paf (0.1 μM), endotoxin (100 $\mu g\,ml^{-1}$), fMLP (1 μM) and A23187 (10 μM). The upper panel indicates the absolute generation of 6-oxo-$PGF_{1\alpha}$ in non-pretreated macrophages during a 60 min period. The lower panel represents the generation of 6-oxo-$PGF_{1\alpha}$ in CV 6209-pretreated macrophages and the data are expressed as a percentage of the generation by the corresponding non-pretreated macrophages: CV6209 0.1 μM open columns; 1 μM hatched columns; 10 μM closed columns. Data are presented as the means with s.e.mean shown by vertical bars ($n = 8$). * $P < 0.05$, paired Student's t test.

thieno(3,2-f),(1,2,4)-triazolo,(4,3-a),(1,4)-diazepine-2-yl)-1-(4-morpholinyl)-1-propanone), (Boehringer Ingelheim, West Germany).

Results

[^{14}C]-*arachidonic acid release*

Activation of adherent macrophages by all stimuli caused the release of pre-incorporated [^{14}C]-AA into the supernatant (Figure 1). However, there was no detectable conversion of the released [^{14}C]-AA to 5-lipoxygenase products (data not shown). The basal release of [^{14}C]-AA and that induced by A23187 were not affected by WEB 2086 pretreatment. In contrast, WEB 2086 (10–100 μM) significantly reduced ($P < 0.05$, paired t test) the release of [^{14}C]-AA induced by Paf (0.1 μM), endotoxin (100 $\mu g\,ml^{-1}$) and fMLP (1 μM). The extent of the inhibition ranged from 80% for Paf to 30% for fMLP (Figure 1).

PGI_2 generation

Each of the stimuli for [^{14}C]-AA release also elicited an increase in the generation of PGI_2 measured as immunoreactive 6-oxo-$PGF_{1\alpha}$ (Figures 2 and 3). In macrophages pretreated with WEB 2086 (10–100 μM) there was a similar pattern of inhibition for PGI_2 generation and for [^{14}C]-AA release except that the basal PGI_2 generation, but not that of [^{14}C]-AA, was markedly reduced by WEB 2086 (Figure 2). In a separate series of experiments, the potent Paf receptor antagonist, CV 6209 (0.1–10 μM) concentration-dependently inhibited basal and stimulated PGI_2 generation (Figure 3) except that induced by A23187.

Influence of culture medium

Approximately 80% of cells obtained by peritoneal lavage adhered within the 2 h period. This adherence was not dependent on the presence of foetal calf serum (FCS). The basal generation of PGI_2 was considerably higher in cells which adhered in the presence of FCS (Table 1). However, WEB 2086 (100 μM) and CV 6209 (10 μM) inhibited basal 6-oxo-$PGF_{1\alpha}$ generation irrespective of the presence of FCS in the culture medium.

Paf generation

Endotoxin and A23187 consistently stimulated the generation of Paf in macrophages, of which approximately 50% was released into the BSA-containing culture medium (Table 2). In unstimulated macrophages and in those exposed to fMLP, detectable levels of cell-associated Paf (> 0.01 pmol per 10^6 cells h^{-1}) were observed after 60 min, but there was no consistent release of Paf into the extracellular medium. In pooled samples subjected to t.l.c., all the platelet aggregating activity co-eluted with authentic Paf.

Temporal relationship between Paf and 6-oxo-$PGF_{1\alpha}$ generation

In fMLP (1 μM)-stimulated macrophages, cell-associated Paf was detectable after 6s (limit of detection 0.03 pmol per 10^6 cells h^{-1}), reached a maximum at 2 min and returned towards control within 20 min. An increase in 6-oxo-$PGF_{1\alpha}$ was observed at 30 s and levels peaked at 2–5 min after fMLP (Figure 4). All the Paf synthesized by the fMLP-stimulated macrophages remained cell-associated and no Paf was detected in unstimulated macrophages during the 20 min period.

Superoxide generation

Paf (0.1 μM) and fMLP (0.01 μM) stimulated generation of superoxide anion during a 90 min incubation (Table 3). WEB 2086 (10 μM) completely inhibited the response to Paf and reduced that to fMLP. However, this concentration of WEB 2086 had no free radical scavenging activity since it did not inhibit superoxide generation by the hypoxanthine (100 μM)/xanthine oxidase (25 mu ml^{-1})

Table 1 The influence of the culture medium on the inhibition of basal PGI_2 generation by WEB 2086 and CV 6209

Culture medium	*6-oxo-$PGF_{1\alpha}$* (pmol per 10^6 h^{-1})	*WEB 2086* 100 μM (% inhibition)	*CV 6209* 10 μM
RPMI 1640[1] (20% FCS)	2.74 ± 0.89	96 ± 2	94 ± 2
RPMI 1640 (no FCS)	0.81 ± 0.20	80 ± 8	70 ± 10

$n = 4$.
[1] Peritoneal cells from individual animals were divided into 2 aliquots and adhered in the presence or absence of FCS. After removal of the non-adherent cells by washing, incubations of each group were carried out in RPMI 1640 (0.25% BSA).
* $P < 0.05$, paired t test.

Table 2 Generation of Paf by macrophages during a 60 min incubation with endotoxin, formyl-methionyl-leucyl-phenylalanine (fMLP) and A23187

Stimulus	*Paf* (pmol per 10^6 cells h^{-1}) *Cell-associated*	*Supernatant*
–	0.026 ± 0.009	ND
fMLP 1 μM	0.025 ± 0.012	ND
Endotoxin 100 $\mu g\,ml^{-1}$	0.087 ± 0.023	0.091 ± 0.045
A23187 10 μM	1.428 ± 0.605	1.769 ± 0.554

$n = 8$.
ND = none detected.

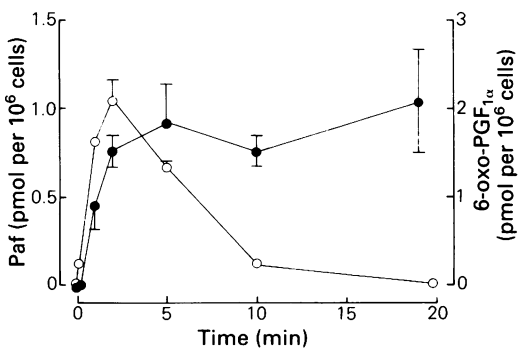


Figure 4 Temporal relationship between Paf (open symbols) and 6-oxo-$PGF_{1\alpha}$ (closed symbols) generation in fMLP (1 μM)-stimulated macrophages. The data are presented as the means and s.e.mean are shown by vertical bars where the latter exceed the size of the symbol ($n = 4$).

Table 3 Effects of WEB 2086 (10 μM) on superoxide anion generation by adherent macrophages

Stimulus	*Superoxide anion* (nmol per 10^6 1.5 h^{-1})	*WEB 2086* (10 μM) (% inhibition)
Paf 0.1 μM	2.43 ± 0.77	106 ± 7*
fMLP 0.01 μM	3.73 ± 1.19	28 ± 5*

* $P < 0.05$, paired t test

system (control, 5.48; WEB 2086 10 μM, 5.78 nmol per ml h^{-1}).

Discussion

It is well established that much of the Paf generated by pro-inflammatory cells remains intracellular (Henson, 1987), but it is not known whether this intracellular Paf has any functional consequences. Our observations suggest a role for Paf in signal transduction. Peritoneal macrophages from healthy guinea-pigs mobilized arachidonic acid (AA) in response to several stimuli, by a mechanism which was susceptible to inhibition by two structurally dissimilar, potent and specific Paf receptor antagonists, WEB 2086 (Casals-Stenzel *et al.*, 1987) and CV 6209 (Terashita *et al.*, 1987). The same stimuli caused intracellular Paf formation. These data suggest that intracellular Paf releases AA from its phosphatide stores, leading to eicosanoid generation. We have recently proposed that intracellular Paf has a role in AA mobilization in cultured endothelial cells (Stewart *et al.*, 1989). fMLP-stimulated superoxide anion generation was also reduced by WEB 2086 indicating that Paf generation may have a wider role in macrophages.

Guinea-pig resident peritoneal macrophages incorporated [^{14}C]-AA which was released on subsequent stimulation with optimal concentrations of Paf, fMLP, endotoxin and A23187. WEB 2086 inhibited the release of [^{14}C]-AA by all stimuli except A23187. However, the inhibition was most marked for endotoxin-stimulated macrophages. This effect cannot be ascribed to inhibition of cyclo-oxygenase since indomethacin was present throughout these experiments. Inhibition of lipoxygenase is unlikely to explain the reduction in [^{14}C]-AA release for there was no detectable conversion of released [^{14}C]-AA to [^{14}C]-5-lipoxygenase products. It may be concluded that the inhibition takes place at the level of the generation of non-esterified AA. The stimuli used in this study have been reported to elicit significant leukotriene formation in other macrophage populations (Rouzer *et al.*, 1982; Rhodes *et al.*, 1985; Williams *et al.*, 1986; Schade *et al.*, 1987; Kouzan *et al.*, 1988). The present findings may be explained by our use of [^{14}C]-AA whereas other workers have mainly

used the higher specific activity [^{3}H]-AA (Rouzer *et al.*, 1982; Kouzan *et al.*, 1988) or radioimmunoassay (Rhodes *et al.*, 1985; Williams *et al.*, 1986) which may provide greater sensitivity. Alternatively, the 5-lipoxygenase pathway may not be expressed in the guinea-pig resident peritoneal macrophage population.

Data from the metabolism of pre-incorporated [^{14}C]-AA predicted that stimulated release of cyclo-oxygenase products from macrophages should also be susceptible to inhibition by WEB 2086 since it is well established that the formation of cyclo-oxygenase products is limited by the availability of non-esterified arachidonic acid. WEB 2086 inhibited PGI_2 generation and [^{14}C]-AA release by stimulated macrophages to similar extents. Although the maximum inhibition of PGI_2 generation depended on the stimulus used, with each stimulus the inhibition by WEB 2086 and CV 6209 was similar. These observations support the proposed role of intracellular Paf in regulating non-esterified AA levels.

Superoxide anion generation by macrophages provided an AA-independent and functionally important response to examine our hypothesis that Paf has a second messenger role in this cell type. Superoxide anion generation induced by fMLP was reduced by WEB 2086 which suggests that the involvement of Paf in signal transduction processes extends beyond regulation of eicosanoid generation. Incomplete inhibition of the response to fMLP suggests that there are other mechanisms involved in the intracellular pathway for superoxide anion generation. Intracellular Paf may be responsible for endotoxin-induced priming of enhanced fMLP-induced superoxide anion generation in human PMNLs (Worthen *et al.*, 1988). Thus, Paf may act to upregulate other transduction pathways. Superoxide anion production by guinea-pig alveolar macrophages in response to fMLP is inhibited by another Paf receptor antagonist, Ro 19-3704 (Lagente *et al.*, 1988). These authors suggested that this effect was due to an ability to scavenge free radicals but did not directly test this hypothesis. WEB 2086 does not have free radical scavenging activity since it had no effect on superoxide anion generation by hypoxanthine/xanthine oxidase nor does it inhibit A23187-induced superoxide anion generation in rabbit PMNLs (Stewart, unpublished observations).

An important consideration in the present study is the specificity of the antagonists. WEB 2086 is a triazolodiazepine which is reported to lack the sedative/hypnotic properties of the other compounds in this class (Casals-Stenzel & Weber, 1987; Casals-Stenzel *et al.*, 1987). CV 6209 is a recently developed phospholipid analogue of Paf which has structural similarity to an earlier compound, CV 3988 (Terashita *et al.*, 1983). Phospholipid analogue antagonists of Paf have the potential for non-specific actions due to physicochemical interactions with lipid membranes. However, at the concentrations used in the present study CV 6209 is devoid of such actions (Terashita *et al.*, 1987). Furthermore, there is indirect evidence that neither WEB 2086 nor CV 6209 are inhibitors of phospholipase, cyclo-oxygenase or PGI_2 synthetase since they failed to inhibit PGI_2 generation or mobilization of pre-incorporated [^{14}C]-AA induced by a maximally effective concentration of A23187. In addition, none of BN 52021 (Nunez *et al.*, 1986), L-652,731 (Hwang *et al.*, 1985), WEB 2086 (Casals-Stenzel *et al.*, 1987) or CV 6209 (Terashita *et al.*, 1987) has significant inhibitory actions on cyclo-oxygenase activity at concentrations which inhibit basal PGI_2 generation (Stewart & Dusting, 1988, this study). Further evidence against a non-specific action of WEB 2086 or CV 6209 is apparent from the ability of these compounds to inhibit differentially a range of stimuli which produce a similar extent of mobilization of AA.

The failure of WEB 2086 to inhibit the basal release of pre-incorporated [^{14}C]-AA may be explained by a non-uniform labelling of phospholipid pools by [^{14}C]-AA. There is evidence for the existence of multiple pools of arachidonic acid in macrophages (Humes *et al.*, 1982; Tanaka & Shibata, 1985; Schade *et al.*, 1987) consistent with the possibility that, under the conditions of this study, [^{14}C]-AA may not label the pool of AA which contributes to basal eicosanoid production.

The inhibition of basal PGI_2 generation by Paf receptor antagonists implied that Paf was formed under the conditions of adherence. It was also considered possible that the FCS used in the culture medium could contain Paf or stimuli for its generation. Nevertheless, cells adhered in the absence of FCS were equally susceptible to the inhibitory actions of the Paf receptor antagonists, even though they produced less PGI_2. Cell-associated, but not extracellular Paf was observed in basal adherent macrophages giving support to the suggestion that macrophages become partly activated during adherence (Kouzan *et al.*, 1987) and providing an explanation of the inhibition of 'basal' PGI_2 generation by the Paf antagonists.

Even though the ionophore A23187 was the most effective stimulus for Paf generation, neither antagonist inhibited A23187-induced AA mobilization. This observation appears to conflict with the suggestion that intracellular Paf generation is a key event in AA mobilization. However, unlike physiological stimuli, A23187 causes persistent and large increases in intracellular Ca^{2+} which would be expected to activate directly the Ca^{2+}-dependent enzyme, phospholipase A_2 irrespective of any action of intracellular Paf. It is

also possible that the lack of inhibition was due to the use of a maximally effective concentration of A23187. The amount of intracellular Paf generated by A23187, which greatly exceeded that produced by the more physiological stimuli, may have surmounted the antagonism by WEB 2086 and CV 6209. Variations in the extents of inhibition of the other stimuli may be related to differing mechanisms of activation of macrophages.

Henson and co-workers have suggested that Paf accumulates in cells in concentrations ($\sim 10^{-5}$ M) sufficient to have direct physicochemical actions on membranes (Harris *et al.*, 1985; Henson, 1987). In the present study, the stimuli which were susceptible to inhibition by the Paf antagonists elicited synthesis of cell-associated Paf ($\sim$0.2 pmol per 10^6) which would lead to intracellular concentrations of the order of 10^{-8}–10^{-7} M, well in excess of exogenous concentrations required to activate macrophage AA metabolism (Stewart & Dusting, 1988). In contrast, the extracellular Paf concentrations ($< 10^{-9}$ M) were below the EC_{50} for activation of AA metabolism in macrophages stimulated with endotoxin and extracellular Paf could not be detected in basal or fMLP-stimulated macrophages. The low or undetectable concentrations of Paf in these supernatants may be a result of its rapid metabolism by macrophages (Roubin *et al.*, 1986). These observations on the distribution of Paf are not consistent with an extracellular site of action.

The time-course of fMLP-stimulated Paf and PGI_2 generation provides evidence which is consistent with an intracellular action since Paf was detectable within 6 s of exposure to fMLP whereas PGI_2 was not detected in the extracellular medium until 30 s. However, the interpretation of this temporal relationship as evidence for a causal link between these lipids may be influenced by the relative detection limits of the assays. It could be suggested that since both Paf and PGI_2 synthesis are dependent on phospholipase A_2 activity these lipids should be synthesized simultaneously. However, phospholipase A_2 activity is clearly not essential for Paf synthesis in rat peritoneal macrophages even though inhibitors of phospholipase A_2 reduce the release of the immediate precursor, lyso-Paf (Parente & Flower, 1985). It would appear that resting lyso-Paf levels are adequate to support Paf synthesis, at least over short periods. In addition, it is possible that the alternative, physiological pathway involving choline phosphotransferase (Braquet *et al.*, 1987) may subserve the generation of intracellular Paf. In this pathway, the requirement for phospholipase A_2 activity is further removed from the ultimate synthetic step than it is for the acetyltransferase pathway. The apparent rapid metabolism of intracellular Paf by guinea-pig macrophages would confer greater control on Paf as an intracellular messenger and is a common feature of other second messengers (Berridge, 1984).

In conclusion, we have shown that a number of compounds which stimulate macrophages also stimulate the generation of Paf within the cells and this is an important prelude to AA mobilization and eicosanoid generation. These data raise the possibility that Paf has the role of a second messenger or alters the gain of other signal transduction processes within these cells.

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Different time courses of the blockade of sodium current by lignocaine and SUN 1165 in single myocytes isolated from guinea-pig atrium

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1 The time course of the blockade of sodium currents (I_{Na}) by the antiarrhythmic agents, lignocaine and SUN 1165, was studied in single myocytes isolated enzymatically from guinea-pig atrium, by a new concentration-jump termed as a 'concentration-clamp' technique. This technique combines an intracellular perfusion and a rapid exchange of external solution surrounding the voltage-clamped myocyte within 2 ms.

2 Lignocaine (3.7×10^{-5} M to 3.7×10^{-4} M) inhibited the peak amplitude of I_{Na} in a concentration-dependent fashion. It took 2 to 5 s to reach apparent steady-state inhibition at the concentrations used. Complete recovery from the inhibition also took 2 to 5 s after washing out the agent. In contrast, the inhibitory effect of SUN 1165 (1×10^{-5} M) on the peak I_{Na} gradually progressed and reached a steady-state level about 2 min after the start of drug-application. The recovery required more than 10 min after washing out of the agent.

3 In cardiomyocytes treated with scorpion toxin (5 $\mu g\,ml^{-1}$), the inactivation of I_{Na} was greatly inhibited, resulting in the relatively persistent Na inward current (persistent I_{Na}) during the depolarizing pulse. Lignocaine (1.1×10^{-4} M) applied during the depolarizing pulse, reduced in a single-exponential fashion the amplitude of the persistent I_{Na} in milliseconds. On the other hand, lignocaine applied several tens of milliseconds before the depolarizing pulse induced only a small reduction of the peak amplitude of the first persistent I_{Na}. When SUN 1165 (1×10^{-5} M) was applied during the depolarizing pulse, there was only a small instantaneous reduction of the amplitude of the persistent I_{Na}, although it did inhibit time-dependently, the peak I_{Na}. Both agents accelerated the decay phase of the persistent I_{Na} in a time-dependent manner.

4 These results suggest that lignocaine and SUN 1165 may preferentially interact with the open-state of the sodium channel rather than with the rested one, although SUN 1165 does so much more slowly.

Introduction

N-(2,6-dimethylphenyl)-8-pyrrolizidine acetamide hydrochloride hemihydrate (SUN 1165), has been demonstrated to possess a potent antiarrhythmic action in *in vivo* and *in vitro* studies (Aisaka *et al.*, 1985; Hidaka *et al.*, 1985; Inomata & Ishihara, 1988). SUN 1165 bears a structural resemblance to lignocaine, but it has a special feature (pyrrolizidine) which is not present in commonly used antiarrhythmic agents (Miyano *et al.*, 1985). Voltage-clamp studies with isolated single cardiomyocytes have clearly shown that the effects of SUN 1165 on the sodium current (I_{Na}) resemble those of lignocaine and that its blocking action on the I_{Na} is the main mechanism of antiarrhythmic action (Yatani & Akaike, 1984; Inomata *et al.*, 1987). There may be a common basis for the mechanism of action of both antiarrhythmics that involves a voltage- and use-dependent inhibition of sodium channels (Class I-type), although they have been classified as different subtypes in terms of onset and offset kinetics of

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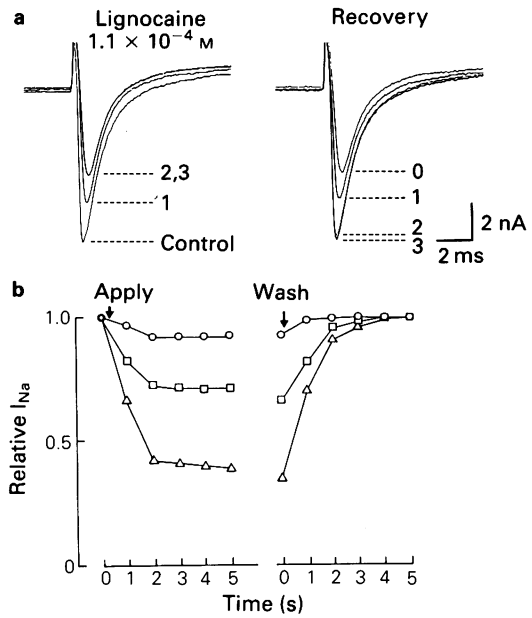


Figure 1 Time-course of the blocking action of I_{Na} and the recovery from the blockade of I_{Na} by lignocaine in single guinea-pig atrial cells. (a) The superimposed original I_{Na} traces after the application (left) and the washing out (right) of 1.1×10^{-4} M lignocaine. Each number shown at the right side of the current traces indicates the time (s) after the application or the washing out of the agent. (b) The time courses of the inhibitory action of lignocaine on peak I_{Na} after the application and the washing out of several concentrations of the agent: (○) 3.7×10^{-5} M; (□) 1.1×10^{-4} M; (△) 3.7×10^{-4} M. Each symbol indicates the mean of five experiments. Amplitude of peak I_{Na} was regarded as the difference between the maximum peak level and the terminal level.

the blocking action on the maximum rate of rise of the action potential (Campbell, 1983; Pallandi & Campbell, 1988; Hattori *et al.*, 1986; 1988).

The introduction of a 'concentration-clamp' technique which combines intracellular perfusion and a rapid solution change with a time constant of a few ms for the cell-attached condition of isolated cells has made it possible to analyse the precise time course of drug action and the interaction between the ionic channels (open and closed states) and drugs (Akaike *et al.*, 1986; Oyama *et al.*, 1987; 1988; Inomata *et al.*, 1988). Thus, it is now possible to estimate accurately the changes of sodium ion permeability induced by antiarrhythmic agents occurring in the millisecond order. Consequently, in order to characterize more precisely the inhibitory action of SUN 1165 on the sodium channels of cardiomyocytes, a comparison of the effects on I_{Na} between lignocaine and SUN 1165 was made by use of the 'concentration-clamp' technique.

Methods

The enzymatic dispersal procedure used for preparation of single atrial myocytes was essentially similar to that previously described (Matsuda *et al.*, 1982). In brief, the hearts of male guinea-pigs weighing 300 to 500 g were used. The aorta was cannulated and the heart was perfused with nominally Ca^{2+}-free Tyrode solution until the spontaneous contractions stopped. Thereafter, the perfusate was changed to the Ca^{2+}-free solution containing 0.4 mg ml^{-1} collagenase (Sigma, Type I) for about 45 min. Then the enzyme was washed out with a high-K storage solution composed of (in mM): KCl 85, K_2HPO_4 30, $MgSO_4$ 5, Na_2ATP 5, pyruvate 5, creatine 5, taurine 20, glucose 20, EGTA 0.2. The pH of the storage solution was adjusted to 7.4. The temperature of all solutions was kept at 37°C. The right atrium was dispersed in the storage solution with forceps. The dispersed single myocytes were left in the storage solution for later use at room temperature of 24 to 26°C.

Experiments were performed on viable rod-shaped myocytes with a 'concentration-clamp' technique (Akaike *et al.*, 1986). A single myocyte was sucked onto the tip of a suction pipette (inner diameter of 2 to 3 μm at the tip) and dialysed intracellulary with the internal solution (pH 7.4) containing (in mM): KOH 130, L-aspartate 130, KH_2PO_4 2.5, Na_2ATP 2, pyruvate 5, glucose 5.5 and HEPES 10. The tip of the suction pipette with the cell attached was inserted into a plastic tube through a circular hole (see Figure 1 of Akaike *et al.*, 1986). The lower end of this tube could be directly exposed to the external solution by vertically moving a stage holding the dishes containing solutions (see Figure 1 of Akaike *et al.*, 1986). The composition of the external solution (pH 7.4) was as follows (in mM): NaCl 137, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 0.53, NaH_2PO_4 0.05, $NaHCO_3$ 11.9, glucose 5.5, HEPES 5. A negative pressure of −3 cmHg applied to the upper end of the plastic tube was controlled by an electromagnetic valve, driven by 24 V d.c. supplied by a stimulator. The time constant of the solution exchange around an atrial myocyte was about 2 ms.

The ionic current was recorded by a single electrode voltage-clamp system (Ishizuka *et al.*, 1984). Both membrane current and potential were monitored on a digital oscilloscope (National, VP-5730A) and simultaneously stored on the FM-tape recorder (Sony, A-45) for further analysis. All experiments

were carried out at a room temperature of 24 to 26°C. The drugs used were SUN 1165 (Suntory), lignocaine (Sigma) and scorpion toxin (from *Leiurus quinquestriatus*, Sigma). All drugs were dissolved in the external solution just before use.

Results

Effect on the sodium current

The sodium current (I_{Na}) was elicited by a depolarizing pulse, lasting for 20 ms, from a holding potential of −80 mV to −20 mV at a frequency of 1 Hz. No use-dependent changes in the I_{Na} were observed at this stimulus frequency. Lignocaine was applied to voltage-clamped single myocytes 800 ms before the depolarizing pulse. As shown in Figure 1, the inhibition of the peak amplitude of I_{Na} by lignocaine rapidly progressed at any concentration used in this study and reached a steady-state level within 2 to 5 s after the drug application. The degree of inhibition on the peak amplitude of I_{Na} became more profound as the concentration of lignocaine was increased. The peak amplitudes of I_{Na} at the steady-state inhibition, relative to the control, were 0.94 ± 0.04 (mean ± s.e. of five experiments) at 3.7×10^{-5} M lignocaine, 0.71 ± 0.06 at 1.1×10^{-4} M and 0.39 ± 0.07 at 3.7×10^{-4} M. The recovery from the inhibition was complete within 2 to 5 s after washing out of lignocaine at any concentration used. In contrast to lignocaine, SUN 1165 was slow in inhibiting the peak amplitude of I_{Na} and it took 2 min or more to reach the steady-state inhibition as shown in Figure 2. The concentration (1×10^{-5} M) of SUN 1165 used in this study was close to the maximum value of effective plasma concentrations required for terminating ventricular arrhythmia in dogs (Ishihara *et al.*, 1984). Complete recovery from the inhibition was also observed, but it required more than 10 min after washing out the agent.

Effect on the persistent sodium current

The action of a drug that shortens the time to peak and hastens the decay phase has been accounted for by a model in which a drug blocks the open-state of channels (Yeh & Narahashi, 1977). However, the decay phase of I_{Na} is too rapid to make an exact evaluation of drug interaction with the open-state of Na channels. Scorpion toxin is known to prolong the decay phase of I_{Na} without affecting the sodium current activation kinetics in nerve axon (Narahashi *et al.*, 1972). Therefore, single atrial myocytes were treated with scorpion toxin ($5\ \mu g\ ml^{-1}$) for about 3 min in order to produce a relatively-persistent sodium current (persistent I_{Na}). In the subsequent

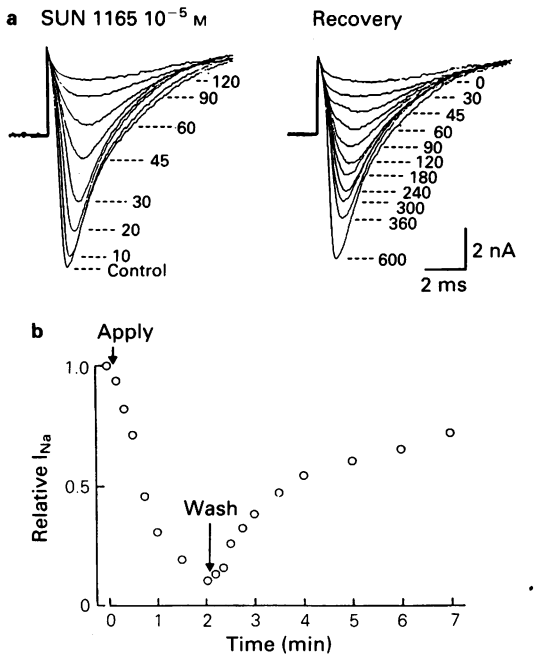


Figure 2 Time-course of inhibitory action of SUN 1165 on the peak I_{Na} in single guinea-pig atrial cells. (a) The superimposed original I_{Na} traces after the application (left) and the washing out (right) of 1×10^{-5} M SUN 1165. Each number shown at the right side of the current traces indicates the time (s) after the application or the washing out of the agent. (b) The time course of inhibitory action of 1×10^{-5} M SUN 1165 after the application and the washing out of the agent.

experiments, the persistent I_{Na} was elicited by applying a depolarizing pulse of 150 ms in duration to −25 mV from a holding potential of −85 mV every 5 s (Figure 3a). There were no use-dependent changes in the current under these conditions.

The persistent I_{Na} induced in this way, was suddenly deflected from its predicted decay time course when lignocaine at a concentration of 1.1×10^{-4} M was applied to the preparation during the depolarizing pulse as shown in Figure 3b. Thus, there was no detectable delay in onset of the inhibitory action of lignocaine. The reduction in the current amplitude of persistent I_{Na} by 1.1×10^{-4} M lignocaine progressed in a single-exponential fashion with the time constant of 39 ± 2 ms (mean ± s.e. of eight experiments). The deflection of the current by lignocaine became more pronounced as the concentration was increased and the time constant of the reduction in the amplitude of the persistent I_{Na} also decreased in a concentration-dependent manner (not shown). When 1.1×10^{-4} M lignocaine was applied during

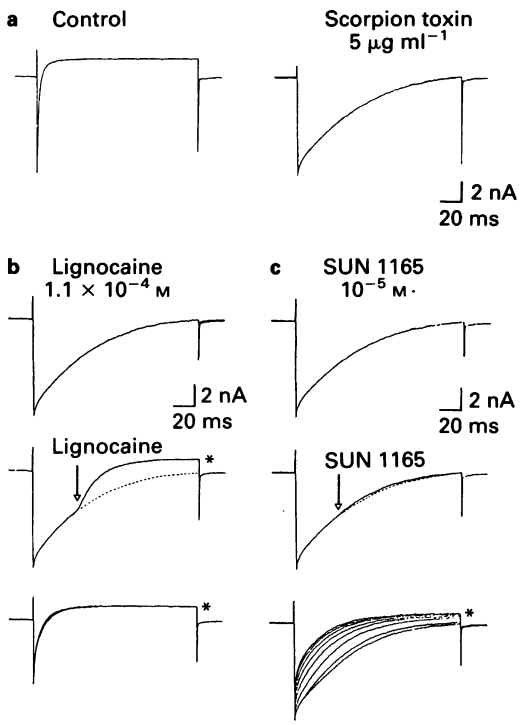


Figure 3 (a) The inhibition of sodium channel inactivation by scorpion toxin in a single atrial cell of guinea-pig. Original current trace in the control (left) and in the presence of scorpion toxin 5 $\mu g ml^{-1}$ (right). (b) The inhibitory action of 1.1×10^{-4} M lignocaine on the I_{Na} of scorpion toxin-treated single atrial cells. Current traces show the control (upper), effect of lignocaine applied during the depolarizing pulse (middle), and the second and third pulses after the application of lignocaine (lower), respectively. (c) The inhibitory action of 1×10^{-5} M SUN 1165 on the I_{Na} of the scorpion toxin-treated single atrial cells. Current traces show the control (upper), effect of SUN 1165 applied during the depolarizing pulse (middle), and the second to 9th pulses after the application of the agent (lower), respectively. The outward deflection indicated by the asterisk occurred because of no correction for the leakage current.

the depolarizing pulse, the amplitude of the persistent I_{Na} at 30 and 80 ms after the application of the drug, relative to control, was 0.51 ± 0.02 and 0.17 ± 0.01 (mean $\pm$ s.e. of four experiments), respectively. On the other hand, when 1.1×10^{-4} M lignocaine was applied 30 and 80 ms before the depolarizing pulse, the peak amplitudes of the first persistent I_{Na} were 0.96 ± 0.01 and 0.93 ± 0.04 (mean $\pm$ s.e. of four experiments), respectively. The steady-state inhibition by 1.1×10^{-4} M lignocaine was observed from the 2nd depolarizing pulse. The decay phase of persistent I_{Na} was greatly accelerated in the continued presence of lignocaine (Figure 3b). The half decay time decreased from 41 ms to 3 ms when the drug effect reached a steady-state.

SUN 1165 at a concentration of 1×10^{-5} M applied during the depolarizing pulse affected only slightly the amplitude of the persistent I_{Na} (Figure 3c). However, the agent again slowly reduced the peak current (see Figure 2). The inhibition of the persistent I_{Na} by SUN 1165 was also associated with an accelerated decay phase of the current. In the continued presence of 1×10^{-5} M SUN 1165, the time for half decay of the persistent I_{Na} decreased from 40 ms in control to 10 ms at the 10th depolarizing pulse (about 45 to 50 s after the start of drug-application).

Discussion

In the present study, we found that both lignocaine and SUN 1165 produced a final reduction of the peak amplitude of I_{Na} in single cardiomyocytes, but the time courses to a steady-state in their inhibitory actions were quite different from each other (compare Figure 1 and 2). Lignocaine blocked the peak I_{Na} in the order of seconds while SUN 1165 required minutes when both agents were applied to the preparation before a depolarizing pulse. Similarly although the recovery from inhibition was complete in both cases, the time required for recovery from the inhibition was again in seconds for lignocaine and minutes for SUN 1165. These results suggests that the rates of binding to and release from the sodium channels are much slower for SUN 1165 than for lignocaine.

In order to know more certainly whether lignocaine and SUN 1165 block open Na channels, we used scorpion toxin to keep the channels open for a considerably longer time. We succeeded in recording a persistent I_{Na} from cardiomyocytes treated with scorpion toxin (Figure 3a) and examined the effects of these drugs on it. We found that the rate of inhibition by lignocaine occurred in a few milliseconds when the agent was applied during the depolarizing pulse, which was not the case for SUN 1165 (Figure 3). The prompt acceleration of the rate of inhibition of the persistent I_{Na} by lignocaine is reminiscent of the rapid interaction between the drug and open sodium channels and indicates that lignocaine preferentially blocks the open state of Na channels rather than the rested one because the time course of the inhibitory action of the agent, as shown in Figure 1 and also in scorpion toxin-treated cells, is much slower when the agent is applied before the depolarizing pulse. In contrast, SUN 1165 had almost no instantaneous effect on the persistent I_{Na},

although, in the continued presence of SUN 1165, the decay phase of persistent I_{Na} was greatly accelerated when the drug effect on the peak amplitude of the persistent I_{Na} reached the steady state. These results suggest that SUN 1165 also blocks the open state of sodium channels.

However, it may be argued that both lignocaine and SUN 1165 cause a simple displacement of scorpion toxin from its binding site which results in an apparent acceleration of the decay phase of I_{Na}. This seems unlikely because Class I-type antiarrhythmic agents such as procainamide do not inhibit the sea anemone toxin binding to the sodium channels (Sheldon *et al.*, 1987) to which scorpion toxin also binds (Catterall & Beress, 1978). It is also possible that scorpion toxin makes another channel carry the additional inward current. This is also unlikely because the membrane potential at which the maximum peak current amplitude was produced and the reversal potential does not change in the presence of scorpion toxin (Narahashi *et al.*, 1972). Thus, the marked acceleration of the rate of inhibition of the persistent I_{Na} by lignocaine as well as by SUN 1165 is undoubtedly due to the blockade of open sodium channels by these agents.

In general, the clinically useful antiarrhythmic agents have a low affinity for the rested state of sodium channels, while they possess a higher affinity for the open or inactivated state (Hondeghem & Katzung, 1984). An inhibitory action of both lignocaine and SUN 1165 on the inactivated state of sodium channels was previously demonstrated in isolated single cardiomyocytes (Lee *et al.*, 1981; Yatani *et al.*, 1984; Inomata *et al.*, 1987). From the present results which were obtained by applying a 'concentration-clamp' technique that made it possible to analyse the precise time course of drug action, we suggest that both lignocaine and SUN 1165 bind preferentially to the open rather than the rested state of the sodium channel although SUN 1165 does so slowly.

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Endothelin induces an increase in renal vascular resistance and a fall in glomerular filtration rate in the rabbit isolated perfused kidney

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1 The effects of endothelin infusion on renal vascular resistance (RVR), glomerular filtration rate (GFR) and the interaction with locally generated endothelium-derived relaxant factor (EDRF) were studied in the rabbit isolated perfused kidney (IPK). For comparison the effects of infusions of angiotensin II (AII) and noradrenaline (NA) were also assessed.

2 Each kidney was perfused at a constant rate of 10 ml min^{-1} and alterations in RVR determined by measuring changes in perfusion pressure. GFR was determined by the clearance of [^{51}Cr]-EDTA, using timed urine collections.

3 Endothelin (10^{-11}–10^{-9} M) produced a dose-related increase in RVR. Endothelin was approximately 30 times more potent in molar terms than AII and 500 times more than NA at inducing a 50 mmHg increase in perfusion pressure.

4 Endothelin appeared to be a weak inducer of EDRF release in the IPK as EDRF inhibitors methylene blue (10 μM) or haemoglobin (10 μM) only slightly augmented the increase in RVR at a given concentration of endothelin. In contrast the effect of NA on RVR was significantly increased by methylene blue (10 μM) whereas that induced by AII was not affected.

5 Endothelin infusion produced a significant, dose-dependent decrease in GFR of the IPK, contrasting with an increase in GFR during AII infusion and a minimal effect of NA on GFR. This supports evidence that AII is predominantly a constrictor of efferent glomerular arterioles and that NA constricts both afferent and efferent glomerular vessels. We suggest that the vasoconstrictive effect of endothelin in the kidney is predominantly preglomerular, which explains its effect on GFR.

Introduction

The importance of the endothelium in the regulation of vascular tone has become increasingly evident in recent years (Vanhoutte, 1988). The endothelium releases a variety of vasoactive factors including the prostaglandins prostacyclin (PGI_2) and prostaglandin E_2 (PGE_2) (MacIntyre *et al.*, 1978; Ali *et al.*, 1980), endothelium-derived relaxant factor (EDRF) (Furchgott & Zawadski, 1980) and the recently described vasoactive peptide endothelin (Yanagisawa *et al.*, 1988). The precise role these factors play in regulating vascular tone in health and disease is, as yet, unclear.

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The 21 amino acid peptide endothelin is a very potent vasoconstrictor *in vitro* (Yanagisawa *et al.*, 1988); in molar terms it is 20–50 times more potent than angiotensin II which was previously one of the most potent vasoconstrictor peptides known. Its effect *in vivo* is less clear as, in some vascular beds, it is a vasoconstrictor whereas, in others, it is a vasodilator (Lippton *et al.*, 1988; Wright & Fozard, 1988). The precise site of origin of endothelin is unclear as the endothelium of the microcirculation seems to be unable to synthesize endothelin (Yanagisawa *et al.*, 1988). However, endothelin is capable of producing an effect distal to, and therefore downstream of, its site of release (Yanagisawa *et al.*, 1988), suggesting that it may have a role in the control of vascular tone within the microcirculation.

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The role of endothelin in the control of blood flow within individual organs and its interaction with other endothelium-derived vasoactive factors such as EDRF is unknown. We have investigated the effects of endothelin infusion on renal vascular resistance and glomerular filtration rate (GFR) in a rabbit isolated perfused kidney model (IPK), and compared it with the known vasoconstrictors angiotensin II (AII) and noradrenaline (NA). We have also studied the interaction between these vasoactive factors and EDRF generated within the kidney.

Methods

Isolated perfused kidney

Each rabbit was anaesthetized with pentobarbitone (Rhone Poulenc) 40–60 mg kg^{-1} i.v. and ventilated with air following surgical tracheal intubation. Frusemide (4 mg), which exerts a protective effect against hypoxic damage to the kidney (Myers & Moran, 1986), was administered intravenously at induction of anaesthesia. Laparotomy via a midline incision was performed and the left kidney exposed. The left renal artery was cannulated directly with polythene tubing (Portex 800/100/260/100), such that the average warm ischaemic time was 30–60 s. The kidney was initially perfused *in situ* at 4°C with 100 ml of KHB/AA which contained indomethacin 5.6 μM. Cold perfusate was used as we found that cooling the kidney prior to handling and removal improved function of the IPK. The left ureter was cannulated with polythene tubing (Portex 800/100/340/100) and the kidney was then removed and placed in an organ chamber where it was perfused with KHB/AA (containing indomethacin 5.6 μM) warmed to 37°C and gassed with 95% O_2/5% CO_2. Venous effluent was discarded, i.e. a non return system was used. Each kidney was perfused at a fixed rate of 10 ml min^{-1} throughout each experiment and the perfusion pressure at the renal artery was monitored continuously via a Statham pressure transducer. Drugs were infused at 0.12 ml min^{-1} into the circuit prior to the main perfusion pump.

Changes in perfusion pressure were measured as the maximal increase in pressure (mmHg) during infusion of each agent and results expressed as mean ± s.e.mean.

In some kidneys a vascular tone was induced with a continuous infusion of noradrenaline 150 nM (Edwards, 1983) and the perfusion rate adjusted to produce a perfusion pressure of 90 mmHg. The responses to infusion of endothelin (range 10^{-15}–10^{-10} M) were assessed.

Measurement of renal function in IPK

GFR of each isolated kidney was determined by clearance of [^{51}Cr]-EDTA. [^{51}Cr]-EDTA (0.5 MBq) was added to the KHB/AA and urine was collected over 5 min intervals, volume being measured gravimetrically. Urine and perfusate counts were determined and GFR calculated (Ross *et al.*, 1973). Each agent was infused until a steady perfusion pressure had been achieved for 5 min and then GFR was measured for a further 5 min. After each agent was stopped baseline perfusion pressure was reached and after a further 5 min GFR was again measured. Results are calculated as percentage change in GFR during infusion of each agent (GFR during infusion compared with the mean of GFR pre and post infusion) and expressed as mean ± s.e.mean.

Materials

The isolated perfused kidney (IPK) was perfused with Krebs-Henseleit buffer supplemented with 20 essential and non-essential amino acids (KHB/AA) (Epstein *et al.*, 1982). Albumin or other oncotic agents were not added to the perfusate as a non return system was employed; large quantities of perfusate were used in each experiment. Hypo-osmolar perfusate does not significantly affect vascular responses of GFR in the IPK, osmolality of the perfusate being more important for tubular function (Ratcliffe *et al.*, 1986). The perfusate had the following final electrolyte concentrations (mmol l^{-1}): NaCl 118, KCl 4.75, $MgSO_4$ 1.19, $NaHCO_3$ 25, K_2HPO_3 1.19, $CaCl_2$ 1.9, glucose 11.1. The amino acids were added by using 100 ml of Vamin 14 Electrolyte Free solution (Kabi-Vitrium) per 10 l of final solution (KHB/AA); this produced the required final concentrations (Epstein *et al.*, 1982). Indomethacin (Sigma) was dissolved in absolute alcohol to a concentration of 2 mg ml^{-1} and then added to KHB/AA to a final concentration of 5.6 μM (2 μg ml^{-1}) which is sufficient to inhibit completely cyclo-oxygenase activity (Ferreira *et al.*, 1971). Buffer was then filtered through a 0.45 μm filter (Millipore, UK).

Porcine endothelin (Peptide Institute, Osaka) was dissolved in 0.9% saline to a final concentration of 50 μM and stored in aliquots at −20°C. For each experiment an aliquot was diluted in 0.9% saline to appropriate concentrations, samples being stored on ice before use. Methylene blue (Sigma) was dissolved in 0.9% saline for each experiment. Haemoglobin (Sigma) was dissolved in distilled water to a concentration of 1 mM, converted to the ferrous form by addition of sodium dithionite 10 mM and then dialysed twice against 100 volumes of distilled water for 2 h to remove the sodium dithionite (Martin *et al.*, 1985). The solution was then stored at −20°C for up

to 2 weeks before use, further dilutions were made in 0.9% saline. AII (Sigma) was dissolved in ethanol to a concentration of 1 mM and stored at −20°C, further dilutions were in 0.9% saline. NA (Sigma) was dissolved in 0.9% saline to appropriate concentrations on the day of each experiment. All concentrations refer to final concentration in the KHB/AA in the IPK.

Statistics

Multifactor analysis of variance with a least-squares difference test was used to compare responses with and without the EDRF inhibitors methylene blue and haemoglobin. $P < 0.05$ was accepted as indicating statistical significance.

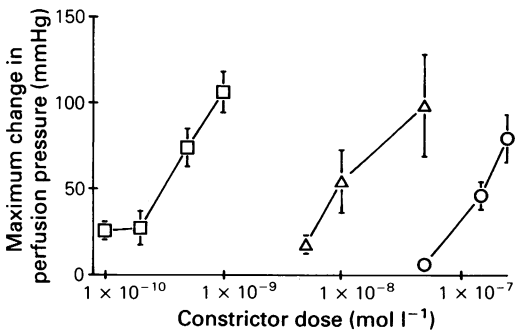


Figure 1 Maximum change in perfusion pressure of the rabbit isolated perfused kidney induced by the constrictor agents (□) endothelin ($n = 6$), (△) angiotensin II ($n = 8$) and (○) noradrenaline ($n = 8$). Each point represents the mean and vertical lines show s.e.mean.

Results

Basal perfusion pressure, prior to infusion of vasoactive agents, was 50.0 ± 4.2 mmHg (mean ± s.e.mean).

Endothelin was a potent vasoconstrictor (Figure 1), producing a detectable increase in perfusion pressure at a concentration of 10^{-10} M (range 10^{-10} to 10^{-9} M). In comparison AII and NA, given alone, produced similar vasoconstrictor responses in the concentration ranges 5–50 × 10^{-9} M and 50–250 × 10^{-9} M respectively (Figure 1). The calculated dose of endothelin required to produce a 50 mmHg increase in perfusion pressure was 3.4 × 10^{-10} M whereas those for AII and NA were 9.8 × 10^{-9} M and 170 × 10^{-9} M, respectively. The duration of effect of equipotent doses of endothelin, AII and NA was similar.

Simultaneous infusion of the EDRF inhibitor methylene blue 10 μM did not affect the constrictor response to endothelin (Figure 1a), although haemoglobin 10 μM did significantly increase the constrictor response to endothelin ($P < 0.01$) (Figure 2a). In our model, methylene blue 10 μM significantly increased the constrictor response to NA ($P < 0.01$) but not to AII (Figure 2c,b).

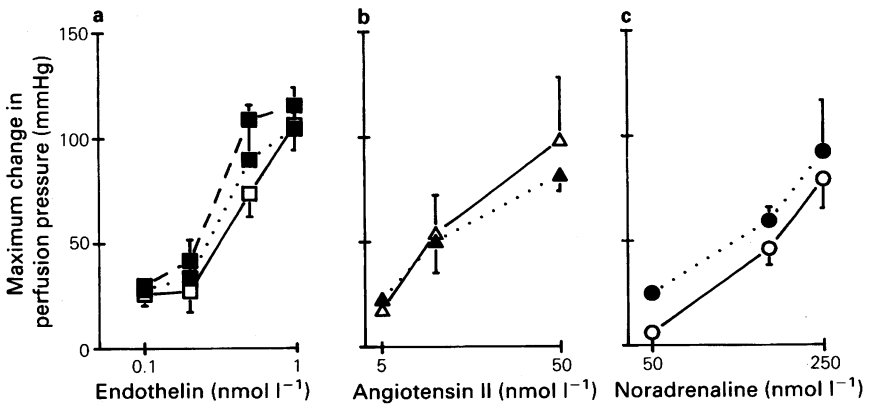


Figure 2 Maximum change in perfusion pressure of the rabbit isolated perfused kidney induced by the constrictor agents in the absence and presence of inhibitors of EDRF. (a) (□) Endothelin alone ($n = 6$), (■.....■) endothelin + methylene blue (10 μM) ($n = 6$), (■– – –■) endothelin + haemoglobin (10 μM) ($n = 5$) ($P < 0.01$ vs endothelin alone). (b) (△) Angiotensin II alone ($n = 8$), (▲) angiotensin II + methylene blue (10 μM) ($n = 6$) (c) (○) noradrenaline alone ($n = 8$), (●) noradrenaline + methylene blue (10 μM) ($n = 6$) ($P < 0.01$ vs noradrenaline alone). Each point represents the mean and vertical lines show s.e.mean.

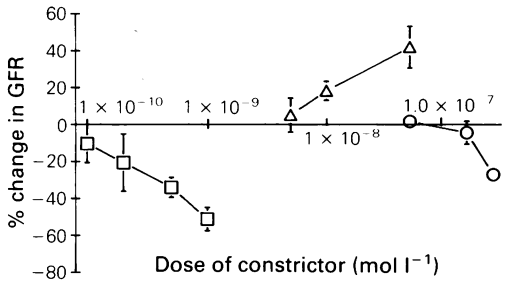


Figure 3 Effect of the constrictor agents endothelin (□) ($n = 6$), angiotensin II (△) ($n = 8$) and noradrenaline (○) ($n = 8$) on the glomerular filtration rate (GFR) of the rabbit isolated perfused kidney. Each point represents the mean and vertical lines show s.e.mean.

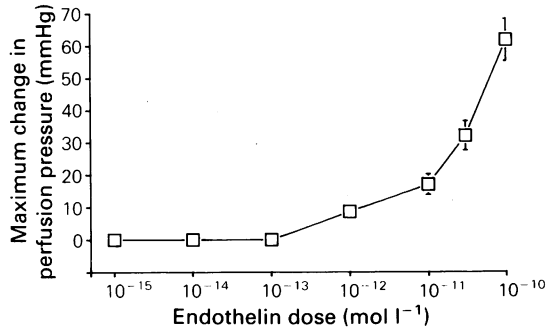


Figure 5 Effect of endothelin on perfusion pressure of rabbit isolated perfused kidneys preconstricted with noradrenaline (150 nM). Each point represents the mean ($n = 6$) and vertical lines show s.e.mean.

Endothelin infusion produced a marked and dose-dependent decrease in GFR of the IPK; 10^{-9} M induced a 52% fall in GFR (Figure 3). Higher doses of endothelin (e.g. 10^{-8} M) produced a virtual cessation of urinary flow associated with a marked and sustained increase in the perfusion pressure, with no recovery of urine flow during a subsequent hour. NA altered GFR only at higher doses, inducing a slight fall, whereas AII, in contrast, produced a marked dose-dependent increase in GFR (Figure 3).

Simultaneous infusion of the EDRF inhibitors methylene blue and haemoglobin did not affect the changes in GFR that occurred with infusion of endothelin (Figure 4).

When vascular tone was induced in the IPK by a continuous infusion of NA 150 nM, the vasoconstrictive effect of endothelin was detectable at concentrations as low as 10^{-12} M; lower concentrations of endothelin, down to 10^{-15} M, resulted in neither vasoconstriction nor vasodilatation (Figure 5).

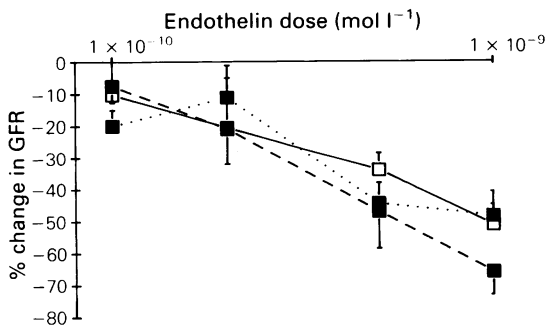


Figure 4 Effect of the EDRF inhibitors methylene blue and haemoglobin on the change in glomerular filtration rate (GFR) induced by different doses of endothelin. (□——□) Endothelin alone ($n = 6$), (■.....■) endothelin + methylene blue (10 μM) ($n = 6$), (■- - -■) endothelin + haemoglobin (10 μM) ($n = 5$). Each point represents the mean and vertical lines show s.e.mean.

Discussion

Our results indicate that endothelin is a very potent vasoconstrictor in the rabbit IPK, being approximately 30 times more potent in molar terms than AII and 500 times more potent than NA. Experiments *in vivo* suggest that endothelin may be a vasodilator in some vascular beds, including, at least initially, the kidney (Lippton *et al.*, 1988; Wright & Fozard, 1988). We detected no evidence of vasodilatation due to endothelin in the IPK. Isolated organs, including the kidney, have a very poor vascular tone when a cell-free perfusate is employed and, in such a model, any vasodilator response may not be detected. However, when a vascular tone was induced in the IPK with NA, which constricts both pre- and post-glomerular vessels (Edwards, 1983), endothelin, at concentrations as low as 10^{-15}–10^{-13} M, failed to produce a vasodilator response. This suggests that, at least in the rabbit IPK, vasodilatation is not an effect of endothelin. The short lived vasodilatation to endothelin that occurs *in vivo* may reflect a reflex response of the kidney which is lost in the IPK model.

Although endothelin *in vivo* has a prolonged duration of action, in the IPK we did not find that its effect was any more longer lasting than either AII or NA. This may merely reflect a washout effect in a non return perfusion system *in vitro*. However, the plasma half-life of endothelin *in vivo* is probably very short as approximately 60% is removed by the lungs

during a single pass (de Nucci *et al.*, 1988). Therefore the effect *in vivo* may be the result of an interaction of endothelin with some blood-derived or neural component, both of which are absent in the IPK. It is unlikely that the prolonged effect *in vivo* is due to a sustained transduction mechanism in vascular smooth muscle as this would presumably also be seen in the IPK.

Endothelin also produced a substantial fall in GFR in the IPK, in contrast with the increase in GFR that occurred with equipotent concentrations of AII. This effect of endothelin is in agreement with recent evidence in a rat IPK model where endothelin was a renal vasoconstrictor and lead to a substantial fall in GFR (Firth *et al.*, 1988). AII is both a contractor of rat mesangial cells (Ausiello *et al.*, 1980) and a constrictor predominantly of the efferent glomerular arteriole in the rabbit (Edwards, 1983) and the rat (Myers *et al.*, 1975). These two actions of AII have opposing effects on the GFR; mesangial cell contraction leads to a fall in the filtration coefficient (K_f) and therefore GFR, whereas increased efferent arteriolar tone increases the transglomerular basement membrane pressure (ΔP) and therefore GFR. As GFR increased with AII infusion, the effect of increased ΔP must outwiegh the fall in K_f. NA increases symmetrically the tone in both pre and post glomerular arterioles (Edwards, 1983), which should maintain ΔP at a relatively constant level. The minor fall in GFR of the IPK that occurred only with higher concentrations of NA supports this.

As the GFR fell substantially with endothelin infusion, this suggests that endothelin is predominantly a constrictor of preglomerular vessels, resulting in a fall in ΔP and therefore in GFR. This is particularly true in our IPK model where the perfusate flow rate is fixed and therefore unable to influence GFR. Recent evidence in cultured mesangial cells of the rat and an *in vivo* rat model suggests that endothelin both contracts mesangial cells and *in vivo* produces a substantial fall in K_f (Badr *et al.*, 1989). This is somewhat surprising since AII, as already stated, is a potent contractor of mesangial cells and yet does not produce a fall in GFR, presumably indicating that K_f does not fall substantially. This suggests that the effect of endothelin in reducing K_f may in some way be separate from its ability to contract mesangial cells. Alternatively, endothelin may be stimulating intrarenal AII generation, thereby increasing efferent arteriolar resistance and contracting mesangial cells, possibly in synergy with endothelin. Although endothelin inhibits activation of the renin angiotensin system (RAS) by rat isolated cortical cells (Takagi *et al.*, 1988) and isolated glomeruli (Rakugi *et al.*, 1988), it activates the *RAS in vivo*, producing an increase in plasma renin activity (Miller *et al.*, 1989). The effect of angiotensin converting enzyme inhibitors and angiotensin II antagonists on the response to endothelin both in the IPK and *in vivo* would therefore be of particular interest.

EDRF is involved in the control of renal vascular resistance in the IPK (Bhardwaj & Moore, 1988) and *in vivo* (Conger *et al.*, 1988), although the distribution of its effect and its site of origin is unclear. The relative inability of the EDRF inhibitors methylene blue and haemoglobin to augment the vasoconstrictor response to endothelin suggest that endothelin, like AII and unlike NA, does not, to any great extent, induce release of EDRF. In the rat mesenteric vascular bed endothelin induces the release of EDRF (de Nucci *et al.*, 1988). The interaction between these two potent endothelium-derived vasoactive factors is obviously important and may well vary between vessel beds and species.

In summary, endothelin, in the rabbit IPK, is a potent vasoconstrictor, probably predominantly of preglomerular arterioles, and produces a significant fall in GFR. As such it may play a causal role in the pathogenesis of a variety of conditions where reductions in renal blood flow and GFR occur together, for example acute renal failure (Firth *et al.*, 1988) and cyclosporin nephrotoxicity (Thiel, 1986).

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Effects of verapamil on ischaemia-induced impairment of ATP-dependent calcium extrusion in rat heart sarcolemma

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1 The effects of ischaemia and reperfusion were studied on adenosine 5'-triphosphate (ATP)-dependent $^{45}Ca^{2+}$-transport in rat heart sarcolemma vesicles.

2 The effect of verapamil, 1 μmol l^{-1}, was studied by pretreatment of the hearts during Langendorff-perfusion and *in vitro* by adding the drug after isolation of the vesicles.

3 Without drug pretreatment the Ca^{2+}-uptake appeared to be strongly reduced after 30 and after 60 min of global ischaemia, whereas after 30 min of reperfusion it was restored to slightly above the control level.

4 Verapamil pretreatment during the Langendorff perfusion significantly increased Ca^{2+}-uptake in sarcolemma vesicles both before the onset of ischaemia and after 30 min of reperfusion, whereas no beneficial effect was found on the impaired uptake activity during the ischaemic period.

5 When tested *in vitro* after the isolation of the sarcolemma vesicles, verapamil only inhibited the Ca^{2+}-uptake activity with an IC_{50} of 112 μmol l^{-1}, which was increased to 250 μmol l^{-1} after ischaemia and reperfusion.

6 The present study indicates that pretreatment with verapamil, 1 μmol l^{-1}, of the intact rat heart activates an ATP-dependent Ca^{2+} extrusion process that may contribute to decrease cellular calcium levels in control and, more importantly, in a reperfusion situation. In contrast, *in vitro* only a less potent inhibition of the extrusion process was found, indicating that physiological regulatory mechanisms may be altered in the vesicles.

Introduction

Calcium ions have a central role in the regulation of many cellular processes, ranging from intracellular oxidative metabolism and enzyme activities to muscle contraction, hormone and neurotransmitter release and membrane permeability (Dhalla *et al.*, 1984; Rasmussen & Barrett, 1984). The large Ca^{2+}-concentration gradient between the extra- and intracellular space and the fact that most Ca^{2+}-dependent processes require a relatively narrow concentration range for optimal activity can be handled by a series of high and low affinity Ca^{2+}-transporting and Ca^{2+}-binding proteins (Dhalla *et al.*, 1984; Carafoli, 1987).

In the ischaemic heart the impairment of the Ca^{2+}-homeostasis plays an important role in cell damage (Carafoli, 1987) both in an early and in a later phase. Almost immediately after the onset of ischaemia a lack in O_2 supply and in glucose leads to the deprivation of high energy phosphates and changes in Na^+ and K^+ permeability (Vary *et al.*, 1981; Reimer & Jennings, 1981; Nayler *et al.*, 1987). It was recently proposed by Barry *et al.* (1987) that an early rise of intracellular Ca^{2+} in chick ventricular cells as a result of an impaired Ca^{2+}-resequestration in the sarcoplasmic reticulum might activate Ca^{2+}-ATPases and hasten ATP depletion, leading to a subsequent rigor contraction. This effect was observed to develop in several species within 15 to 60 min of global ischaemia (Cavero *et al.*, 1983; Wexler *et al.*, 1986; Van Amsterdam *et al.*, 1988). In long-term ischaemia the storing capacity of the mitochondria becomes insufficient and membrane permeability for Ca^{2+}-ions increases, resulting in calcium overload and membrane damage, an irreversible kind of injury that ultimately leads to cell necrosis (Carafoli, 1987). Another situation in which

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Ca^{2+}-influx is greatly increased and may play an important role in tissue damage is reperfusion, especially when accompanied by a calcium-paradox situation, in which the tissue has been perfused in the absence of Ca^{2+}-ions (Zimmerman & Hülsmann, 1966; Murphy *et al.*, 1987).

The increased Ca^{2+}-influx during ischaemia is counteracted by the cell by activation of extrusion mechanisms, such as Na^+/Ca^{2+}-exchange and ATP-dependent Ca^{2+}-transport. Verapamil, which was originally introduced as a coronary vasodilator (Haas & Hartfelder, 1962), is best known for inhibition of the slow inward calcium channel (Fleckenstein, 1977). However, in recent years it appeared that the drug affects other processes not directly related to the channel, such as the ATP-dependent Ca^{2+}-transport and Na^+/Ca^{2+}-exchange in plasmalemma and sarcoplasmic reticulum fractions (Mas-Oliva & Nayler, 1980; Erdreich *et al.*, 1983; Wang *et al.*, 1984; Van Amsterdam & Zaagsma, 1986). Previously, we have shown that the rat isolated sarcolemma preparation consists of approximately 50% inside-out and 50% right side-out vesicles (Velema & Zaagsma, 1981) and that more than 90% of the ATP-dependent Ca^{2+}-uptake was localized in the inside-out fraction (Velema *et al.*, 1987), indicating that we are actually measuring a cellular calcium extrusion process.

On the basis of these observations, it was considered of interest to study the effect of ischaemia and reperfusion as well as verapamil pretreatment on the adenosine 5'-phosphate (ATP)-dependent Ca^{2+}-uptake in rat sarcolemma vesicles. In particular, we have investigated the uptake activity after normoxic, ischaemic and reperfusion conditions. The effects of verapamil on this parameter were studied both during the Langendorff perfusion (i.e. prophylactically) and *in vitro*, after the isolation of the sarcolemma vesicles.

Methods

Perfusion of the hearts

The preparation of the hearts has been described previously (Van Amsterdam *et al.*, 1987). Briefly, male Wistar rats (200–240 g) were anaesthetized with diethylether and subsequently given heparin (250 i.u.) through the penile vein. The thorax was opened just below the sternum, the aorta was prepared free of connective tissue and a cannula was introduced *in situ* through a small incision in the aorta wall. The heart was taken out and immediately perfused at a hydrostatic pressure of 8 kPa with a Krebs-Henseleit buffer solution of the following composition ($mmol\,l^{-1}$): NaCl 111, $NaHCO_3$ 25, $MgSO_4$ 1.18, $CaCl_2$ 2.52, NaH_2PO_4 1.28 and D-(+)-glucose 5.55. The buffer solution was gassed with 95% O_2 and 5% CO_2, and kept at 37°C and pH 7.4. For each experiment 10 hearts were prepared in this way within 20 min and perfused simultaneously on a perfusion carousel. The organ chamber was maintained at 37°C with warm, humidified air.

The hearts were left to equilibrate for 30 min. Then, two hearts were removed and eight hearts were perfused in the presence of verapamil, $1\,\mu mol\,l^{-1}$, for 15 min. Again two hearts were taken from the carousel, the flow was stopped and the six remaining hearts were immersed in a large volume of Krebs-Henseleit (at 37°C and gassed with 95% N_2 and 5% CO_2) in order to prevent adiabatic cooling at the heart surface due to evaporation of the buffer solution. Two hearts were taken from the caroussel after 30 and after 60 min of global ischaemia, whereas the last two hearts were subsequently reperfused in the absence of the drug for 30 min. During this reperfusion period the hearts started to beat spontaneously within 5 min.

Isolation of the purified sarcolemma

Heart sarcolemma (SL) vesicles were prepared essentially as described before (Velema & Zaagsma, 1981; Van Amsterdam & Zaagsma, 1986), with a slight modification of the differential centrifugation method. The ventricles (2 at a time) were homogenized in 20 ml Tris buffer, containing Tris-HCl $20\,mmol\,l^{-1}$ and EDTA $1\,mmol\,l^{-1}$ (pH 7.0) at 4°C, in a Virtis blender for four periods of 7 s at 20,000 r.p.m. separated by 7 s intervals. The homogenate was centrifuged for 20 min at 8,800 *g*, with an 8 × 50 aluminum fixed angle rotor in a MSE 65 ultracentrifuge. The supernatants were set apart, whereas the pellets were resuspended by 20 strokes in a glass vibration potter tube and recentrifuged at the same speed. The combined supernatants were centrifuged by two successive 20 min runs at 12,500 *g*, followed by a 60 min run at 44,000 *g*. The resulting pellet was resuspended in 20 ml Tris buffer, containing Tris-oxalate $20\,mmol\,l^{-1}$, KCl $150\,mmol\,l^{-1}$ and EDTA $1\,mmol\,l^{-1}$ (pH 6.8) and recentrifuged at 44,000 *g*. Aliquots of the purified sarcolemma vesicles were taken up in small volumes of HEPES $40\,mmol\,l^{-1}$ and KCL $75\,mmol\,l^{-1}$ KCl at pH 6.8 and stored immediately under liquid nitrogen. Protein was measured by the Lowry method with bovine serum albumin as a reference.

ATP-dependent $^{45}Ca^{2+}$-uptake

ATP-dependent, oxalate-facilitated Ca^{2+}-uptake was determined at a free Ca^{2+}-concentration of $10\,\mu mol\,l^{-1}$ as described by Van Amsterdam &

Zaagsma (1986) using a medium of (mmol l^{-1}): HEPES 40, $MgCl_2$ 10, KCl 75, Tris-oxalate 2.5, EGTA 0.087 and $^{45}CaCl_2$ 0.125 (approx. 10,000 c.p.m. $nmol^{-1}$) at a pH of 6.8. The vesicles were preincubated in a total volume of 300 μl for 10 min at 30°C and the reaction was started by addition of 50 μl of ATP (35 mmol l^{-1}), giving a final ATP concentration of 5 mmol l^{-1}. The incubation reaction was terminated after 20 min by rapid suction filtration of the mixture through Millipore HAWP filters (pore size 0.45 μm), followed by 3 washes with 2 ml of ice cold HEPES buffer. The filters were solubilized in 3.5 ml Lumagel and counted in a Beckman LS 1701 scintillation counter. The difference of the $^{45}Ca^{2+}$ taken up in the presence and absence of ATP was taken as specific Ca^{2+}-uptake. Nonspecific uptake under control conditions represented about 10% of total uptake (Figure 1).

Drugs

$^{45}Ca^{2+}Cl_2$ was obtained from New England Nuclear, Boston (U.S.A.). Verapamil hydrochloride was a gift from Knoll, Ludwigshafen (F.R.G.). All other reagents were of analytical grade. Verapamil was dissolved at 5 mmol l^{-1} in ultrafiltered water and diluted in the appropriate incubation buffer solution.

Statistics

Differences between control and treated groups were evaluated by means of one-way analysis of variance (Grimm, 1973). Differences were considered to be significant at $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***).

Results

Purity of the membranes

Previously (Velema *et al.*, 1983) we have characterized in detail the purity of the SL preparation. Because of an alteration in the isolation procedure, involving a change from 600 to 150 mmol l^{-1} KCl in the extraction of the contractile proteins, we have compared the ouabain-sensitive Na^+/K^+-ATPase activity in both sarcolemma fractions. We found an activity of 103 ± 10 μmol Pi mg^{-1} h^{-1} ($n = 5$) in the former and 102 ± 2 μmol Pi mg^{-1} h^{-1} ($n = 4$) in the latter sarcolemma preparation, indicating a purification factor of 13 to 15 with respect to the homogenate (7–8 μmol Pi mg^{-1} h^{-1}), which was not different for the two isolation procedures.

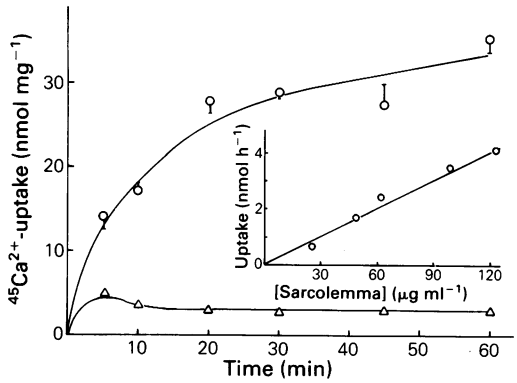


Figure 1 Time-dependent $^{45}Ca^{2+}$-uptake in rat cardiac sarcolemma vesicles in the presence (○) and absence (△) of ATP. Inset: dependence of $^{45}Ca^{2+}$-uptake on the protein concentration. ATP and free Ca^{2+}-ion concentrations were 5 mmol l^{-1} and 10 μmol l^{-1} respectively.

Characterization and modulation of the Ca^{2+}-uptake process

The relation between the ATP-dependent ^{45}Ca-uptake process and the protein concentration is shown in Figure 1 (inset), indicating that up to 120 μg ml^{-1} sarcolemma no deviation from linearity was observed. Figure 1 shows the time course of the uptake in the purified sarcolemma vesicles of normoxic hearts. A standard incubation period of 20 min at approximately 60 μg ml^{-1} was chosen for the subsequent experiments.

The level of the ATP-dependent $^{45}Ca^{2+}$-uptake appeared to be dependent on the heart perfusion. When global ischaemia was started by stopping the coronary flow, the specific Ca^{2+}-uptake declined from 31.44 ± 4.14 nmol mg^{-1} h^{-1} to 11.01 ± 2.07 nmol mg^{-1} h^{-1} after 30 min, and then remained stable until 60 min of ischaemia (Figure 2). At reperfusion, the activity of the Ca^{2+}-transport system was restored to even higher values than before the onset of ischaemia and amounted to 42.18 ± 5.04 nmol mg^{-1} h^{-1} after 30 min of reperfusion.

When the hearts were pretreated for 15 min with verapamil (1 μmol l^{-1}) the mere presence of the drug induced a 110% increase in the ATP-dependent $^{45}Ca^{2+}$-uptake to 65.97 nmol mg^{-1} h^{-1}. However, the uptake in membranes isolated after 30 and 60 min of global ischaemia in the presence of the drug was comparable to that from the corresponding drug-free membranes. At reperfusion, which was performed with buffer-solution without verapamil in all preparations, the uptake in the verapamil-treated membranes was significantly higher than in the non-treated membranes (72.54 and 31.44 nmol mg^{-1} h^{-1}, respectively.

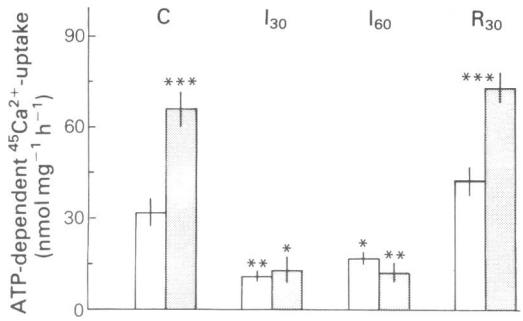


Figure 2 ATP-dependent $^{45}Ca^{2+}$-uptake in rat cardiac sarcolemma vesicles. C = control, I_{30} and I_{60} = uptake after 30 and 60 min of global ischaemia, R_{30} = uptake after 30 min of reperfusion. Open bars represent control conditions ($n = 15$), shaded bars pretreatment with 1 μmol l^{-1} verapamil ($n = 14$). Data are mean values (s.e.mean shown by vertical lines) derived from triplicate experiments from 4–5 separate membrane batches. Differences with respect to control were considered significant at * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

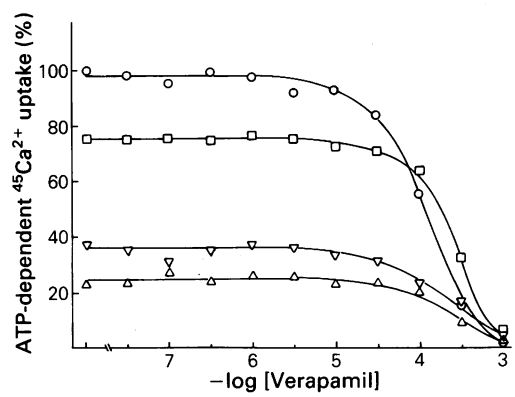


Figure 3 Effect of verapamil on ATP-dependent $^{45}Ca^{2+}$ uptake in rat cardiac sarcolemma vesicles from membranes before (○), after 30 (△) and 60 (▽) min of global ischaemia and after 60 min of global ischaemia plus 30 min of reperfusion (□). Data represent the mean from two triplicate experiments, performed on separate membrane batches.

As we have previously shown (Van Amsterdam & Zaagsma, 1986), the direct inhibitory effect of verapamil on the ATP-dependent $^{45}Ca^{2+}$-uptake in sarcolemma membranes is observed from 10 to 1000 μmol l^{-1} verapamil, which is a much higher concentration than that required for the inhibition of the slow calcium current. Because of the potent stimulant effect of 1 μmol l^{-1} verapamil pretreatment on sarcolemmal Ca^{2+}-uptake, we have also studied the effect of the drug *in vitro* on sarcolemma vesicles from normoxic, ischaemic and reperfused hearts. As is shown in Figure 3, the ATP-dependent $^{45}Ca^{2+}$-uptake is not increased at any concentration of verapamil ranging from 30 nmol l^{-1} to 1 mmol l^{-1}. In fact, the uptake system is only inhibited by the drug, starting at 10–30 μmol l^{-1}. The IC_{50} values for this inhibition were 250 μmol l^{-1} for the ischaemic and the reperfusion fraction, whereas the control vesicles were inhibited slightly more potently with an IC_{50} value of 112 μmol l^{-1}.

Discussion

The results of this study show that 30 and 60 min of global ischaemia reduce the activity of the ATP-dependent $^{45}Ca^{2+}$-uptake in rat heart sarcolemma vesicles by 50% and more, whereas after 30 min of reperfusion this process is completely restored (Figure 2). The reduction of this Ca^{2+}-extrusion process in ischaemic conditions may be a factor contributing to the development of cell damage, because the cellular Ca^{2+} concentration is elevated. While the concentration of ATP in the *in vitro* determination of the enzyme activity is constant and does not represent the rate limiting step, some other mechanism that may be indirectly related to the low level of high-energy phosphates occurring during ischaemia (Bersohn & Shine, 1983; Lavanchy *et al.*, 1986) is likely to reduce the enzyme activity. This process, however, is not irreversible, because after 30 min of reperfusion, uptake was restored to control levels. An autoregulatory mechanism at the cardiac level, restoring Ca^{2+}-pump activity at reperfusion may be a possible explanation of this phenomenon.

Verapamil at negative inotropic concentrations may have diverse effects on the ATP-dependent $^{45}Ca^{2+}$-uptake in heart sarcolemma vesicles:

When the drug is given at 1 μmol l^{-1} for 15 min during a Langendorff perfusion, a concentration at which the negative inotropic effect of verapamil reduces contractility to about 5% of control (Van Amsterdam *et al.*, 1987), the activity of the uptake is 2.1 times higher than the drug-free control membranes (Figure 2). Therefore, we can conclude that verapamil not only inhibits the slow inward calcium current but also promotes the outward calcium flux when given in an *ex vivo* organ perfusion. These two different mechanisms cooperate in lowering the intracellular Ca^{2+}-concentration.

The stimulant (protective) effect of verapamil was no longer apparent, however, when the hearts were made ischaemic by a complete cessation of the coronary flow. In this case the Ca^{2+}-uptake activity of both the drug-free and the verapamil-pretreated vesicles was lowered to 30–50% of the control value. A

possible explanation may be that the ischaemia-induced impairment of the enzyme activity has reached a level which can no longer be modulated by verapamil. It could also be imagined that accumulation of verapamil in the tissue before and during the ischaemic period would gradually result in such high intracellular concentrations that the Ca^{2+}-pump is being inhibited again, which would counteract a possible stimulant effect. However, Pang & Sperelakis (1983) have shown in cat papillary muscle and chick embryonic ventricular muscle that with [^{3}H]-verapamil, 1 μmol l^{-1}, even after 120 min incubation, T/M ratios did not exceed 11.6. Therefore, accumulated verapamil will not reach the intracellular concentration level, required for direct inhibition of the sarcolemmal ATP-dependent Ca^{2+}-extrusion (see below).

After 60 min of global ischaemia and 30 min of reperfusion, the ATP-dependent uptake activity in the sarcolemma vesicles was increased by 34% to 42.18 nmol $mg^{-1} h^{-1}$ in drug-free vesicles, whereas after pretreatment with verapamil 1 μmol l^{-1} the increase was 131%, resulting in 72.54 nmol $mg^{-1} h^{-1}$.

In contrast to the potent stimulant effect on the uptake by verapamil when given before the isolation of the membranes, the *in vitro* activity of the drug in sarcolemma vesicles, isolated from drug-free perfused hearts, is of a completely different kind. In this case, only a concentration-dependent inhibition which started at 10 μmol l^{-1} verapamil and showed an IC_{50} value of 112 μmol l^{-1} was observed (Figure 3). These data are in agreement with the inhibitory effect of 0.1 to 1 mmol l^{-1} verapamil on Ca^{2+}-uptake and Ca^{2+}-ATPase activities in sarcoplasmic reticulum and plasma membranes reported by other authors (Wang *et al.*, 1984; Van Amsterdam & Zaagsma, 1986; Kim & Raess, 1988). The first authors also reported a stimulant action of verapamil on the Ca^{2+}-ATPase but not on the Ca^{2+}-uptake in rabbit skeletal muscle SR. Mas Oliva & Nayler (1980) described a stereoselective inhibition of ATP-stimulated Ca^{2+}-uptake at 1 μmol l^{-1} verapamil, which was achieved within a very short period of 5 s in rabbit cardiac SL. However, this was not observed in rat heart SL (Van Amsterdam & Zaagsma, 1986). Mostly, a stimulatory activity on microsomal Ca^{2+}-transport is seen with dihydropyridine calcium antagonists, but usually at concentratins from 10–1000 μmol l^{-1} (Wang *et al.*, 1984; Movsesian *et al.*, 1985) and not at therapeutic concentrations (Skattebøl & Triggle, 1987). An exception was reported by David-Dufilho *et al.* (1984) who observed a significant stimulation of ATP-dependent Ca^{2+}-uptake in cardiac sarcolemma vesicles of spontaneously hypertensive rats by 0.1–10 μmol l^{-1} nifedipine, whereas the stimulant in normotensive control rats was observed only at higher concentrations, indicating that in pathological conditions changes in drug sensitivity may occur.

The effect of ischaemia and reperfusion on the *in vitro* potency of verapamil is minor. No stimulation of the activity took place at any concentration of the drug, whereas the IC_{50} value increased to 250 μmol l^{-1} for both the ischaemic conditions and the reperfusion membranes.

The pronounced differences between the effects of verapamil when given to the intact organ or to isolated sarcolemma vesicles may be related to the more complete and complex level of organization in the intact organ, giving a possibility for the drug to activate other regulatory and adaptation mechanisms. One possible explanation is the regulation of the Ca^{2+}-uptake by calmodulin (Lamers *et al.*, 1985), that may be more active in intact cells in which its concentration is higher than in isolated organelles. Verapamil could strengthen the interaction of calmodulin with the uptake enzyme, thereby preserving its binding during the isolation procedure of the sarcolemma which would enhance the activity of the Ca^{2+}-pump. During ischaemia, however, this interaction may be impaired and therefore becomes less sensitive to verapamil treatment.

In conclusion the data show that verapamil pretreatment at a negative inotropic concentration, resulting in a decreased energy expenditure (Nayler *et al.*, 1987), also leads to an increased Ca^{2+}-extrusion both in control and reperfusion conditions. Therefore, verapamil is able to lower the intracellular Ca^{2+}-concentration not only by inhibition of the influx through the slow channel but also by stimulating its extrusion from the cell. Both processes might be expected to concur in protecting the cell from ischaemic damage, especially during reperfusion.

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Actions of anaesthetics and avermectin on $GABA_A$ chloride channels in mammalian dorsal root ganglion neurones

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1 The γ-aminobutyric acid (GABA)-mimetic actions of some anaesthetics and the antehelminthic avermectin B_{1a} were examined on freshly isolated mammalian dorsal root ganglion (DRG) neurones by use of suction electrodes and a single electrode voltage clamp.

2 Pentobarbitone (60 μM–3 mM), chloralose (600 μM–1 mM), etomidate (10–100 μM), alphaxalone (10–60 μM) and avermectin (10–60 μM) directly activated chloride channels in GABA-sensitive DRG neurones. The agonist action was sensitive to block by bicuculline and picrotoxinin.

3 Steady-state current-voltage (I-V) curves for the anaesthetics were either linear, or rectified in the opposite direction to steady-state I-V curves obtained with GABA. Current relaxations in response to voltage jumps were also of the opposite direction. An extra surge of current ('bounce') was commonly observed on washout of some of these agonists.

4 Pentobarbitone was ineffective as an agonist at alkali pH (10.4 and 9.4), but was approximately twice as effective at acid (5.4) than at normal (7.4) pH values.

5 These results suggest that some anaesthetics and avermectin are capable of 'blocking' GABA channels in addition to activating them.

Introduction

Several general anaesthetics enhance the actions of γ-aminobutyric acid (GABA) both in the central and peripheral nervous systems. In the case of pentobarbitone and some other anaesthetics, at least part of this action may be explained by an increase in duration of the open-time of GABA-activated channels (e.g. Jackson *et al.*, 1982). However, it has also been shown that a few anaesthetic drugs act on vertebrate neurones in a manner similar to GABA (Nicoll, 1975; Harrison & Simmonds, 1984), and this action is antagonized by bicuculline and picrotoxin. It has been suggested that these substances, which bear no obvious structural relation to the classical GABA-mimetic agonists, can directly activate GABA receptor-channel complexes. It is not known whether the agonist action occurs at the receptor site or at the ion channel. The present study examines the actions of various anaesthetic substances and the antehelminthic avermectin on $GABA_A$ channels in freshly isolated mammalian dorsal root ganglion (DRG) neurones, in an attempt to examine their kinetic behaviour in response to rapid jumps in membrane voltage and compare these responses to those obtained with GABA and structurally related agonists (Robertson, 1989).

Some of these results have been communicated previously (Robertson, 1985; 1986).

Methods

Experiments were conducted on freshly isolated dorsal root ganglion neurones using voltage clamp methods. Full details of our experimental methods are given in Robertson & Taylor (1986) and Robertson (1989). Briefly, ganglia (thoracic, lumbar and sacral) were obtained by dorsal laminectomy of adult rats and cats. Cells were cleaned enzymatically (with 0.5% trypsin and 0.5% collagenase), and recordings performed with a suction microelectrode in conjunction with a single electrode voltage clamp system (ASF2, Axon Instruments). Clamp 'switching'

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rates of 60–65 kHz were achieved as a consequence of low electrode resistance (~0.3 MΩ) and by reducing input capacitance. Voltage clamp traces were captured and subsequently analyzed on computer.

The external solution used in recording agonist-induce chloride responses was as follows (in mM): choline chloride, 135, CsCl 5, $MgSO_4$ 10, HEPES 5 and glucose 10; adjusted to pH 7.4 with CsOH. The suction electrode (intracellular) solution contained (mM): CsCl 140, HEPES 5, EGTA 2; adjusted to pH 7.4 with CsOH. Experiments were conducted at 22–24°C.

GABA was obtained from Sigma Chemical Company. Sodium pentobarbitone (Prosana or Abbott Pharmaceuticals) and etomidate (Janssen Pharmaceuticals) were kindly provided by Prof. David Curtis. α-Chloralose (Fluka) was a gift from Prof. W. Levick. Avermectin B1a (Merck Sharp and Dohme Laboratories) was dissolved in 2% dimethyl sulphoxide (DMSO) to give a final concentration of 10 μM drug and 0.1% DMSO for experiments. Alphaxalone (Glaxo Group Research) was dissolved and stored in 0.2 mM ethanol. Drugs were bath applied and solutions switched rapidly (less than 1 s), revealing transient components of responses.

Results

γ-Aminobutyric acid

Application of GABA (usually 60 μM) produced an inward current that reversed at the equilibrium potential for chloride ions predicted by the activity of chloride in the intracellular and extracellular solutions. Current relaxations were measured in response to voltage jumps in control and drug solutions and subtracted on-line to give the net agonist-induced current. The net agonist-induced current is shown in all figures. The instantaneous current, measured at the point where the command voltage is achieved, was linear in symmetrical chloride solutions (Figure 1a), while the steady-state current (measured at the end of the voltage step) showed rectification (Figure 1b). The direction of the rectification was consistent with a reduction in the number of open channels at hyperpolarized potentials, due to the reduction in GABA channel open time as the membrane voltage was made more negative (e.g. Robertson, 1989).

Pentobarbitone

Racemic pentobarbitone was bath applied to DRG neurones at concentrations between 60 μM and 3 mM. In every cell where pentobarbitone was effective ($n = 53$), GABA responses could also be elicited.

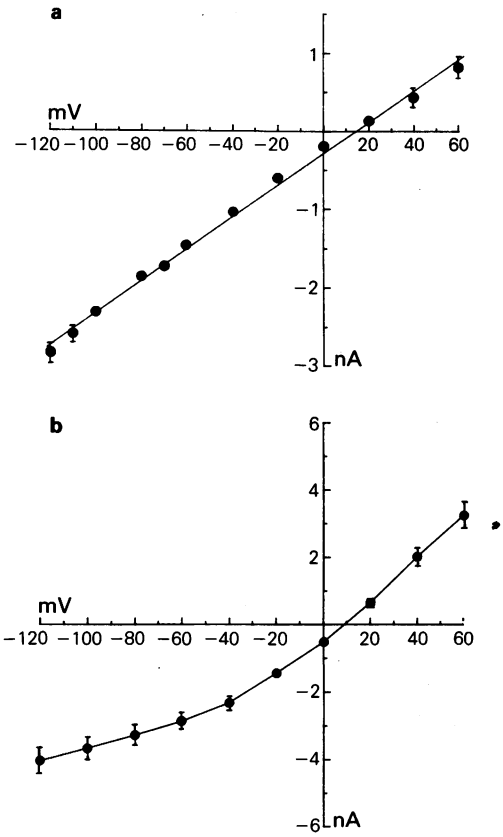


Figure 1 (a) Instantaneous current-voltage relation for 60 μM GABA with 140 mM chloride on both sides of the cell membrane. Data points are mean for 8 cells (with s.e.mean shown by vertical bars) normalized to −1 nA at −40 mV. (b) Steady-state current voltage relation for the same cells. The rectification in this curve is due to the voltage sensitivity of GABA channel lifetime. Data points also mean with s.e. shown by vertical bars.

However, if cells were unresponsive to GABA then no response to pentobarbitone was obtained ($n = 10$).

Where pentobarbitone was applied at the same concentration as GABA, the anaesthetic was less effective than GABA (60 μM pentobarbitone was only 10% as effective as 60 μM GABA, $n = 3$; 1 mM pentobarbitone was 40% as effective as 1 mM GABA, $n = 3$). The agonist action of pentobarbitone was rapidly and reversibly blocked by 50 μM of either bicuculline methiodide or picrotoxinin.

In four experiments the effects of 60 μM GABA and 1 mM pentobarbitone applied separately and together were assessed. GABA (60 μM) alone evoked on average −1.2 nA of inward current, 1 mM pento-

barbitone produced −2.1 nA, but together these agonists produced a mean of −11.1 nA of current.

When pentobarbitone concentration was increased to 600 μM and above, the current response increased rapidly to a peak and then quickly declined to a plateau level. However, an unusual response was observed when the drug was washed out of the bath. Immediately after the solution switch, a marked transient increase in current was observed. This 'bounce' of current then decayed to pre-drug baseline level. In contrast, current responses to high concentrations of GABA showed desensitization only, and never 'bounce' on washout.

Current responses to 1 mM pentobarbitone at −40 and +40 mV are illustrated in Figure 2a. At least part of the decline in peak current in Figure 2a was due to desensitization, but there may also have been a contribution from channel 'block' by pentobarbitone (see below).

Voltage jumps were performed before and during pentobarbitone exposure to measure the voltage-dependence of the agonist action of pentobarbitone on GABA channels. Figure 2b compares the relaxations produced by 60 μM GABA and 1 mM pentobarbitone upon a potential step from −40 to −100 mV. The current relaxations for the two agonists have very different characteristics. The GABA response showed the usual exponential decrease in current to a new, lower steady state value, while the pentobarbitone response showed a more rapid relaxation in the opposite direction, such that current increased to a new steady state value after the instantaneous jump. At the end of the hyperpolarizing voltage step, the relaxation for the two agonists were again opposite in direction, consistent with a reduction in the average number of open channels during the hyperpolarizing voltage step with GABA, and an increase in the number of open channels on the same voltage step with pentobarbitone. These unusual relaxations were a typical feature with pentobarbitone at concentrations greater than 100 μM.

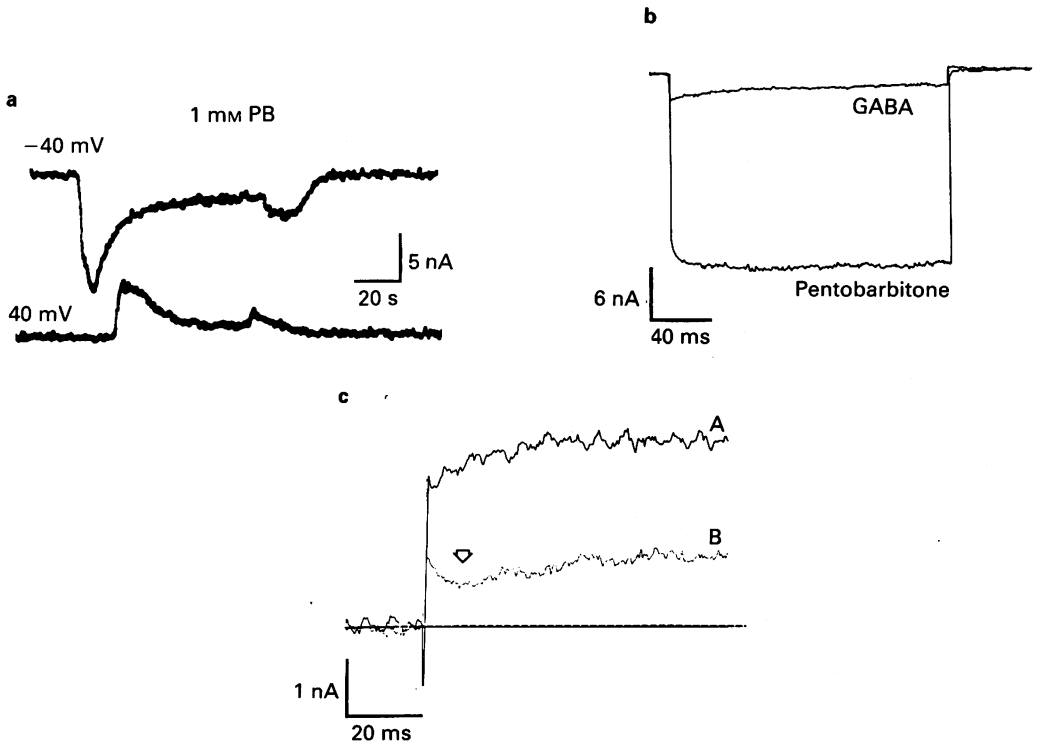


Figure 2 (a). Agonist action of 1 mM pentobarbitone at two potentials. Pentobarbitone produces a large increase in current, which declines to a plateau, and is followed by a secondary increase in current ('bounce') on washout of the drug. (b) Voltage jump relaxations with 60 μM GABA and 1 mM pentobarbitone in the same cell on a 60 mV hyperpolarizing step from −40 mV. The relaxations are opposite in direction for the two agonists. (c) Relaxations in response to depolarizing steps to +60 mV from −40 mV with 60 μM GABA (A) and 600 μM pentobarbitone (B). Note that the GABA-induced current increases steadily after the instantaneous current, while the outward current in pentobarbitone initially decreases (open arrow), before increasing to a new steady state value.

Figure 2c illustrates expanded relaxations in response to depolarizing voltage jumps with 600 μM pentobarbitone and 60 μM GABA on the same cell. The GABA current increased steadily after the instantaneous current, but the current induced by pentobarbitone showed an initial sharp decrease after the ohmic current, and then increased steadily. (The open arrow highlights this phenomenon). Similar behaviour was observed with 10 other cells exposed to pentobarbitone.

As a consequence of the different modes of current relaxation, the steady state current-voltage relationship for cells exposed to 600 μM pentobarbitone showed rectification in the opposite direction from channels gated by GABA or GABA-like agonists, with less current at positive membrane potentials.

Since pentobarbitone is 75% in the uncharged form at physiological pH and 25% in an anionic form, experiments were conducted to test which form of the anaesthetic was responsible for the reduced current at positive potentials, that is to determine whether the anionic or neutral forms were responsible for the agonist action of pentobarbitone.

Experiments were performed measuring the response to 1 mM pentobarbitone at pH 7.4, then changing pH to 10.4 or 9.4, and comparing the response at the new alkaline pH. In two separate experiments pentobarbitone produced -4.4 and -7.0 nA at pH 7.4, but there was no response to this agonist at pH 10.4. Similarly, in four experiments at pH 7.4 and 9.4, pentobarbitone produced a mean response of -5.5 nA at pH 7.4, but a mean current of only -0.2 nA at pH 9.4. (In three applications pentobarbitone caused no current change at pH 9.4, the other cell showed a maximum current that was only 10% of the full response at pH 7.4). One of these experiments is illustrated in Figure 3a.

Changes in holding current in control solutions at alkaline pH were negligible. In order to determine if the GABA receptor/channel complex itself was disturbed by alkaline solutions, experiments were conducted to measure the effectiveness of 60 μM GABA at pH 9.4. In all three experiments, GABA responses were $84 \pm 2.6\%$, (mean $\pm$s.e.) of their value at pH 7.4, suggesting that the receptor/channel was not substantially altered at pH 9.4.

Further experiments were carried out with pentobarbitone at pH 5.4. In 6 cells with 8 applications of pentobarbitone at pH 7.4 and pH 5.4, 1 mM pentobarbitone produced 1.2 to 2.9 fold more current at acid pH values. The mean increase in current was 1.9 ± 0.3 fold at -40 mV. The steady state I-V relationship from 4 cells with paired applications of 1 mM pentobarbitone at both pH values is illustrated in Figure 3b. Pentobarbitone was more effective at pH 5.4 at all potentials examined; however current responses still rectified at positive potentials. Additionally, the bounce of current observed upon washout of pentobarbitone was still present at pH 5.4.

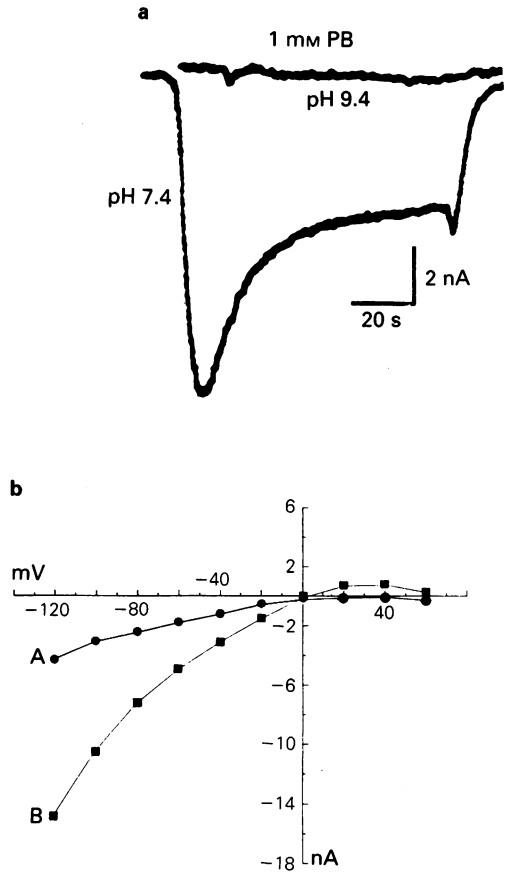


Figure 3 (a) The agonist action of pentobarbitone at normal and alkali pH. Pentobarbitone (1 mM) elicited a large inward current at pH 7.4, but was ineffective at pH 9.4. On reapplication of the drug at pH 7.4, the usual inward current was observed (not shown). Cell holding potential -40 mV. (b) Steady state I-V for 4 cells with 1 mM pentobarbitone applied at pH 7.4 (A) and pH 5.4 (B).

Chloralose

α-Chloralose activated GABA receptor/channels at concentrations of 100 μM and above ($n = 34$). Current responses to 600 μM chloralose were blocked by both 50 μM picrotoxinin and 50 μM bicuculline methiodide. Chloralose responses were not observed on GABA-insensitive cells ($n = 5$). When the potency of 60 μM GABA and 600 μM chloralose were compared on the same cells ($n = 7$), GABA produced a

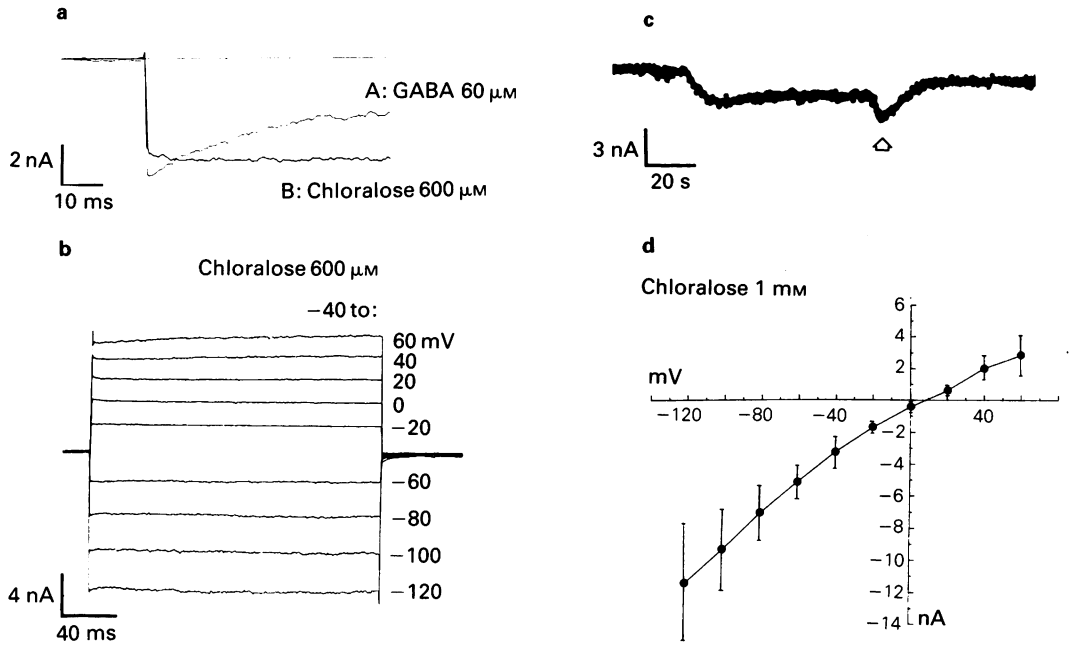


Figure 4 (a) Voltage jump relaxations on a −80 mV step from −40 mV with 60 μM GABA (A) and 600 μM chloralase (B). (b) A family of current relaxations from another cell with 600 μM chloralose, stepping from −40 mV to the potentials indicated at the right hand side of the current traces. (c) The response of a DRG neurone to 1 mM chloralose. Note the decline after the initial peak and the bounce (arrow) on washout of this anaesthetic agonist. Clamp potential −40 mV. (d) Steady-state current voltage relation for 1 mM chloralose. Points are the mean values from 3 cells.

mean inward current of -2.25 ± 0.47 nA, and 600 μM chloralose gave -2.51 ± 0.4 nA.

There was no evidence for 'bounce' or desensitization with 600 μM chloralose. Voltage jump relaxations (Figures 4a and b) in 600 μM chloralose did not show the form of relaxations observed when GABA was used to activate the channels. As with pentobarbitone, if relaxations were seen, they were of the opposite direction to those obtained with GABA (i.e. increasing current on hyperpolarizing steps).

With higher concentrations of chloralose (1 mM), current responses showed a decline after an initial peak, and a slight bounce after removal of the agonist (see Figure 4c). The steady state I-V for 1 mM chloralose on 3 cells is shown in Figure 4d.

Etomidate

The non-barbiturate anaesthetic etomidate was effective in eliciting conductance changes on DRG neurones at concentrations of 30, 60 and 100 μM ($n = 28$). These responses reversed at the chloride equilibrium potential and were blocked by the GABA antagonists bicuculline methiodide and picrotoxinin (50 μM), and cells unresponsive to GABA did not respond to etomidate ($n = 6$). In 11 cells where 60 μM etomidate was compared to 60 μM GABA, the average response in etomidate was -1.27 ± 0.16 nA and -2.24 ± 0.39 nA in GABA. However, these figures represent only an approximate index of potency, since as Figure 5 shows, there was a large and rapid bounce of current as eto-

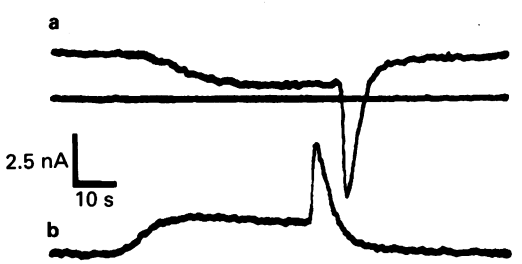


Figure 5 Response to 60 μM etomidate at −40 mV (a) and +40 mV (b). The current rises slowly to a plateau, and there is a large transient increase in current when etomidate is washed out of the bath. The middle trace is the clamp potential during exposure to the anaesthetic.

midate was washed out of the recording bath. The bounce could be as large as four times the 'steady state' (plateau) response. In the cell illustrated in Figure 5, 60 μM GABA produced -4.6 nA of inward current at -40 mV, but 60 μM etomidate gave a plateau current of -1.5 nA followed by a bounce reaching -6 nA. If etomidate was applied for long periods (greater than 2 min) the plateau response declined slightly, but a large bounce was still observed on washout.

In 3 experiments where GABA and etomidate were applied separately, then additively, the response to the two agonists was greater than the algebraic sum of the individual responses.

Current responses to voltage jumps in 60 μM GABA and 100 μM etomidate are illustrated in Figure 6. As was observed for pentobarbitone and chloralose, these currents did not 'relax' in the manner of GABA-induced responses. The voltage jump responses in etomidate were essentially flat, with a small increase in current being observed on hyperpolarizing voltage jumps. The steady state current voltage relation in 60 μM etomidate is shown in Figure 6b. There appeared to be little overall voltage-dependence of the etomidate response, and any voltage sensitivity was opposite to that seen with GABA-induced responses.

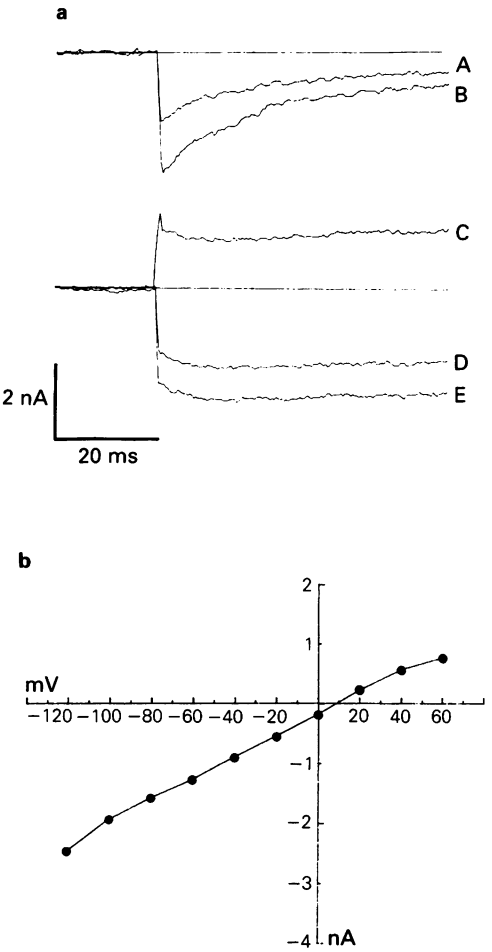


Figure 6 (a) Voltage jump relaxations in response to hyperpolarizing steps from -40 mV in 60 μM GABA (A: step to -120 mV; B: step to -100 mV) and 100 μM etomidate in the same cell. Jumps in etomidate are to $+60$ mV (C), -100 mV (D) and -120 mV (E). The outward relaxations on hyperpolarizing steps in etomidate are quite dissimilar to those responses in GABA, as is the reduction in current after the instantaneous current in trace (C). (b) Steady state current voltage relation in 60 μM etomidate (mean of 7 applications). Note the linearity of the I-V.

Alphaxalone

The steroid anaesthetic alphaxalone (10 to 60 μM) activated a chloride conductance in 20 DRG neurones, and these responses were blocked by 10 and 50 μM picrotoxinin. Cells that were not responsive to GABA did not produce currents in response to alphaxalone ($n = 4$). Current responses to 10 μM alphaxalone showed considerable desensitization with only occasional bounce on removal of the agonist.

In 4 cells the currents in response to 10 μM alphaxalone and 60 μM GABA were compared: the anaesthetic produced a mean current of -2.3 ± 0.7 nA while GABA gave a mean current of -5.6 ± 2.1 nA.

Voltage jump relaxations with alphaxalone were again mainly flat, with small fast relaxations in the opposite direction to GABA currents.

Avermectin B_{1a}

The insecticide avermectin B_{1a}(AVM) is structurally quite dissimilar to any of the other anaesthetic agonist-like compounds examined, and has been reported to have GABA-like agonist properties in some invertebrate preparations. At 10 μM, AVM activated picrotoxinin-sensitive chloride channels in all 5 cells tested; these cells were also GABA-sensitive. Responses to AVM were typically much slower in onset and decay than current responses to the other agonists examined, often taking minutes to reach a peak response. The chloride currents induced by AVM were however fully reversible; no irreversible increases in Cl^- permeability were seen (see Duce & Scott 1985). AVM 10 μM elicited currents of amplitudes between -1 and -5 nA, with the mean response being -3.1 ± 0.9 nA, compared to -8.3 ± 2.3 nA with 60 μM GABA on these same

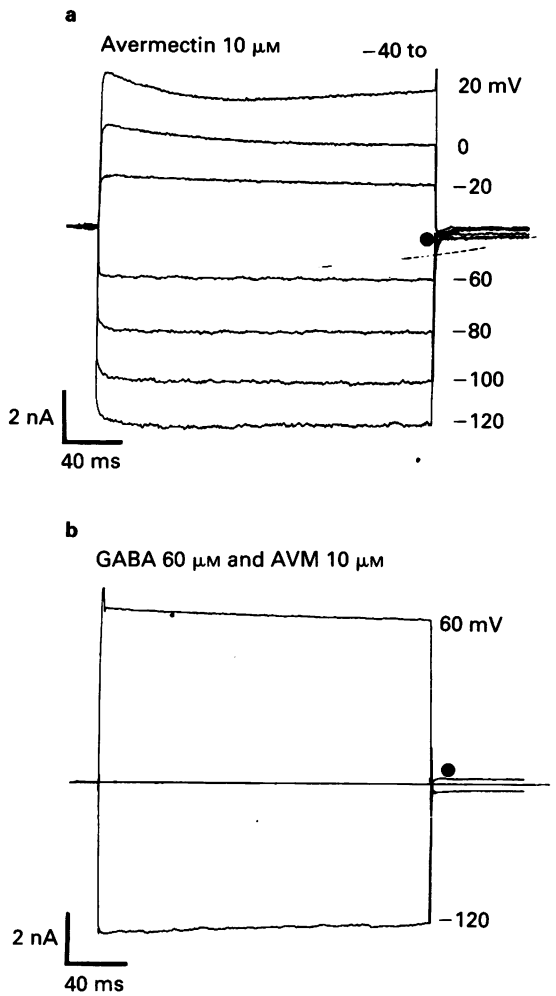


Figure 7 (a) Relaxations in response to voltage jumps in 10 μM avermectin are very different from those seen with GABA. Currents were obtained in response to steps from −40 mV to the potentials indicated on the right hand side of the figure. Note that the current increases at the beginning of the hyperpolarizing step, and then relaxes back to the baseline again at the end of the step (indicated by ●). (b) Simultaneous application of 10 μM avermectin (AVM) and 60 μM GABA results in altered current relaxations in response to potential steps. In the relaxation obtained on a step from −40 to −120 mV, AVM markedly slows the inward relaxation due to GABA channel activation, and slows the 'off' relaxation at the end of the voltage step (●).

cells. Despite the slow rise and decline of the AVM-induced current, this could be very rapidly and completely blocked by including the GABA antagonist picrotoxinin in the perfusate. Block by the antagonist was fully reversible. No tests of bicuculline sensitivity were made.

Current responses to voltage jumps in AVM are illustrated in Figure 7a and the steady state current voltage relationships was approximately linear. AVM currents displayed small outward relaxations on hyperpolarizing potential steps, and inward relaxations on depolarizing steps. These results are consistent with there being an increase in the number of conducting channels at more negative potentials.

No obvious bounce was detected on washing AVM out of the bath, instead the current declined slowly back to control values. In one experiment, 10 μM AVM was applied with 60 μM GABA (Figure 7b). AVM appeared to slow down the normal GABA relaxations at hyperpolarized potentials considerably. This can be seen with the slow change in current amplitude during the hyperpolarizing voltage step.

Due to the poor solubility of AVM, this substance was dissolved in 0.1% DMSO. In 3 experiments, application of DMSO alone had no effect on the resting conductance of DRG neurones, or on GABA responses.

Halothane, ketamine and diazepam

No conductance change was observed on applications of halothane (1 mM, $n = 3$), 1 mM ketamine ($n = 3$), or 100 nM and 20 μM diazepam ($n = 6$), although all of these neurones responded vigorously to 60 μM GABA.

Discussion and conclusions

Pentobarbitone activated GABA channels in DRG neurones at concentrations as low as 60 μM, and up to 3 mM. The anaesthetic range for pentobarbitone is approximately 100–200 μM (Richards 1972), and it is possible that the GABA-mimetic action of this drug plays a role during surgical anaesthesia. Addition of pentobarbitone and GABA together produced currents that were 3–4 times greater than the algebraic sum of individual GABA and pentobarbitone responses, due both to the potentiation of GABA responses as well as the agonist action of pentobarbitone. Similar observations of the agonist-like effects of pentobarbitone have previously been made on a variety of neurones (Nicoll, 1975; Nicoll & Wojtowicz, 1978; Barker & Ransom, 1978; Connors, 1981; Akaike *et al.*, 1985).

As well as exerting agonist actions, the presence of 'bounce' on washout of pentobarbitone suggested that this compound may also be blocking GABA receptor/channels. Bounce has also been observed with pentobarbitone by Akaike *et al.* (1985, 1987),

and these authors have proposed a channel blocking mechanism to account for this phenomenon. Adams (1975) observed a bounce on washout of high concentrations of certain nicotinic agonists from frog muscle and suggested that these current surges were due to the agonist binding to 'hypothetical non-receptor sites' and that the agonists were released from these reservoirs during washout. This possibility was excluded during the present experiments by changing the solution very rapidly around a single DRG neurone. The results obtained here were more consistent with a rapid blocking of the GABA receptor/channel by pentobarbitone, with these channels becoming unblocked and available to carry current during washout of the agonist. Peters *et al.* (1988) have also observed a marked bounce on washout of high concentrations of pentobarbitone, and suggest these high concentrations had blocking activity on $GABA_A$ chloride channels. A depressant or blocking action of pentobarbitone has been well described at acetylcholine (ACh)-gated chloride channels in invertebrates (Adams *et al.*, 1982; Judge & Norman, 1982; Wachtel & Wilson, 1983). In the present experiments the characteristics of the pentobarbitone induced currents during voltage jumps (where the current increased on a hyperpolarizing step) and the consequent curvature of the anaesthetics' steady-state I-V relationship suggests that there may be a greater degree of block by this agonist at positive membrane potentials. Since pentobarbitone exists in both anionic and neutral forms at pH 7.4, it was possible that the anionic form might be entering the channel and transiently reducing ionic conductance at positive potentials. In experiments with alkaline solutions, pentobarbitone produced little or no conductance change. At this pH most of the anaesthetic is in the anionic form, and may have been completely blocking the channels. However, at pH 5.4, where over 99% of the drug would be uncharged, the steady state I-V still rectified significantly at positive potentials and bounce was still observed on washout of the agonist. The simplest interpretation of these results is that agonism and blocking are associated with the neutral and not the anionic form of pentobarbitone.

Etomidate was the most potent anaesthetic in activating GABA receptor/channels, indeed this compound had a potency comparable to GABA when the blocking action of etomidate was taken into consideration. The GABA-agonist action of etomidate has previously been observed by Evans & Hill (1978), who found that the agonist effects were apparent at concentrations similar to those measured in the rat brain during hypnotic and behavioural effects. The marked bounce on washout and the shape of the voltage jump relaxations in etomidate can also be interpreted in terms of a sequential channel block by this anaesthetic after activation. Indeed computer simulations of the current induced by etomidate (unpublished observations) would suggest that at 60 μM etomidate blocks about 80% of the activated receptor/channels at steady state. It is suggested that the channel blocking kinetics have opposite sensitivity to membrane potential than the normal kinetics seen when GABA or related agonists activate the channel (Robertson, 1989). Judge & Norman (1982) found that 100–500 μM etomidate depressed ACh-evoked chloride responses in *Helix* neurones, while Cullen & Martin (1982) have reported that metomidate (a close analogue of etomidate) depressed GABA-induced chloride responses in lamprey medullary neurones.

The general anaesthetic α-chloralose was also effective in activating GABA channels directly. Similar results have been obtained previously by Nicoll & Wojtowicz (1980) and Nicoll & Madison (1982). This anaesthetic also produced steady state currents and relaxations opposite in direction to those seen with GABA, which are consistent with some form of blocking behaviour. Chloralose is well known for its excitatory actions during anaesthesia, and the basis for this action should be examined at the electrophysiological level. It is possible that the GABA blocking action of chloralose becomes significant at higher doses, leading to a temporary reduction in synaptic inhibition causing an increase in excitability (e.g. see Nicoll, 1972).

Harrison & Simmonds (1984) have observed that low doses of the steroid anaesthetic, alphaxalone, potentiated the depolarizing responses to GABA and muscimol in rat cuneate neurones. These authors also found that doses of 10 μM and above alphaxalone could directly activate GABA receptor/channels, and that this action was only seen with the α- and not the β-isomer. The experiments reported here show that alphaxalone also activates GABA channels directly on DRG neurones. This supports observations made by Barker *et al.* (1987) and by Cottrell *et al.* (1987) on central neurones and chromaffin cells respectively. (Barker *et al.*, 1987 have also observed 'bounce' on washout of alphaxalone; see Figure 9 of their paper).

Avermectin B_{1a} activated GABA channels in DRG neurones, although with a much slower time course than the other agonists tested. The activation was rapidly and completely antagonized by picrotoxinin, suggesting that this large macrocyclic compound is capable of activating and opening GABA channels directly. However, Duce & Scott (1985) have reported that AVM can induce increases in chloride permeability in locust muscle fibres that are insensitive to GABA, and suggest that AVM has other, less specific membrane actions. In this connection, it is interesting to note that Martin & Pen-

nington (1989) have recently reported that AVM can induce cation channels in patches of *Ascaris* muscle. It would seem then that AVM probably has other 'nonselective' actions on biological membranes, and further experiments on mammalian neurones would be necessary in order to determine if these occur in higher animals.

AVM is a potent antehelmintic and insecticide, and its mode of action has been explained by the GABA agonist like actions of this compound (see e.g. Wang & Pong, 1982). Avermectin binds specifically to brain membranes, and enhances GABA binding (Wang & Pong, 1982). Supavilai & Karobath (1981) have also suggested that avermectin enhances benzodiazepine binding to a novel modulatory site on the GABA-benzodiazepine receptor/complex. Whether these effects are correlated with the slow agonist actions of this compound is not yet known. Because of the slow rise times of the avermectin responses observed in the present experiments, it is possible that this compound is acting by stabilizing spontaneous openings of GABA channels (see e.g. Jackson, 1986, for details of this phenomenon in ACh receptors). However, preliminary experiments with application of GABA antagonists and large voltage gradients (designed to reveal any current generated by spontaneous GABA channel openings) suggest that any contribution from such spontaneous openings to the background conductance of DRG neurones would be vanishingly small. Additionally, other agents which stabilize GABA channels in an open conformation should also act as agonists on this basis, however neither ketamine nor halothane, which potentiate GABA responses in central neurones (Scholfield, 1980; Gage & Robertson, 1984) showed any GABA receptor agonist action.

The benzodiazepine diazepam was also tested for potential agonist activity since Barker and colleagues (Barker, 1983; Barker *et al.*, 1984) have reported that diazepam is an extremely potent agonist at GABA receptor channels. In contrast to results obtained on central neurones in the present study, 100 nM and 20 μM diazepam were completely ineffective in eliciting a conductance change, even though GABA responses were quite marked in these neurones. It may be that in cultured central neurones benzodiazepines may be enhancing the action of endogeneously released GABA.

The activation of GABA channels by anaesthetic agents whose chemical structures vary widely and are not easily reconciled with the classical requirements for GABA agonists (Curtis & Watkins, 1960) is unusual for the agonist-gated channels studied to date. It will be interesting to determine precisely how these anaesthetics activate GABA channels, and whether this occurs by interactions at the receptor site, or if the ionophore is opened directly. Given the complex nature of the interaction of anaesthetics with $GABA_A$ channels (modulation, agonism and blocking), it would be necessary to use single channel techniques to resolve their kinetics properly. Perhaps such studies will tell us more about the molecular processes involved in receptor/channel activation in general.

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Electrophysiological effects of OPC-88117, a new antiarrhythmic agent on papillary muscles and single ventricular myocytes isolated from guinea-pig hearts

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1 The effects of OPC-88117, a new antiarrhythmic agent, on transmembrane action potentials were examined in right ventricular papillary muscles and in single ventricular myocytes isolated from guinea-pig hearts.

2 In papillary muscles, OPC-88117 above 3×10^{-6} M caused a dose-dependent prolongation of action potential duration (APD).

3 OPC-88117 above 3×10^{-5} M caused a significant decrease in the maximum upstroke velocity ($\dot{V}_{max}$) of the action potential without affecting the resting membrane potential. The inhibition of $\dot{V}_{max}$ was enhanced at higher stimulation frequencies.

4 In the presence of OPC-88117, trains of stimuli at rates $\geqslant 1.0$ Hz led to a use-dependent inhibition of $\dot{V}_{max}$ with rapid onset. The time constant for the recovery of $\dot{V}_{max}$ from the use-dependent block was 456 ms.

5 The curves relating membrane potential and $\dot{V}_{max}$ were shifted by OPC-88117 to the direction of more negative potentials (9 mV at 10^{-4} M).

6 In single ventricular myocytes treated with OPC-88117 ($1–3 \times 10^{-4}$ M), the $\dot{V}_{max}$ of test action potentials preceded by conditioning clamp pulses to 0 mV was decreased progressively as the clamp pulse duration was prolonged.

7 These findings suggest that the primary electrophysiological effect of OPC-88117 on the cardiac muscle cell is prolongation of APD (Class III action) and that at high concentrations, it may also possess a lignocaine-like sodium channel inhibitory effect (Class I action).

Introduction

OPC-88117 (8-methyl-3-(4-methyl-1-piperazinyl)-2(1H)-quinolinone hydrochloride) is a newly synthesized antiarrhythmic compound (Figure 1). *In vivo* experiments in anaesthetized dogs have demonstrated that OPC-88117 given intravenously has a potent inhibitory action against ventricular arrhythmias induced by catecholamines or cardiac glycosides. The antiarrhythmic potency of this substance is comparable to or higher than lignocaine, procainamide, disopyramide or mexiletine (unpublished data). Preliminary experiments in guinea-pig isolated ventricular muscles have shown that OPC-88117 prolongs the action potential duration and suppresses the maximum upstroke velocity ($\dot{V}_{max}$) of the action potential without affecting the resting membrane potential. These findings seem to suggest that OPC-88117 is a unique antiarrhythmic drug having characteristics both of Class I and Class III drugs (Vaughan-Williams, 1970; 1984; Hauswirth & Singh, 1979). However the precise mode of action of OPC-88117 on cardiac muscle cells in relation to its antiarrhythmic activity remains to be elucidated.

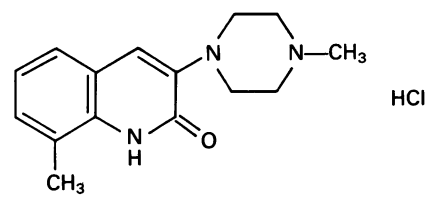


Figure 1 Chemical structure of OPC-88117.

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In the present study, the effects of OPC-88117 on the transmembrane action potential were investigated in right ventricular papillary muscles, as well as in single ventricular myocytes isolated from guina-pig hearts. The modulation of drug-induced $\dot{V}_{max}$ inhibition by stimulation frequencies or by membrane potential level was studied most extensively to compare the characteristics of its sodium channel blocking action with other Class I drugs.

Methods

Papillary muscles

Guinea-pigs of either sex weighing 200–250 g were killed by a blow on the head and the hearts were quickly removed. Papillary muscles 2 to 3 mm in length and 0.3 to 0.4 mm in diameter were dissected from the right ventricle. The preparation was mounted in the tissue bath and superfused continuously with Krebs-Ringer solution kept at 34°C and gassed with 95% O_2 and 5% CO_2. The composition of the solution was as follows (in mM): NaCl 120.3, KCl 4.0, $CaCl_2$ 1.2, $MgSO_4$ 1.3, $NaHCO_3$ 25.2 and glucose 5.5 (pH 7.4). Equipment for stimulation and for recording transmembrane action potentials was the same as described by us previously (Kodama *et al.*, 1985; Toyama *et al.*, 1987).

To study the use-dependent effects of OPC-88117 on the maximum upstroke velocity ($\dot{V}_{max}$) of action potentials, the preparation was stimulated repetitively at varying rates ranging from 0.2 to 2.5 Hz. Rest periods of 30 s, which were sufficient to ensure full recovery from the rate-dependent decrease in $\dot{V}_{max}$ were interposed between the trains of stimuli. This experimental protocol is able to detect the existence of two types of $\dot{V}_{max}$ inhibition by OPC-88117, tonic and use-dependent block. The former is defined by the decrease of $\dot{V}_{max}$ of the first action potential preceded by the rest period, and the latter is the decrease of $\dot{V}_{max}$ during the train (from the value of first action potential to the new steady-state level).

The recovery of $\dot{V}_{max}$ from the use-dependent block was studied by applying a single test stimulus at various coupling intervals following a stimulation train for 20 s at 1.0 Hz. The intensity of the test stimuli was adjusted to obtain a constant latency from the stimulus artifact to the initiation of the action potential upstroke.

To determine the relationship between membrane potential and $\dot{V}_{max}$, the papillary muscle was stimulated at an interstimulus interval of 30 s and the resting potential was made less negative by adding KCl to the medium in 1–2 mM steps up to a final K^+ concentration of 20 mM. After an equilibration period of 7 to 8 min for each concentration of K^+, an action potential was recorded to measure the $\dot{V}_{max}$.

Single ventricular myocytes

Single ventricular myocytes were isolated enzymatically by a procedure similar to that described by Watanabe *et al.* (1985). In brief, hearts were quickly removed from guinea-pigs and perfused on a Langendorff apparatus with the following solutions in sequence: (1) Ca^{2+}-free Krebs solution for 5 min, (2) enzyme solution containing collagenase (2 mg ml^{-1}, Sigma, type V) and protease (0.2 mg ml^{-1}, Sigma, type XIV) for 5 min, and (3) Ca^{2+}-free Krebs solution for 5 min. The left ventricle was then cut into small pieces in Ca^{2+}-free Krebs solution to disperse single myocytes. A few drops of cell suspension were placed in a recording chamber attached to an inverted microscope. The chamber was perfused at a rate of 2 ml min^{-1} with normal Krebs solution of the following composition (mM): NaCl 136.9, Kcl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 0.5, NaH_2PO_4 0.33, HEPES 5.0 and glucose 5.0; pH was adjusted to 7.4 by adding NaOH, and the solution was equilibrated by 100% O_2. The temperature was maintained at 35°C.

Following the increase in calcium concentration of the medium to 1.8 mM (normal Krebs solution), 30 to 40% of myocytes deteriorated into round-shaped cells due to irreversible contracture. The remaining cells were tolerant to calcium, their intact rod-shape was maintained without spontaneous beating, and the experiments were carried out with these myocytes.

Single suction pipette electrodes were used for membrane potential recording as well as for whole-cell voltage clamp. The pipettes were fire polished to a tip diameter of 1.5 μm and filled with internal solution to give a resistance ranging from 2 to 3 MΩ. The pipette solution consisted of (mM): KCl 120.0, NaH_2PO_4 10.0, EGTA 1.0, MgATP 5.0 and HEPES 10.0; pH was adjusted to 7.2 by adding KOH. Action potentials were recorded by the current-clamp mode by passing a short stimulus current (less than 4 ms in duration) through the electrodes. Transition from the voltage-clamp mode to the current-clamp mode was regulated by a pulse generator through an electronic relay. Details of the experimental protocols are given under Results.

Drugs and data analysis

OPC-88117 (Otsuka Pharmaceutical Co. Ltd., Osaka, Japan) was dissolved in deionized water and diluted with superfusate (Krebs solutions) to achieve the final concentration required.

Values are presented as the means ± s.e. unless otherwise stated. Data were analysed by t test, analysis of variance, Dunnett's test, and regression analysis. Differences were considered significant at $P < 0.05$.

Results

Action potentials of papillary muscle

Effects of OPC-88117 (3×10^{-6}–3×10^{-4} M) on the membrane action potential configuration were examined in eight papillary muscles constantly stimulated at 1.0 Hz (Figure 2, Table 1). Following exposure to OPC-88117 at 3×10^{-6} M for 30 min, no significant changes were observed. OPC-88117 at 10^{-5} M caused a significant prolongation of action potential duration at 80% repolarization (APD_{80}), while other parameters were still unaffected. At 3×10^{-5} M, APD_{80} was prolonged further, and a prolongation of action potential duration at 30% repolarization (APD_{30}) was also observed. In addition, the maximum upstroke velocity ($\dot{V}_{max}$) was decreased significantly. At the higher concentration, the prolongation of APD_{80} and decrease in $\dot{V}_{max}$ were enhanced in a dose-dependent manner, whereas APD_{30} was shortened to a level below control. The resting membrane potential (RP) was unchanged even at 3×10^{-4} M OPC-88117. IC_{20} of $\dot{V}_{max}$ inhibition by OPC-88117, which was obtained by interpolation in a graph of log molar drug concentrations versus responses, was 9.4×10^{-5} M.

Effects of OPC-88117 on the contour of membrane action potentials were also examined at a low (0.5 Hz) and a high (2.0 Hz) stimulation frequency and compared with those at 1.0 Hz. The results obtained are summarized in Figure 3. The prolongation of APD_{80} by OPC-88117 was similar in its extent at the three different stimulation frequencies. In contrast, the $\dot{V}_{max}$ inhibition was largely influenced by the stimulation frequency. Thus, at 0.5 Hz OPC-88117 caused no significant decrease in $\dot{V}_{max}$ even at 10^{-4} M, whereas at 2.0 Hz the OPC-88117-induced reduction of $\dot{V}_{max}$ was much more marked than that observed at 1.0 Hz.

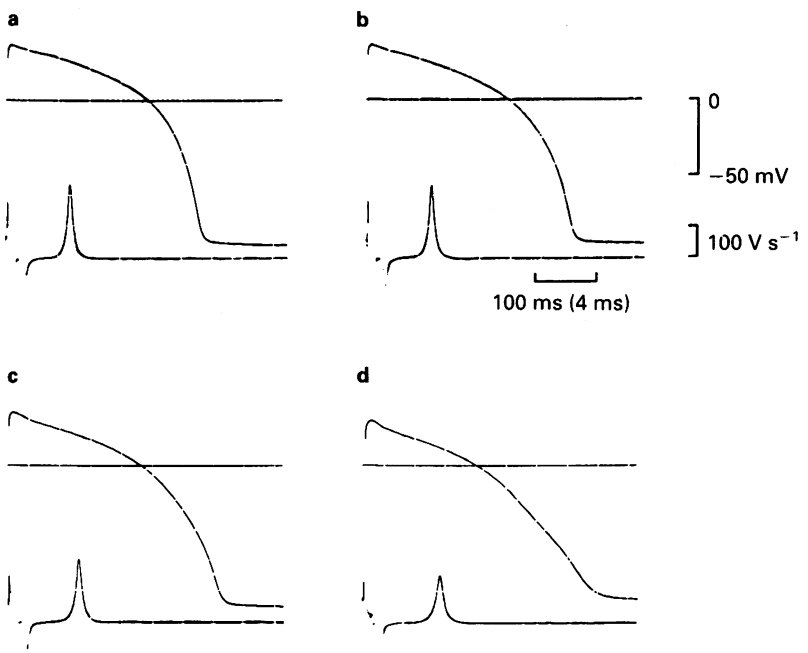


Figure 2 Effects of OPC-88117 on transmembrane action potentials of papillary muscles. (a), Control; (b) OPC-88117 (3×10^{-5} M); (c) OPC-88117 (10^{-4} M); (d) OPC-88117 (3×10^{-4} M). Upper trace is membrane action potential and lower trace is the differentiated upstroke spike of the action potential ($\dot{V}_{max}$). $\dot{V}_{max}$ was recorded at a faster sweep velocity. The preparation was constantly stimulated at 1.0 Hz. Membrane potential was recorded just before and 30 min after the drug application at each concentration.

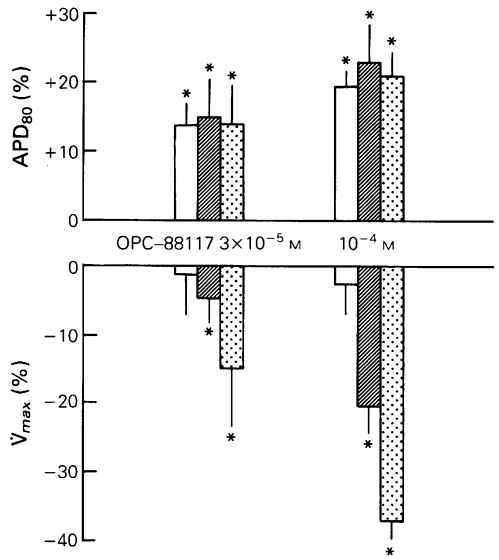


Figure 3 Influence of stimulation frequency on the effects of OPC-88117 in papillary muscles. Effects of OPC-88117 on the action potential duration at 80% repolarization (APD_{80}) and on the maximum upstroke velocity ($\dot{V}_{max}$) at three different stimulation frequencies (0.5 Hz (open columns), 1.0 Hz (hatched columns) and 2.0 Hz (stippled columns)) were compared. Values are presented as % change from control (means ± s.e., $n = 5$). *Significantly different from control at $P < 0.05$.

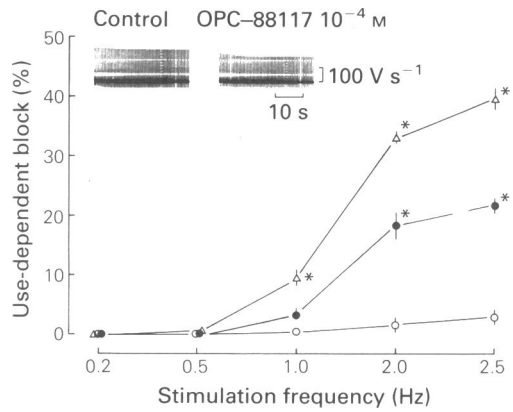


Figure 4 Use-dependent block of $\dot{V}_{max}$ by OPC-88117 in papillary muscles. The traces show differentiated upstroke spikes of action potential during stimulation trains at 2.0 Hz in previously quiescent tissue. The records were obtained before and 30 min after application of OPC-88117 10^{-4} M.

The graph shows the relationship between stimulation frequency and the intensity of the use-dependent block. Ordinate scale: percentage decrease of $\dot{V}_{max}$ from the first action potential of stimulation trains to the new steady state level. Data were obtained before (○), and 30 min after application of OPC-88117 at 3×10^{-5} M (●) and at 10^{-4} M (△). Values are presented as means of five preparations; vertical lines show s.e.mean. *The change was statistically significant at $P < 0.05$.

Use-dependent effects on $\dot{V}_{max}$

The frequency-dependent inhibition of $\dot{V}_{max}$ by OPC-88117 was tested more extensively by trains of stimulation at different frequencies, which were separated from each other by a rest period of 30 s. In untreated papillary muscles, the value of $\dot{V}_{max}$ was almost unchanged at rates of stimulation trains from 0.2 to 2.5 Hz. After the treatment with OPC-88117 for 30 min, $\dot{V}_{max}$ of the first action potential in each train was slightly decreased; there was a tonic block of $\dot{V}_{max}$ by 3.0 ± 0.8% at 3×10^{-5} M ($n = 5$) and 5.1 ± 1.3% at 10^{-4} M ($n = 5$). During stimulation by trains at rates ⩾1.0 Hz, a rapid decrease in $\dot{V}_{max}$ approaching a new steady level within the 10th to 15th beats (use-dependent block) was observed. The use-dependent block of $\dot{V}_{max}$ by OPC-88117 was greater at higher stimulation frequencies and at higher drug concentrations (Figure 4).

The recovery of $\dot{V}_{max}$ from the use-dependent block was studied by adding a single test stimulus at various coupling intervals following a stimulation train for 20 s at 1.0 Hz. Before the application of OPC-88117, $\dot{V}_{max}$ of the test action potential recovered almost completely within 100 ms of the diastolic interval (the interval from the end of the last action potential to the beginning of the test action potential). After treatment with OPC-88117 (10^{-4} M), much slower $\dot{V}_{max}$ recovery was observed. Representative results are shown in Figure 5, where fractional reduction of $\dot{V}_{max}$ of test action potentials was plotted against the diastolic interval in a semilogarithmic graph. In the presence of OPC-88117, the recovery time course of $\dot{V}_{max}$ with a diastolic interval longer than 100 ms was approximated by a single exponential function. The average time constant was calculated to be 456 ± 28 ms ($n = 5$).

Voltage-dependent effects on $\dot{V}_{max}$

The effects of OPC-88117 on the relationship between $\dot{V}_{max}$ and the resting membrane potential from which the action potential originated were examined in five papillary muscles, driven at an interstimulus interval of 30 s. The slow rate of stimulation was used to eliminate the use-dependent

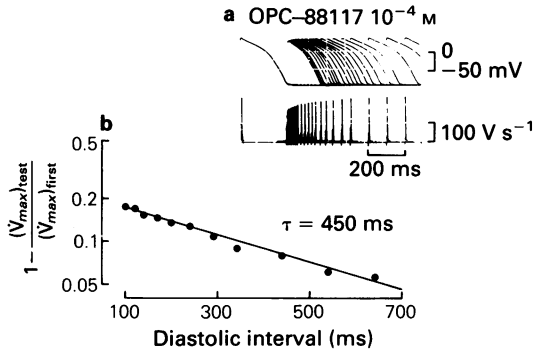


Figure 5 Offset of the use-dependent block of $\dot{V}_{max}$ by OPC-88117 in papillary muscle. (a) Superimposed records of action potentials (upper trace) and their differentiated upstroke spikes (lower trace) 30 min after application of OPC-88117 (10^{-4} M). The preparation was stimulated at 1.0 Hz for 20 s after a rest period of 30 s. After cessation of the stimulation train, a single test stimulus was applied with various coupling intervals. (b) The recovery process of $\dot{V}_{max}$ of test action potential. Ordinate scale: fractional $\dot{V}_{max}$ reduction of test action potential as compared with $\dot{V}_{max}$ of the first action potential. Abscissa scale: diastolic interval, which was measured from the end (at 95% repolarization) of the last action potential induced by the stimulation train to the upstroke of the test action potential. The time course was approximated by single exponential function with a time constant (τ) of 450 ms.

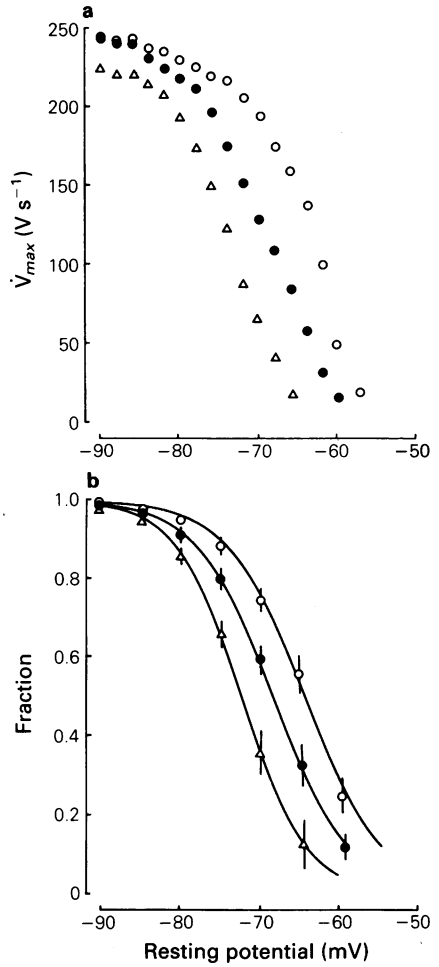


Figure 6 Effects of OPC-88117 on the relationship between resting membrane potential and $\dot{V}_{max}$ in papillary muscles driven with an interstimulus interval of 30 s. Values were obtained before (○) and 30 to 40 min after application of OPC-88117 at 3×10^{-5} M (●) and at 10^{-4} M (△). Absolute values in one experiment are shown in (a). Means of the normalized values ($n = 5$) are illustrated in (b); vertical lines indicate s.e.mean.

depression of $\dot{V}_{max}$ by OPC-88117. The membrane potential was depolarized in steps from the original resting level at 4 mM $[K^+]_0$ to about −60 mV, by increasing the K^+ concentration in the medium. The decrease of $\dot{V}_{max}$ by OPC-88117 (3×10^{-5} M, 10^{-4} M) was more pronounced at less negative membrane potentials (Figure 6a). A fraction of $\dot{V}_{max}$ was calculated in each experiment by normalizing the data with the value at 4 mM $[K^+]_0$, and means ± s.e. were obtained. As shown in Figure 6b, at a level of 50% reduction of $\dot{V}_{max}$, the average curve obtained following the drug application was shifted by 5 mV, at 3×10^{-5} M and by 9 mV, at 10^{-4} M OPC-88117, along the voltage axis in the direction of hyperpolarization.

$\dot{V}_{max}$ of single ventricular myocytes

In single ventricular myocytes, the effects of conditioning clamp pulses on $\dot{V}_{max}$ of subsequent test action potentials were examined, in order to determine whether the use-dependence of the inhibition of $\dot{V}_{max}$ by OPC-88117 is due to the blockade of an activated or an inactivated sodium channel.

Figure 7 shows the results of experiments with a single clamp pulse to 0 mV. Following a rest period of 30 s, the membrane potential was clamped up from the resting level (holding potential of −82 mV) to 0 mV for 10 to 1000 ms. At the end of the conditioning clamp pulse, the membrane potential was clamped back to the holding voltage for 100 ms, which is long enough for a drug-free channel to reactivate fully (Carmeliet & Vereecke, 1979; Ebihara &

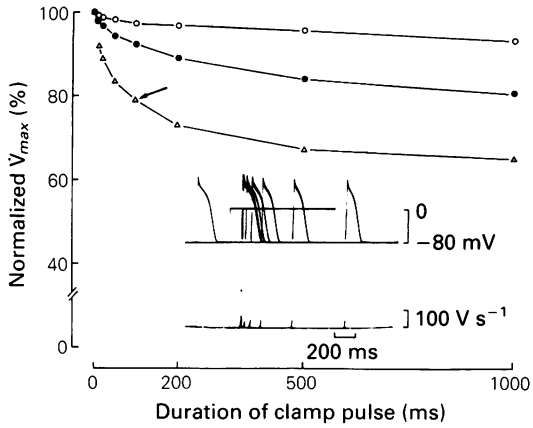


Figure 7 Effect of 0 mV conditioning clamp pulse on the inhibition of $\dot{V}_{max}$ induced by OPC-88117 in a single ventricular myocyte. Ordinate scale: $\dot{V}_{max}$ of test action potential normalized by the value of action potential without clamp pulse (reference level) in each condition. Data were obtained before (○) and 10 min after addition of OPC-88117 at 10^{-4} M (●) or at 3×10^{-4} M (△). An arrow indicates time constant (105 ms) for the exponential decay of $\dot{V}_{max}$. Inset shows superimposed records obtained 10 min after addition of OPC-88117 at 3×10^{-4} M. Action potential at extreme left was elicited without clamp pulse as a reference. Right side superimposed action potentials were preceded by 0 mV clamp pulse of 10, 20, 50, 100, 200, 500 and 1000 ms in duration with a coupling interval of 100 ms.

Johnson, 1980), but short enough so that only partial dissociation of the drug from the blocked channel occurs (Grant *et al.*, 1984). The voltage-clamp was then released, and a stimulus was applied to elicit a test action potential.

In untreated control myocytes, such a clamp pulse with a duration less than 200 ms had no significant effect on the $\dot{V}_{max}$ of the test action potential. However, further prolongation of the clamp pulse duration resulted in a slight but significant decrease in $\dot{V}_{max}$. A clamp pulse of 1000 ms in duration decreased $\dot{V}_{max}$ by 6.1 ± 1.2% ($n = 5$) from the value of action potentials without conditioning clamp pulse (reference level). Treatment of the myocyte with OPC-88117 at 10^{-4} M or 3×10^{-4} M for 10 min did not affect the resting potential (RP). $\dot{V}_{max}$ of reference action potential was slightly (3–8%) decreased compared with the value before drug application. In such myocytes, a conditioning clamp pulse caused a progressive decrease in $\dot{V}_{max}$ of the test action potential as the clamp pulse duration was prolonged (Figure 7). At 10^{-4} M OPC-88117, the decrease in $\dot{V}_{max}$ from the reference value was significant when the clamp pulse duration was longer than 50 ms, and reached 20.1 ± 0.8% ($n = 5$) at 1000 ms. At 3×10^{-4} M OPC-88117, similar clamp pulses caused a greater decrease in $\dot{V}_{max}$. Thus, a slight but significant decrease of $\dot{V}_{max}$ (5.8 ± 1.1%, $n = 5$) from the reference value was observed even at a clamp pulse of 10 ms, and it reached an almost steady level (34 ± 2.9%, $n = 5$) at 500 ms. At this concentration, the change in $\dot{V}_{max}$ with prolongation of clamp pulse duration in each experiment was well fitted to a single exponential function. The average time constant was 106 ± 8 ms ($n = 5$).

The recovery process of $\dot{V}_{max}$ following a conditioning clamp pulse to 0 mV for 1000 ms was examined, by introducing a test action potential with various coupling intervals during which the membrane potential was clamped back at the resting level (−82 mV). Before the application of OPC-88117, $\dot{V}_{max}$ of test action potentials recovered rapidly and followed a single exponential function with a time constant of 31 ± 2 ms ($n = 5$). In the presence of OPC-88117 (10^{-4} M, 3×10^{-4} M), the recovery process of $\dot{V}_{max}$ was approximated by a dual exponential function (Figure 8). The time constant of the early fast component (τ), and the late slow component (τ_2) were calculated by using the peeling-off method (Riggs, 1963). Values of τ_1 at 10^{-4} M and 3×10^{-4} M OPC-88117 were 29 ± 2 ms ($n = 5$) and 30 ± 4 ms ($n = 4$), respectively. These values were similar to the recovery time constant of $\dot{V}_{max}$ before the drug application. On the other hand, τ_2 values at 10^{-4} M and 3×10^{-4} M OPC-88117 were 466 ± 11 ms ($n = 5$) and 461 ± 13 ms ($n = 4$), respectively. There was no statistical difference between the two values.

Discussion

The present experiments in guinea-pig papillary muscles have revealed that OPC-88117 above 3×10^{-6} M causes a dose-dependent prolongation of action potential duration (APD) at the late repolarization phase. At concentrations above 3×10^{-5} M, OPC-88117 also caused a significant decreased in the maximum upstroke velocity ($\dot{V}_{max}$) of action potential without affecting the resting membrane potential (RP). The prolongation of APD was not affected by stimulation frequencies ranging from 0.5 to 2.0 Hz, whereas the inhibition of $\dot{V}_{max}$ was enhanced at higher frequencies.

The prolongation of APD by OPC-88117 can be attributed to either an increase of inward currents or a decrease of outward currents during the repolarization phase (Carmeliet & Vereecke, 1979). The contribution of the slow calcium inward current (I_{Ca}) or

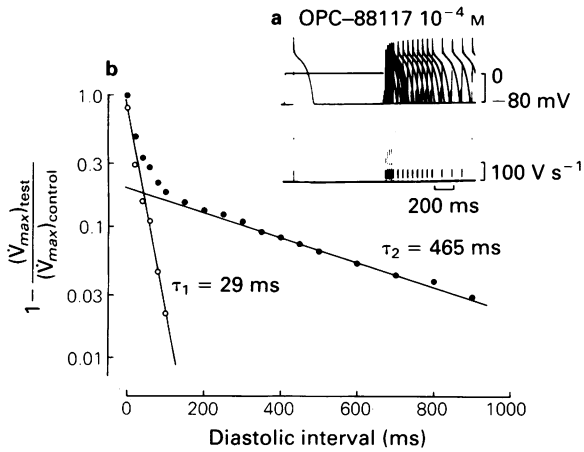


Figure 8 Recovery of $\dot{V}_{max}$ in single ventricular myocyte treated with OPC-88117. (a) Superimposed records of action potentials (upper trace) elicited after a conditioning clamp pulse of 1000 ms in duration and their differentiated upstroke spikes (lower trace) 10 min after application of OPC-88117 10^{-4} M. The coupling interval between the clamp pulse and the test action potential, during which the cell was clamped to the resting potential level (−82 mV), was varied, and each clamp pulse was preceded by a 30 s rest period. (b) Recovery process of $\dot{V}_{max}$ of test action potential. Ordinate scale, fractional $\dot{V}_{max}$ reduction of test action potential without conditioning clamp pulse (reference level). Abscissa scale, coupling interval between the clamp pulse and the test action potential. The time course was approximated by a dual exponential function with τ_1 of 29 ms and τ_2 of 465 ms.

the fast sodium inward current (I_{Na}) seems unlikely for the following reasons. An enhancement of I_{Ca} would be reflected in a prolongation of APD at the early repolarization phase (Carmeliet & Vereecke, 1979). However, the present data showed that APD_{30} was shortened by OPC-88117 at concentrations above 10^{-4} M. OPC-88117-induced prolongation of APD_{80} was preserved even when the $\dot{V}_{max}$ of the action potential, which reflects the sodium channel availability (Gintant *et al.*, 1983; Grant *et al.*, 1984), was markedly inhibited by this substance. The prolongation of APD could, therefore, be ascribed to other ionic mechanisms including a decrease of time-dependent or time-independent K^+ outward currents. Nevertheless, further experimental studies using the voltage-clamp technique are required to clarify this point.

The inhibition of $\dot{V}_{max}$ by OPC-88117 was enhanced by the higher stimulation frequency. The frequency-dependence or use-dependence of this effect can be interpreted within the framework of the 'modulated receptor hypothesis', proposed by Hondeghem & Katzung (1977; 1980) to explain the interaction between local anaesthetic type (Class I) antiarrhythmic drugs and cardiac sodium channels. According to this hypothesis, the reduction of I_{Na} is due to the accumulation of drug-associated nonconducting channels (blocked channels). If OPC-88117, like most of Class I antiarrhythmic drugs (Grant *et al.*, 1984; Hondeghem & Katzung, 1984), has a higher affinity for the receptor of an activated or inactivated channel than for that of a resting channel, an accumulation of blocked channels during the stimulation train leading to a use-dependent inhibition of $\dot{V}_{max}$ would be expected. The small tonic block of $\dot{V}_{max}$ by OPC-88117 in normally polarized papillary muscles is consistent with such an assumption.

The use-dependent block of $\dot{V}_{max}$ by OPC-88117 was observed during stimulation trains at rates $\geqslant$1.0 Hz, and its onset kinetics were very rapid. These characteristics are similar to those described for fast kinetic Class I drugs such as lignocaine, mexiletine and tocainide (Campbell, 1980; 1983a,b). OPC-88117 caused a slow phase of $\dot{V}_{max}$ recovery following stimulation trains, reflecting the dissociation of the drug from the inactivated or the resting sodium channels. The recovery time constant (456 ms) was comparable to those obtained for lignocaine, mexiletine and tocainide (200–500 ms) (Campbell 1980; 1983a). These observations may suggest that binding and unbinding kinetics of OPC-88117 molecules to the cardiac sodium channel receptors are similar to those of fast-kinetic Class I drugs.

The relationship of $\dot{V}_{max}$ to membrane potential was investigated in papillary muscles stimulated with an interstimulus interval of 30 s. Under such experimental conditions, a decrease in $\dot{V}_{max}$ may reflect only the tonic block by OPC-88117, while the use-dependent block may be negligible. The present results have revealed that the decrease in $\dot{V}_{max}$ as a result of tonic block by OPC-88117 is more pronounced at less negative membrane potentials. This effect could be due to a high affinity of this drug for inactivated sodium channels (Grant *et al.*, 1984).

Recently, we have shown that Class I antiarrhythmic drugs can be subdivided into two groups in terms of their sodium channel blocking phase during the conditioning clamp pulse to 0 mV; one 'transient' and one maintained (Kodama *et al.*, 1987a; Courtney, 1988). The former group of drugs (quinidine and disopyramide) may block the sodium channel mainly during its activated state, corresponding to the upstroke phase of the action potential, while the latter group (lignocaine, mexiletine, tocainide and aprindine) may do so predominantly during the inactivated state which corresponds to

the plateau phase of the action potential. In the present experiments with OPC-88117, we tested such a 'state-dependency' of sodium channel block by using a suction pipette whole-cell clamp technique in single ventricular myocytes. The protocols employed were similar to those in our previous experiments using single sucrose-gap voltage clamp technique (Kodama *et al.*, 1987a,b). In the presence of OPC-88117 (10^{-4} M, 3×10^{-4} M), the $\dot{V}_{max}$ of the test action potential, which was elicited 100 ms after the termination of 0 mV clamp, decreased progressively as the preceding clamp pulse duration was prolonged. At 3×10^{-4} M OPC-88117, the decay of $\dot{V}_{max}$ was well fitted to a single exponential function with a time constant of 106 ms. These findings may indicate that OPC-88117 blocks the sodium channel primarily when it is in the inactivated state. Time constants for the onset of the inactivated channel block by OPC-88117 are comparable to those for lignocaine and mexiletine but appreciably shorter than aprindine (Kodama *et al.*, 1987a,b).

In myocytes treated with OPC-88117 (10^{-4} M, 3×10^{-4} M), the recovery of $\dot{V}_{max}$ of a test action potential, following a 1000 ms conditioning clamp to 0 mV, was expressed by a dual exponential function. The time constant for the early fast component (τ_1) was similar to that for $\dot{V}_{max}$ recovery in untreated control myocytes. It may, therefore, reflect the repriming process (recovery from inactivation) of drug-free (unblocked) sodium channels. The time constant for the late slow component (τ_2) was concordant with the recovery time constant of $\dot{V}_{max}$ from the use-dependent block by OPC-88117 in papillary muscles. Accordingly, it may reflect the dissociation of the drug molecules (unbinding) from the sodium channels.

The *in vitro* concentrations of OPC-88117 used in the present study to prolong APD_{80} of guinea-pig papillary muscles were well within the effective i.v. doses used in dog experiments (unpublished data), whereas those concentrations needed to inhibit $\dot{V}_{max}$ were somewhat higher. Accordingly, it seems reasonable to conclude that the primary electrophysiological effect of OPC-88117 on cardiac muscle is prolongation of APD (Class III action) and that, at a higher concentration, it may also possess an inhibitory effect on the fast sodium channels (Class I action) which is similar to lignocaine and mexiletine in terms of use- and channel state-dependence.

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Effects of OPC-88117, a new antiarrhythmic agent, on the electrophysiological properties of rabbit isolated hearts

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1 Effects of a new antiarrhythmic agent, OPC-88117, on the conduction properties and excitability of Langendorff-perfused rabbit hearts were compared with those of lignocaine.

2 OPC-88117 above 10^{-5} M caused a significant prolongation of atrio–His bundle conduction time (A–H interval) as well as His bundle–ventricular conduction time (H–V interval). Lignocaine above 10^{-5} M caused a similar prolongation of H–V interval with a minimum change of A–H interval. The conduction time from His bundle stimulus to the left ventricle (St–LV) at a low frequency (1 Hz) was also prolonged by these drugs around 10^{-4} M.

3 OPC-88117 above 3×10^{-6} M prolonged the effective and functional refractory periods of His bundle (ERP_{HB}, FRP_{HB}) as well as the effective refractory period of left ventricular muscle (ERP_{VM}) in a dose-dependent manner. ERP_{HB}, FRP_{HB} and ERP_{VM} were shortened by low concentrations of lignocaine (3×10^{-6}–10^{-5} M), but were prolonged by high concentrations (3×10^{-5}–10^{-4} M).

4 Lignocaine (3×10^{-6}–10^{-4} M) induced an upward displacement of the His bundle refractory curve, indicating a greater intraventricular conduction delay for premature excitation. In experiments with OPC-88117, such an upward displacement was observed only at 10^{-4} M.

5 These results suggest that the primary electrophysiological effect of OPC-88117 is a lengthening of ventricular refractoriness, and that at high concentrations it may also exert lignocaine-like inhibition of ventricular conduction.

Introduction

OPC-88117 (8-methyl-3-(4-methyl-1-piperazinil)-2(1H)-quinolinone hydrochloride) is a newly synthesized antiarrhythmic agent. Previous studies on the transmembrane action potential of ventricular muscle have indicated that the primary electrophysiological effect of OPC-88117 is prolongation of action potential duration (APD), and that at high concentrations it also possesses an inhibitory action on the fast sodium channels (Kodama *et al.*, 1988; Toyama *et al.*, 1989). The latter action is similar to lignocaine in terms of its use- and channel state-dependence. Thus, the onset and offset kinetics of the use-dependent block with OPC-88117 are very fast. Furthermore, this substance blocks the sodium channel mainly during the inactivated state. These findings suggest that OPC-88117 is a unique antiarrhythmic drug having characteristics both of Class III and Class Ib antiarrhythmic drugs (Hauswirth & Singh, 1979; Vaughan Williams, 1984).

In the present study, the effects of OPC-88117 on the cardiac conduction properties and excitability were investigated in the isolated Langendorff-perfused heart of the rabbit, and compared with those of lignocaine, in order to clarify the pharmacological profile of OPC-88117 as an antiarrhythmic drug more extensively.

Methods

Rabbits weighing 1.5 to 2.0 kg were killed by a blow on the head. The heart was quickly removed and a cannula was inserted into the aorta for Langendorff perfusion. The preparation was perfused at a constant flow (20 ml min^{-1}) with Krebs-Ringer solution, gassed with 95% O_2 and 5% CO_2, of the following composition (mM): NaCl 120.0, KCl 4.0, $CaCl_2$ 1.2,

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Table 1 Effects of OPC-88117 and lignocaine on the atrioventricular conduction

		A–H interval (%)	*H–V interval* (%)
OPC-88117	10^{-6} M	103.5 ± 1.7	103.1 ± 0.1
	3×10^{-6} M	104.7 ± 0.8	103.1 ± 0.1
	10^{-5} M	109.2 ± 0.4*	108.2 ± 0.7*
	3×10^{-5} M	121.0 ± 1.5*	118.6 ± 1.5*
	10^{-4} M	A–H block (2/5)	H–V block (3/5)
Lignocaine	10^{-6} M	99.9 ± 3.6	101.4 ± 0.6
	3×10^{-6} M	96.8 ± 1.1	103.7 ± 2.1
	10^{-5} M	100.1 ± 2.7	111.6 ± 3.3*
	3×10^{-5} M	106.9 ± 2.6	124.7 ± 6.7*
	10^{-4} M	120.7 ± 4.4*	164.3 ± 9.8*
	3×10^{-4} M	—	H–V block (5/5)

Values are presented as percentage of control (means ± s.e., $n = 5$) for each drug. A–H interval, atrio–His bundle conduction time; H–V interval, His bundle–ventricle conduction time. Data were obtained 15 min after application of the drug at the respective concentration. * Significantly different from control at $P < 0.05$.

$MgSO_4 \cdot 7H_2O$ 1.3, NaH_2PO_4 1.2, $NaHCO_3$ 24.2 and glucose 5.5 (pH 7.4). The temperature of the perfusate was maintained at 34°C. Bipolar stainless steel electrodes with an interpolar distance of 1.0 mm were inserted through a small incision made in the right atrium so as to record His bundle electrograms (HBE). Another pair of bipolar recording electrodes was placed on the epicardial surface of left ventricle (LV) near the apex. The signals were amplified at a frequency response from 100 to 500 Hz with a time constant of 0.03 s and displayed on a digital storage oscilloscope (Tektronix 5223). A pair of stimulating electrodes (interpolar distance, 1.0 mm) made of stainless wires were placed on the right atrium close to the sinus node region.

In experiments to test the ventricular conduction properties and refractoriness at a slow basic driving frequency, the lower part of the A-V node was ligated with a fine silk thread to produce A-V block, and the heart was driven through a pair of contiguous bipolar electrodes placed at the proximal end of the His bundle (Osaka *et al.*, 1988). When the refractoriness of ventricular working myocardium was tested, the heart was stimulated through bipolar electrodes on the epicardial surface of the left ventricle.

Parameters measured were as follows: atrio–His bundle conduction time (A–H interval), His bundle–ventricular conduction time (H–V interval), conduction time from His bundle stimulus to the left ventricle (St–LV interval), effective refractory period of His bundle (ERP_{HB}), functional refractory period of His bundle (FRP_{HB}) and effective refractory period of left ventricular muscle (ERP_{VM}).

A–H and H–V intervals were measured under constant right atrial stimulation at a cycle length of 400 ms. St–LV interval was measured under constant His-bundle stimulation at a cycle length of 1000 ms. The pulses for stimulation were 2 ms in duration and twice the diastolic threshold in intensity.

To obtain ERP_{HB} and FRP_{HB} a premature stimulus (S_2) was applied through His bundle stimulating electrodes with various coupling intervals, at every tenth basic stimulus (S_1) and a cycle length of 1000 ms, by means of a programmable stimulator. The shortest S_1–S_2 interval captured by left ventricular response (LV) was defined as ERP_{HB}. The FRP_{HB} was defined as the shortest interval between two successive LV excitations. A similar extrastimulus method was used to obtain the ERP_{VM}; the tenth basic stimulus (S_1) at a cycle length of 1000 ms to the left ventricle was followed by a premature stimulus (S_2), and the shortest S_1–S_2 interval captured by ventricular responses was defined as ERP_{VM}.

Control measurements were performed after an equilibration period of 60 min. The effects of drugs were measured 15 min after the heart was perfused with a solution containing OPC-88117 (10^{-6}–10^{-4} M), or lignocaine (10^{-6}–3×10^{-4} M). Values are expressed as means ± s.e. unless otherwise stated. Data were analysed by *t*-test, analysis of variance and Dunnett's test. Differences were considered significant at $P < 0.05$.

Results

Atrio-ventricular conduction

Effects of OPC-88117 and lignocaine on A–H and H–V intervals were tested in each of five preparations, under right atrial stimulation at a constant cycle length of 400 ms. The average control values of A–H and H–V intervals in all 10 preparations were 52.2 ± 2.1 ms and 31.8 ± 0.6 ms, respectively.

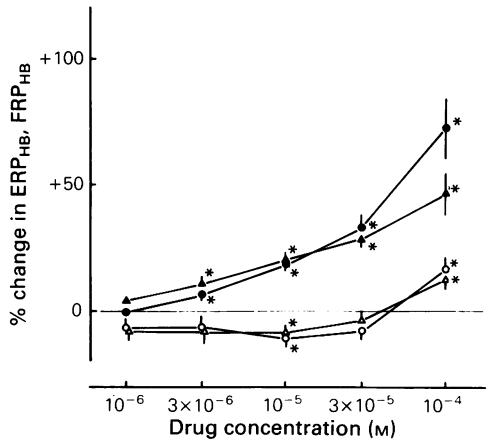


Figure 1 Effects of OPC-88117 on the effective and functional refractory periods of His bundle (ERP_{HB}, FRP_{HB}). Ordinate scale: percentage change from control. Values presented are means of five preparations for OPC-88117 (●; ERP_{HB}, ▲; FRP_{HB}), and five for lignocaine (○; ERP_{HB}, △; FRP_{HB}). Vertical lines show s.e.mean. Data were obtained 15 min after application of the drug at the respective concentrations. * Significantly different from control at $P < 0.05$.

OPC-88117 at concentrations $\leqslant 3 \times 10^{-6}$ M caused no significant changes in these parameters. At 10^{-5} M OPC-88117, a significant prolongation of both A–H and H–V intervals was observed

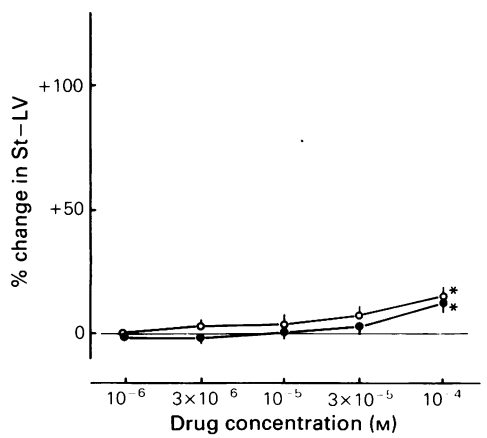


Figure 2 Effects of OPC-88117 (●) and lignocaine (○) on the conduction time from the His bundle stimulus to the left ventricle (St-LV). Ordinate and abscissa scales are as in Figure 1. Values presented are mean of five preparations for each drug with vertical lines showing s.e.mean. Data were obtained 15 min after application of the drug at the respective concentrations. * Significantly different from control at $P < 0.05$.

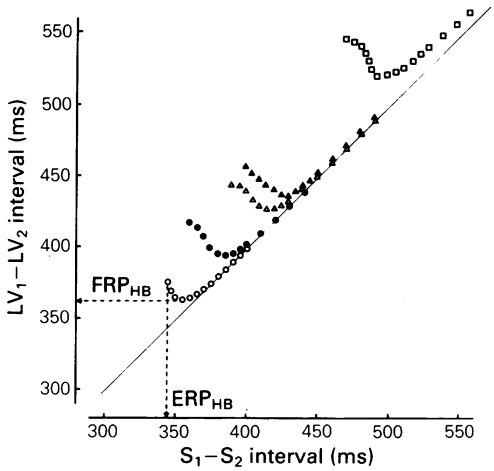


Figure 3 Effects of OPC-88117 on the refractory curve of His bundle. Ordinate scale: the interval between two captured ventricular responses with basic and premature His bundle stimuli ($LV_1 - LV_2$). Abscissa scale: the coupling interval between the basic and premature stimuli ($S_1 - S_2$). Data were obtained before (○), and 15 min after application of the drug at 3×10^{-6} M (●), 10^{-5} M (△), 3×10^{-5} M (▲) and 10^{-4} M (□). ERP_{HB}: effective refractory period of His bundle; FRP_{HB}: functional refractory period of His bundle.

(Table 1). The prolongation was further enhanced at 3×10^{-5} M. At 10^{-4} M OPC-88117, H–V block was induced in three out of five hearts, and the remaining two showed A–H block. Lignocaine at 1 to 3×10^{-5} M caused a significant and dose-dependent prolongation of H–V interval without affecting the A–H interval (Table 1). At 10^{-4} M lignocaine, a slight but significant prolongation of A–H interval was also observed. The higher concentration (3×10^{-4} M) of lignocaine always induced H–V block.

Conduction properties and refractoriness of the ventricle

Effects of the two drugs on ventricular conduction properties and refractoriness were examined in the hearts where A–V block was artificially produced by ligation. Under His bundle stimulation at a basic cycle length (S_1–S_1) of 1000 ms, the control value of St–LV interval was 34.5 ± 1.7 ms ($n = 10$), and those of ERP_{HB} and FRP_{HB} were 327.8 ± 5.9 ms ($n = 10$) and 356.0 ± 4.7 ms ($n = 10$), respectively.

OPC-88117 at concentrations ranging from 3×10^{-6} M to 3×10^{-5} M caused a dose-dependent prolongation of ERP_{HB} and FRP_{HB} without affecting St–LV intervals for basic stimuli (Figures 1 and 2). At 3×10^{-5} M, ERP_{HB} and FRP_{HB} were prolonged by 33.1 ± 4.5% ($n = 5$) and 27.6 ± 3.5% ($n = 5$)

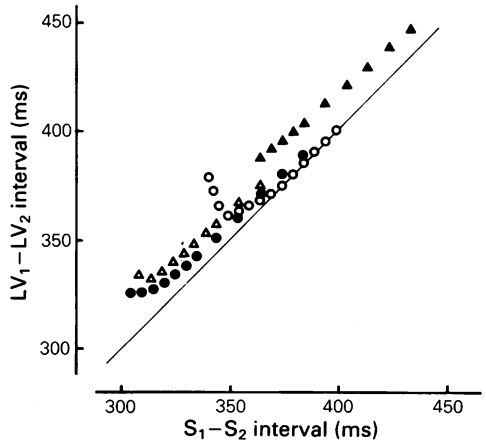


Figure 4 Effects of lignocaine on the refractory curve of His bundle. Ordinate and abscissa scales are as in Figure 3. Data were obtained before (○), and 15 min after application of the drug at 10^{-5} M (●), 3×10^{-5} M (△) and 10^{-4} M (▲).

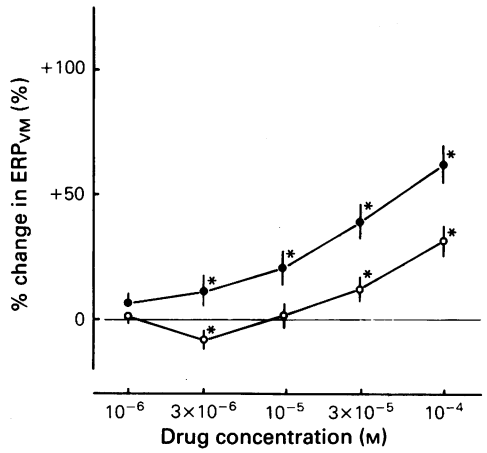


Figure 5 Effects of OPC-88117 (●) and lignocaine (○) on the effective refractory period of left ventricular muscle (ERP_{VM}). Ordinate and abscissa scales are as in Figure 1. Values presented are means of five preparations for each drug with vertical lines showing s.e.mean. Data were obtained 15 min after application of the drug at the respective concentrations. * Significantly different from control at $P < 0.05$.

respectively. At 10^{-4} M OPC-88117, a slight prolongation of St–LV interval was observed in addition to a further prolongation of ERP_{HB} and FRP_{HB}.

Figures 3 and 4 illustrate refractory curves of His bundle. When the coupling interval of premature stimulus ($S_1 - S_2$) was shortened in steps in the control preparations, the intervals between the captured left ventricular responses ($LV_1 - LV_2$) were shortened by similar extents down to $S_1 - S_2$ intervals of 370–380 ms as shown by the data plotted on the identical line. Further shortening of $S_1 - S_2$ resulted in a sharp upward deflection of the curve due to a conduction delay of S_2 excitation. The minimum value of $S_1 - S_2$ and that of $LV_1 - LV_2$ on the curve indicate ERP_{HB} and FRP_{HB} respectively.

In preparations treated with OPC-88117 at concentrations ranging from 3×10^{-6} M to 3×10^{-5} M, the refractory curve shifted towards the right upper direction, giving rise to a dose-dependent prolongation of ERP_{HB} and FRP_{HB}. There was no displacement of the refractory curve from the identical line for the data with $S_1 - S_2$ longer than that causing FRP_{HB}. At 10^{-4} M OPC-88117, however, an appreciable upward displacement of the refractory curve from the identical line was observed.

Lignocaine caused more complex changes of the refractory curve (Figure 4). Thus, at concentrations of $1–3 \times 10^{-5}$ M, a marked shortening of ERP_{HB} and FRP_{HB} was accompanied by an upward displacement of the refractory curve from the identical line. At 10^{-4} M lignocaine, both ERP_{HB} and FRP_{HB} were prolonged to the values above control with a further upward displacement of the refractory curve.

Figure 5 shows the effects of OPC-88117 and lignocaine on ERP_{VM}. The control value of ERP_{VM} was 322.5 ± 4.7 ms ($n = 10$). OPC-88117 above 3×10^{-6} M caused a significant prolongation of ERP_{VM} in a concentration-dependent manner. At 10^{-4} M OPC-88117, ERP_{VM} was prolonged by $60.3 \pm 6.8\%$ ($n = 10$). Lignocaine caused biphasic changes of ERP_{VM} as in the case of ERP_{HB} and FRP_{HB} (Figure 1). Thus, ERP_{VM} was shortened by a low concentration of lignocaine (3×10^{-6} M) while it was prolonged by high concentrations (3×10^{-5} M, 10^{-4} M).

Discussion

The present study showed that OPC-88117 above 10^{-5} M prolonged H–V as well as A–H intervals. At 10^{-4} M OPC-88117, St–LV interval was also prolonged. Such negative dromotropic effects of OPC-88117 on the ventricular conduction may be attributed to the inhibitory action of this substance on the cardiac fast sodium channels.

In our previous experiments in guinea-pig ventricular muscle constantly stimulated at 1.0 Hz, OPC-88117 at concentrations above 3×10^{-5} M caused a significant decrease in the maximum upstroke velocity ($\dot{V}_{max}$) of action potential without affecting the resting potential level. This inhibition of $\dot{V}_{max}$ by OPC-88117 was shown to be augmented at higher stimulation frequencies (Kodama *et al.*, 1988;

Toyama et al., 1989). The different threshold concentration of OPC-88117 required to prolong H–V and St–LV intervals in the rabbit hearts can be explained by the frequency-dependent inhibition of $\dot{V}_{max}$. The H–V interval was measured under right atrial stimulation at a cycle length of 400 ms (2.5 Hz), whereas the St–LV interval was measured under His bundle stimulation at a cycle length of 1000 ms (1 Hz). At the higher stimulation frequency, OPC-88117 even at relatively low concentrations may have caused a considerable decrease in $\dot{V}_{max}$ of action potential, sufficient to reduce the conduction velocity in Purkinje and ventricular muscle fibres.

The lignocaine-induced prolongation of H–V and St–LV may also be interpreted by similar mechanisms. It has been shown by previous investigators that lignocaine at concentrations above 10^{-5} M decreases $\dot{V}_{max}$ of action potential in Purkinje (Davis & Temte, 1969; Bean et al., 1982) as well as in ventricular muscle fibres (Chen et al., 1975; Grant et al., 1980) especially when the tissue was stimulated at rates higher than 1.0 Hz. The frequency-dependent inhibition of $\dot{V}_{max}$ is known to reflect the 'use-dependent' sodium channel blocking action of this drug (Grant et al., 1984; Hondeghem & Katzung, 1984).

The potency of OPC-88117 for the inhibition of ventricular conduction is comparable to lignocaine on a molar basis. As regards the inhibition of A–V nodal conduction, however, OPC-88117 is more potent than lignocaine. Since the electrical activity of A–V node depends primarily on the slow calcium inward current (I_{Si}) (Carmeliet & Vereecke, 1979), OPC-88117, unlike lignocaine, might inhibit I_{Si} as well. Further experimental studies are required to prove this possibility.

The present study has revealed that OPC-88117 causes a marked dose-dependent prolongation of refractory periods of His bundle and left ventricular muscle. The threshold concentration for this action (3×10^{-6} M) was lower than that for the negative dromotropic effect. In guinea-pig papillary muscles, OPC-88117 above 3×10^{-6} M was shown to prolong the action potential duration (APD) in a dose-dependent manner (Kodama et al., 1988; Toyama et al., 1989). The prolongation of ERP_{HB}, FRP_{HB} and ERP_{VM} is, therefore, most likely due to the prolongation of APD in Purkinje and ventricular muscle fibres.

The refractory curves of His bundle in hearts were not displaced by treatment with OPC-88117 at concentrations ranging from 3×10^{-6} M to 3×10^{-5} M (Figure 3), indicating no substantial conduction delay of premature excitation compared with the basic excitation. Accordingly, a prolongation of the 'time-dependent refractoriness', due to the delayed recovery of excitability after full repolarization of action potential, is unlikely with OPC-88117 at concentrations up to 3×10^{-5} M.

Effects of lignocaine on the refractory periods of His bundle and left ventricular muscle are different from those of OPC-88117. ERP_{HB}, FRP_{HB} and ERP_{VM} were shortened by lignocaine at concentrations around 10^{-5} M, whereas they were prolonged at 10^{-4} M. An upward displacement of the refractory curve from the control line was observed at all the concentrations tested, but the extent of displacement was greater at the higher dose. These complex changes may be explained by a combination of APD shortening and a lengthening of the time-dependent refractoriness. It has been demonstrated in Purkinje fibres (Weld & Bigger, 1975) and ventricular muscle preparations (Chen et al., 1975; Grant et al., 1980) that lignocaine causes a marked delay in the recovery kinetics of $\dot{V}_{max}$ during the diastolic interval after full repolarization of action potential. This slow recovery of $\dot{V}_{max}$ at the resting potential level has been attributed to the dissociation of the lignocaine molecule from the sodium channel receptors, leading to a slow recovery of sodium channel availability (Grant et al., 1984; Hondeghem & Katzung, 1984). In the presence of lignocaine, therefore, premature stimuli would be expected to induce action potentials with very low $\dot{V}_{max}$, which would be propagated very slowly to the surrounding myocardium, or would be insufficient to be propagated. At high concentrations of lignocaine, a prolongation of the time-dependent refractoriness due to such a mechanism is thought to overcome the shortening of APD, giving rise to a net prolongation of refractory periods.

In preparations treated with OPC-88117 10^{-4} M, an upward displacement of the refractory curve was observed, as in the case of lignocaine. This is consistent with our previous experiments in guinea-pig papillary muscles, where a marked lignocaine-like delay of the recovery of $\dot{V}_{max}$ after repolarization was demonstrated in preparations treated with OPC-88117 10^{-4} M (Toyama et al., 1989).

The effective plasma concentrations of OPC-88117 needed to suppress ventricular arrhythmias in dogs are 0.5–1.0 $\mu g\,ml^{-1}$, corresponding to 1.7–3.3×10^{-6} M (unpublished data). Accordingly, it seems reasonable to conclude that the primary electrophysiological effects of OPC-88117 may be a lengthening of ventricular refractoriness through APD prolongation. At very high concentrations, this drug may also exert an inhibition of ventricular conduction through a lignocaine-like sodium channel blocking action.

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Cardiac electrophysiological actions of captopril: lack of direct antiarrhythmic effects

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1 Standard microelectrode techniques were used to study the effects of captopril (1, 10 and 100 μM) on action potentials recorded from guinea-pig ventricular cells and sinoatrial node cells.

2 Captopril had no effect on the maximum rate of depolarization ($\dot{V}_{max}$) of ventricular action potentials in cells exposed to either normal Locke solution or 'simulated ischaemic' solution (K^+ = 11.2 mM; pH = 6.4; Po_2 < 80 mmHg), nor was there any augmentation of the normal small decline in $\dot{V}_{max}$ with increasing stimulation rate (range of interstimulus intervals = 2400 ms to 300 ms).

3 Captopril had no effect on the duration of ventricular action potentials, nor did it alter the shortening seen on exposure to simulated ischaemia.

4 Captopril did not alter spontaneous sinus cycle length or any recorded parameter of sinus node action potentials.

5 It is concluded that any antiarrhythmic effects observed during clinical use of captopril are most unlikely to be due to direct actions of the drug on cardiac cell membrane properties.

Introduction

Cardiac failure is a frequent cause of mortality and morbidity. The angiotensin converting enzyme (ACE) inhibitors appear to be of value in alleviating morbidity (Edwards & Padfield, 1985), and at least one of them (enalapril), has now been proven to reduce mortality in this condition (Consensus Trial Study Group, 1987).

In view of the fact that some 35–50% of deaths in chronic cardiac failure are due to ventricular arrhythmias rather than pump failure (Massie & Conway, 1987), it is of interest that enalapril was reported to have made no impact at all on arrhythmic deaths in the Consensus study. There is, however, circumstantial evidence from a number of studies that captopril and possibly enalapril may possess antiarrhythmic properties. Van Gilst *et al.* (1984, 1986) reported that captopril but not enalapril reduced reperfusion arrhythmias in Langendorff-perfused rat hearts following 15 min coronary artery occlusion. Furthermore, at least two controlled, clinical trials of captopril have also shown statistically significant reductions of ventricular arrhythmias (Cleland *et al.*, 1984a,b; Captopril-Digoxin Multicenter Research Group, 1988), and a similar claim has been made for enalapril (Webster *et al.*, 1985).

There are several possible mechanisms by which ACE inhibition might be antiarrhythmic (see Discussion). In addition, captopril but not enalapril, has significant free-radical scavenging ability (Westlin & Mullane, 1988) which could certainly contribute to such an effect. The purpose of the present study, however, was to ascertain whether captopril possesses any direct cellular electrophysiological actions which might potentially be antiarrythmic. To this end, we have used microelectrodes to record action potentials from guinea-pig myocardium during superfusion with normal oxygenated physiological saline and during simulated ischaemia.

Methods

Guinea-pigs were stunned by a blow to the head, their hearts rapidly removed and placed in cold Locke solution. Ventricular myocardial preparations consisted of small strips cut from the right ventricular free wall, pinned to the base of a tissue bath, and superfused with Locke solution (at 36°C) gassed with 95% O_2 and 5% CO_2. The tissue was stimulated by

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constant current pulses of 4 ms duration, from a Grass S11 stimulator, delivered through a bipolar platinum electrode. The stimuli were of sufficient magnitude to maintain a constant latency between the stimulus and the action potential upstroke (Walton & Fozzard, 1979). Sinus node preparations consisted of a small area of the posterior wall of the right atrium, with its borders formed by the crista terminalis, the interatrial septum, and the superior and inferior vena cavae. This preparation beat spontaneously and the area adjacent to the crista terminalis was explored to locate a typical sinoatrial node cell (maximum diastolic potential less than -70 mV, spontaneous diastolic depolarization, and an upstroke dV/dt of less than $10\,V\,s^{-1}$; Campbell, 1987). Once a stable impalement had been maintained for at least 15 min, the experiment was started.

Action potentials were recorded by glass microelectrodes filled with 3 M KCl (resistance 20–30 Mohm) and connected to a high input impedance d.c. amplifier (WPI 750). Action potentials were monitored on an oscilloscope and analyzed by digital techniques modified from previously described methods (Campbell, 1987).

The effects of three concentrations of captopril (supplied by Squibb Australia) on action potential duration and resting and rate-dependent depression of $\dot{V}_{max}$ were investigated, under control and simulated ischaemic conditions. The control Locke solution contained (mM): NaCl 125, KCl 5.6, $CaCl_2$ 2.16, $NaHCO_3$ 25, $MgCl_2$ 1.0, NaH_2PO_4 0.44, glucose 11 (pH 7.4). To simulate ischaemia, the concentration of KCl was doubled to 11.2 mM, glucose was omitted from the solution, and bicarbonate concentration was decreased to 2.5 mM, which reduced pH to 6.4. The solution was gassed with 95% N_2 and 5% CO_2, which decreased the Po_2 of the superfusate from >500 mmHg to <80 mmHg. To eliminate any possible 'order' effect, the sequence of exposure to normal Locke solution and simulated ischaemia, and to captopril-containing and control solutions was randomized.

Data are expressed as mean $\pm$ s.d., and Student's t test was used to test the significance of difference of means of individual pairs of samples.

Results

Effects on maximum rate of depolarization ($\dot{V}_{max}$)

In this series of experiments, continuous impalement was maintained to study $\dot{V}_{max}$ in 'normal' Locke solution and under conditions of simulated ischaemia.

Under ischaemic conditions, the cells depolarized from a control resting potential of -93 ± 3.8 mV to -74 ± 6.2 mV in drug-free solution. The presence of captopril in the superfusate in concentrations of 1, 10 and 100 μM had no effect on this parameter, with the ischaemic solution still causing approximately a 20 mV depolarization (from 90.7 ± 1.2 mV to 71.8 ± 6.2 mV at 100 μM captopril; $n = 5$). Neither the change in action potential amplitude (123 ± 3.8 to 95.4 ± 12.8 mV) nor decrease in the rate of rise of the action potential upstroke ($\dot{V}_{max}$) (reduced from $217 \pm 62\,V\,s^{-1}$ to $150 \pm 68\,V\,s^{-1}$; $n = 5$) caused by the depolarizing effect of the ischaemic conditions was altered by the presence of up to 100 μM captopril in the superfusate. The corresponding values (at 100 μM captopril) were a reduction of action potential amplitude from 115 ± 6.6 mV to 89 ± 12.4 mV, and a decrease in $\dot{V}_{max}$ from 235 ± 47.2 to $111 \pm 29.9\,V\,s^{-1}$. The differences in mean values for control and drug solutions did not reach significance. All the differences between 'nonischaemic' and ischaemic solutions were highly significant ($P < 0.001$).

The depression of V_{max} in the absence of prior stimulation is known as resting block (Campbell, 1983), and was measured by recording $\dot{V}_{max}$ of the first action potential of a train following 30 min exposure of unstimulated tissue to captopril (100 μM). No significant resting block was produced by captopril in either normal Locke solution or in simulated ischaemia.

Rate-dependent depression of $\dot{V}_{max}$ was measured by driving the tissue at a series of interstimulus intervals ranging from 2400 ms down to 300 ms, in both control and ischaemic solutions, and measuring the percentage depression of $\dot{V}_{max}$ during trains of 100 action potentials, In control (non-ischaemic) solution, the presence of captopril had no effect on the depression of $\dot{V}_{max}$ seen during the train of action potentials, with the largest decline occurring at an interstimulus interval of 300 ms. This value was $5.6 \pm 1.8\%$ for drug-free solution, and $5.0 \pm 2.5\%$ in the presence of captopril 100 μM ($n = 5$). The values for depression of $\dot{V}_{max}$ in ischaemic conditions were higher than control due to depolarization of the membrane. At an interstimulus interval of 300 ms, $\dot{V}_{max}$ declined by $35.1 \pm 8.9\%$ in the absence of drug, with a corresponding value for the captopril solution of $37.8 \pm 9.4\%$. There were no significant differences between these values, nor the means of the values at the slower driving rates.

Effects on action potential duration

A separate series of experiments was done to monitor any effects on action potential duration. In these studies, impalement of one ventricular cell was maintained while the effects of captopril in control and ischaemic conditions were studied. Action potential duration was measured at both 50% repol-

Table 1 Effects of captopril (100 μM) on sinus node potentials

	Control	*Captopril*	*30 min Wash*
Cycle length (ms)	345 ± 17.1	347 ± 14.8	345 ± 16.9
Amplitude (mV)	76 ± 10.2	79.3 ± 8.7	77 ± 9.1
Maximum diastolic potential (mV)	−63 ± 12.4	−64.3 ± 8.0	−64.6 ± 9.1
$\dot{V}_{max}$ ($V s^{-1}$)	6.4 ± 3.3	6.7 ± 3.4	6.8 ± 2.2
Repolarization rate ($V s^{-1}$)	−0.95 ± 0.18	−0.96 ± 0.11	−0.98 ± 0.15
Phase 4 slope ($mV s^{-1}$)	88 ± 31	92 ± 38	93 ± 36
Action potential duration (ms)	204 ± 8.3	206 ± 12.9	207 ± 10.1

Exposure times = 30–60 min; $n = 6$, $\dot{V}_{max}$ = maximum rate of depolarization.

arization (APD_{50}) and 90% repolarization (APD_{90}). In the absence of drug there was a marked and rapid (maximal in 15–20 min) reduction in both parameters during simulated ischaemia. APD_{50} fell from 141 ± 20.3 ms to 74 ± 27 ms, and APD_{90} from 162 ± 20.6 ms to 90 ± 31.3 ms ($n = 5$; $P < 0.001$). In the presence of captopril 100 μM, the corresponding values for the same 5 cells in normal Locke solution and simulated ischaemia were APD_{50}: 140 ± 20.0 ms to 64 ± 23.5 ms ($P < 0.001$); APD_{90}: 165 ± 19 ms and 83.2 ± 27.2 ms ($P < 0.001$). None of these values was significantly different from the respective value recorded in the absence of drug.

Sinus node potentials

The effects of 30–60 min exposure to captopril 100 μM were studied during continuous impalement of six sinus node cells exposed to normal Locke solution. No drug effects were noted on any parameter recorded (Table 1). In particular the spontaneous cycle length, and rates of depolarization and repolarization were unaltered, suggesting no effect on either inward calcium or outward potassium currents.

Discussion

The evidence suggesting that ACE inhibitors (and particularly captopril) might be antiarrhythmic has been outlined in the Introduction. It is based partly on a series of *in vitro* experiments showing reduced post-ischaemic reperfusion arrhythmias in rat hearts (van Gilst *et al.*, 1984, 1986; Rochette *et al.*, 1987), and partly on the results of three clinical trials involving patients with moderate to severe congestive cardiac failure (Cleland *et al.*, 1984a,b; Webster *et al.*, 1985; Captopril-Digoxin Group, 1988). In Langendorff-perfused rat hearts, pretreatment with a very high concentration of captopril (369 μM) both abolished serious reperfusion arrhythmias after 15 min of coronary artery ligation and markedly reduced noradrenaline overflow (van Gilst *et al.*, 1986). Enalapril had no such effect, although its active metabolite, enalaprilat was not tested. Rochette *et al.* (1987) confirmed this antiarrhythmic action in a very similar preparation using a lower concentration of captopril (50 μM). At this concentration, which is still well above even peak plasma concentrations seen in therapeutic use (< 10 μM; Kripilani *et al.*, 1980; Singhvi *et al.*, 1982), captopril did not alter the large scale release of noradrenaline accompanying reperfusion.

Two of the clinical trials suggesting antiarrhythmic actions for ACE inhibitors have been extremely small, involving only 14 patients and 19 patients respectively (Cleland *et al.*, 1984a,b; Webster *et al.*, 1985). The Captopril-Digoxin Multicenter study (1988) was significantly larger with approximately 100 patients in each of the captopril, digoxin and placebo groups. The mean number of ventricular premature beats per hour fell 29.4% for the captopril patients ($n = 104$), rose 2.3% for the digoxin group ($n = 96$) and fell 16.1% in the placebo-treated patients ($n = 100$). The reduction with captopril was statistically significant when compared to the digoxin patients but not the placebo group.

However, it is open to question whether the modest reductions in ventricular arrhythmias claimed in these studies truly indicate an antiarrhythmic effect (Morganroth, 1984; Bigger & Rolnitzky, 1985). Certainly no evidence for a reduction in sudden death rates can be gleaned from any of these trials, nor was such an effect noted in the Consensus Study (1987). This may, of course, be simply due to a lack of adequate statistical power in the reports to date (Packer, 1987).

There are certainly a number of theoretical grounds for supposing that ACE inhibitors (and captopril in particular) might be antiarrhythmic. Patients in chronic heart failure treated with these agents generally experience a reduction in sympathetic activity. This is at least partly due to withdrawal of reflexly increased tone, but may also result

from prevention of direct and indirect inotropic and arrhythmogenic properties of angiotensin II (Dzau, 1988; Lindpaintner *et al.*, 1988). Noradrenaline levels in blood may come down initially (Cleland *et al.*, 1984a,b) but this effect seems not to persist (Francis *et al.*, 1986). The improvement in total body potassium balance frequently seen after the addition of ACE inhibitors to patients already on diuretic therapy might also reduce the likelihood of arrhythmias. Finally, captopril, but not enalaprilat, has been shown in dog studies to have significant free-radical scavenging properties at clinically relevant concentrations. Pretreatment with captopril led to marked enhancement of recovery of contractile function during 3 h of reperfusion following 15 min of coronary ischaemia (Westlin & Mullane, 1988). Of more relevance to the present study, the incidence of ventricular fibrillation on reperfusion fell from 6 out of 16 (control) to 0 out of 13 (captopril; $P < 0.05$). Oxygen-free radical production is causally implicated in reperfusion arrhythmias (Pallandi *et al.*, 1987) and other free-radical scavengers have been found to be of benefit in these circumstances (Bernier *et al.*, 1986).

The experiments described in the present study however, were performed to ascertain whether the ACE inhibitor captopril showed evidence of any direct cellular electrophysiological actions which might be antiarrhythmic. In particular, we were interested in identifying any effects on $\dot{V}_{max}$ on action potential duration, or on sinus node potentials. In concentrations up to 100 μM, well above the usual levels found during clinical use, captopril had no significant influence on any of these parameters. Nor were any effects seen in the presence of the 'ischaemic' superfusate, which is probably more representative of the extracellular fluid in the immediate vicinity of arrhythmogenic foci.

It seems reasonable to assume then, that if captopril is exerting any beneficial effect on arrhythmic morbidity and mortality in patients with congestive cardiac failure, such an action is not mediated by any of the established, direct antiarrhythmic mechanisms (Vaughan Williams, 1984). It must be stated, however, that with the possible exception of amiodarone (Dargie *et al.*, 1987; Neri *et al.*, 1987), the available 'traditional' antiarrhythmic agents appear to be of little if any value in preventing or reducing the very high incidence of ventricular tachycardia and sudden death in such patients (Wilson, 1987; Prystowsky, 1988). Indeed the problem of possible proarrhythmic effects of these drugs is a matter for concern (Podrid, 1985).

It may be that the indirect antiarrhythmic properties listed above for captopril and related drugs are ultimately of greater therapeutic benefit in heart failure patients than the direct actions of the traditional antiarrhythmic compounds.

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Binding and functional properties of antimuscarinics of the hexocyclium/sila-hexocyclium and hexahydro-diphenidol/hexahydro-sila-diphenidol type to muscarinic receptor subtypes

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1 In an attempt to assess the structural requirements for the muscarinic receptor selectivity of hexahydro-diphenidol (hexahydro-difenidol) and hexahydro-sila-diphenidol (hexahydro-sila-difenidol), a series of structurally related C/Si pairs were investigated, along with atropine, pirenzepine and methoctramine, for their binding affinities in NB-OK 1 cells as well as in rat heart and pancreas.

2 The action of these antagonists at muscarinic receptors mediating negative inotropic responses in guinea-pig atria and ileal contractions has also been assessed.

3 Antagonist binding data indicated that NB-OK 1 cells (M_1 type) as well as rat heart (cardiac type) and pancreas (glandular/smooth muscle type) possess different muscarinic receptor subtypes.

4 A highly significant correlation was found between the binding affinities of the antagonists to muscarinic receptors in rat heart and pancreas, respectively, and the affinities to muscarinic receptors in guinea-pig atria and ileum. This implies that the muscarinic binding sites in rat heart and the receptors in guinea-pig atria are essentially similar, but different from those in pancreas and ileum.

5 The antimuscarinic potency of hexahydro-diphenidol and hexahydro-sila-diphenidol at the three subtypes was influenced differently by structural modifications (e.g. quaternization). Different selectivity profiles for the antagonists were obtained, which makes these compounds useful tools to investigate further muscarinic receptor heterogeneity. Indeed, the tertiary analogues hexahydro-diphenidol (HHD) and hexahydro-sila-diphenidol (HHSiD) had an M_1 = glandular/smooth muscle > cardiac selectivity profile, whereas the quaternary analogues HHD methiodide and HHSiD methiodide were M_1 preferring (M_1 > glandular/smooth muscle, cardiac).

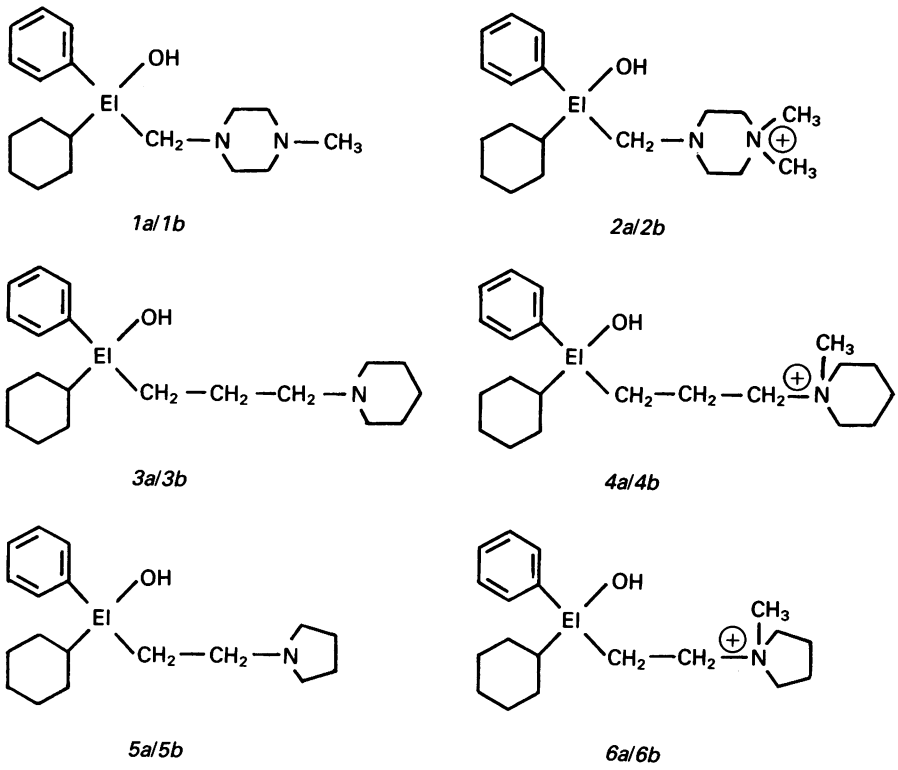
Introduction

Three muscarinic receptors with different selectivities for antagonists have been described in binding studies as well as in *in vitro* and *in vivo* pharmacological assays. M_1 receptors are above all found in the nervous system and are characterized by a high affinity for pirenzepine (Hammer *et al.*, 1980; Birdsall *et al.*, 1980; Brown *et al.*, 1980; Hammer & Giachetti, 1982; Lambrecht *et al.*, 1988b). M_2 receptors, which show low affinity for pirenzepine, have been further subdivided into cardiac type and glandular/smooth muscle subtypes by use of selective antagonists such as 4-diphenylacetoxy-*N*-methyl-piperidine methiodide (4-DAMP) (Barlow *et al.*, 1976; Waelbroeck *et al.*, 1987a; Doods *et al.*, 1987), hexahydro-diphenidol (hexahydro-difenidol) and hexahydro-sila-diphenidol (hexahydro-sila-difenidol) (Mutschler & Lambrecht, 1984; Waelbroeck *et al.*, 1987b; Lambrecht *et al.*, 1988a, c), AF-DX 116 (Hammer *et al.*, 1986; Giachetti *et al.*, 1986; Doods *et al.*, 1987) and methoctramine (Melchiorre *et al.*, 1987; Michel & Whiting, 1988).

Among these selective muscarinic antagonists, hexahydro-diphenidol (*3a*) and hexahydro-sila-diphenidol (*3b*) have been shown to have high affinity

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a: El = C, b: b: El = Si

Figure 1 Structural formula of antagonists studied: demethyl-hexocyclium (*1a*), demethyl-sila-hexocyclium (*1b*), hexocyclium (*2a*), sila-hexocyclium (*2b*), hexahydro-diphenidol (*3a*), hexahydro-sila-diphenidol (*3b*), *N*-methyl-hexahydro-diphenidol (*4a*), *N*-methyl-hexahydro-sila-diphenidol (*4b*), procyclidine (*5a*), sila-procyclidine (*5b*), tricyclamol (*6a*), and sila-tricyclamol (*6b*).

for M_1 receptors in neuronal tissues as well as for glandular/smooth muscle receptors in exocrine glands and smooth muscle, but a much lower affinity for cardiac receptors (Mutschler & Lambrecht, 1984; Waelbroeck *et al.*, 1987b; Lambrecht *et al.*, 1988a,c). In order to assess the structural requirements for the observed selectivity of *3a/3b*, the binding characteristics of a series of C/Si pairs structurally related to *3a/3b* were determined (compounds *1a/1b*, *2a/2b*, *4a/4b–6a/6b*; Figure 1). These experiments were carried out using a NB-OK 1 cell line (M_1 receptors type) and rat heart (cardiac receptors type) and pancreas (glandular/smooth muscle receptors type) homogenates (Waelbroeck *et al.*, 1987a,b,c). Furthermore the binding affinities of compounds *1a/1b–6a/6b* to glandular/smooth muscle and cardiac receptor types were compared with the functional antimuscarinic properties in guinea-pig isolated ileum and atria. Atropine, pirenzepine and methoctramine were used as reference drugs.

The experiments were carried out using the racemates of compounds *1a/1b* to *6a/6b* but some data of functional experiments with the individual enantiomers of *5a/5b* and *6a/6b* have been published elsewhere (Tacke *et al.*, 1986; 1987a) and binding studies are in progress.

Methods

Radioligand binding studies

Tissue preparations Male Wistar rats (200–250 g) were killed by decapitation and the pancreas and heart were immediately removed.

The pancreas was minced with scissors and homogenized in 8 ml per pancreas of 300 mM sucrose enriched with 0.2 mg ml^{-1} bacitracin and 500 kallikrein inhibitor units ml^{-1} of aprotinin, with a glass-

Teflon homogenizer (7 up and down strokes). The resulting homogenate was filtered on two layers of medical gauze, and immediately diluted 11 fold with the incubation buffer (66 mM sodium phosphate buffer (pH 7.4) enriched with 2.6 mM $MgCl_2$ and with 1.32% bovine serum albumin, 0.24 mg ml^{-1} bacitracin and 600 kallikrein inhibitor units ml^{-1} aprotinin).

The hearts were rinsed in isotonic NaCl, then homogenized in 2.5 ml of Tris-HCl buffer (pH 7.5) enriched with 250 mM sucrose, in an Ultraturrax homogenizer (5 s at maximum setting). The resulting homogenate was diluted 8 fold with the same buffer and filtered on two layers of medical gauze.

NB-OK 1 cells were cultured in RPMI 1640 medium, enriched with 10% foetal calf serum, 100 units ml^{-1} penicillin and 100 $\mu g\,ml^{-1}$ streptomycin, as described previously (Waelbroeck *et al.*, 1988). For [^{3}H]-N-methylscopolamine ([^{3}H]-NMS) binding experiments, the cells were rinsed, detached and centrifuged in 20 mM sodium phosphate buffer (pH 7.4) containing 156 mM NaCl and 1 mM EDTA, resuspended and homogenized in 20 mM Tris-HCl buffer (pH 7.5) enriched with 5 mM $MgCl_2$ and stored in liquid nitrogen.

Binding experiments: On rat pancreas homogenates, 1 ml of diluted homogenate (800 to 1000 μg protein per assay) was added to 200 μl of [^{3}H]-NMS and unlabelled drugs (in water) to obtain final concentrations of 50 mM sodium phosphate buffer (pH 7.4), 2 mM $MgCl_2$, 1% bovine serum albumin, 0.2 mg ml^{-1} bacitracin and 500 kallikrein inhibitor units ml^{-1} of aprotinin. The [^{3}H]-NMS concentration was 240 pM (with a K_D value of 120 pM in pancreas). This incubation was prolonged for 4 h at 25°C to allow equilibration of tracer binding to pancreas receptors (Waelbroeck *et al.*, 1987b).

On rat cardiac homogenates, 80 μl of homogenate (400 to 500 μg of protein) was used per assay, in a total volume of 1.2 ml containing (final concentrations): 50 mM sodium phosphate buffer (pH 7.4), 2 mM $MgCl_2$, 1% bovine serum albumin, and the indicated concentrations of [^{3}H]-NMS, and unlabelled drugs. The [^{3}H]-NMS concentration was 1.0 nM (with a K_D value of 0.5 nM in heart). The incubation period was 2 h at 25°C, sufficient to allow equilibrium binding of [^{3}H]-NMS in this tissue (Waelbroeck *et al.*, 1987a,b).

On human NB-OK 1 homogenates, 80 μl of homogenate (80 μg of protein) was used per assay, in a total volume of 1.2 ml containing (final concentrations): 50 mM sodium phosphate buffer (pH 7.4), 2 mM $MgCl_2$, 1% bovine serum albumin, and the indicated concentrations of [^{3}H]-NMS, and unlabelled drugs. The [^{3}H]-NMS concentration was 240 pM (with a K_D value of 120 pM in NB-OK 1 homogenates). An incubation period of 2 h at 25°C was sufficient to allow equilibrium binding of [^{3}H]-NMS in this tissue (Waelbroeck *et al.*, 1988).

Non-specific [^{3}H]-NMS binding, using pancreas, heart and NB-OK 1 homogenates was defined as [^{3}H]-NMS binding in the presence of 1 μM atropine. In our binding conditions, non-specific binding represented less than 5% of total tracer binding, and was fully accounted for by binding to GF/C glass-fibre filters (see below).

To separate bound and free [^{3}H]-NMS, in pancreas, heart or NB-OK 1 homogenates, 2 ml of ice-cold 50 mM sodium phosphate buffer (pH 7.4) was added to each sample, followed by immediate filtration on GF/C glass-fibre filters (Whatman, Maidstone, Kent, England) presoaked overnight in 0.05% polyethyleneimine. The filters were rinsed three times with the same phosphate buffer, dried, and the radioactivity counted by liquid scintillation.

Analysis of binding data All competition curves were repeated at least three times, in duplicate. IC_{50} values were determined by a computer-aided procedure described by Richardson & Humrich (1984), assuming the existence of only one receptor subtype. Experimental data points were within 3% of the expected values, assuming that the molecules investigated competed with [^{3}H]-NMS for binding to a single site.

K_i values were determined from IC_{50} values, by the Cheng & Prusoff (1973) equation which assumes competitive inhibition of tracer binding to a single receptor subtype. The [^{3}H]-NMS K_D value for the three systems investigated was determined in separate experiments, as described by Waelbroeck *et al.* (1987a,b; 1988). The pK_i values, indicated in Table 1, corresponded to $-\log K_i$ values.

The standard deviation of pIC_{50} ($-\log IC_{50}$) determination was equal to or below 0.10 log unit. Repeated determinations of [^{3}H]-NMS K_D values were within 10% of each other. This error should be added to errors in IC_{50} determinations, since [^{3}H]-NMS K_D values were used to calculate pK_i values. We therefore estimated the standard deviation of pK_i values as being of approximately 0.15 log unit (40% of K_i value).

Pharmacological studies

General considerations Guinea-pigs (300–450 g) of either sex were killed by cervical dislocation and the organs required were set up under 0.5 g tension in 6 ml organ baths containing physiological salt solution (PSS) and gassed with 5% CO_2/95% O_2. The composition of PSS was as follows (mM): NaCl 137.0, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1.05, $NaHCO_3$ 11.9, NaH_2PO_4 0.42, glucose 5.6. All experiments

were conducted at pH 7.4, 32°C. Arecaidine propargyl ester (Mutschler & Lambrecht, 1984; Barlow & Weston-Smith, 1985), except where indicated, was used as the agonist.

After 1 h equilibration, concentration-response curves were obtained by cumulative addition of the agonists (van Rossum, 1963). When these responses were regular the tissues were exposed to a solution of antagonist and each concentration of antagonist was allowed to equilibrate before repeating the concentration-response curve.

Guinea-pig isolated ileum Strips of ileal longitudinal muscle (1.5 cm) were prepared according to Paton & Zar (1968). Tissue responses were measured as isotonic contractions with an electromechanical transducer connected to a Hellige amplifier and a Rikadenki recorder. The responses were then calculated as a percentage of the maximum response obtained to arecaidine propargyl ester and plotted against the logarithm of the agonist concentration, and EC_{50} values were determined for the control and the antagonist shifted concentration-response curves.

Guinea-pig isolated atria Left atria were electrically paced, by use of platinum electrodes (2 Hz, 3 ms duration, supramaximal voltage). Atrial responses to the agonists were measured as changes in isometric tension, and these effects were expressed as the percentage inhibition of the force of contraction. EC_{50} values were determined as described for the ileum.

Antagonist affinities (pA_2) Concentration-response curves were repeated in the presence of at least 3 concentrations (log interval = 0.48) of antagonists, allowing 15–45 min equilibration time. Each concentration of antagonist was tested 3 to 5 times and dose-ratios at EC_{50} values of agonists were calculated. These dose-ratios were pooled for each concentration of antagonist and the slope of the Arunlakshana and Schild plots were determined by linear regression using the method of least squares. pA_2 values were estimated by fitting to the data the best straight line with a slope of unity (Arunlakshana & Schild, 1959; Tallarida *et al.*, 1979).

Statistical analysis The data are presented as means ± s.e.mean of n experiments. Differences between mean values were tested for statistical significance by means of Student's t test, accepting $P < 0.05$ as being significant.

Compounds

[^{3}H]-N-methylscopolamine ([^{3}H]-NMS, 72 Ci mmol^{-1}) was obtained from Amersham International (Bucks, England). Atropine, carbachol, bacitracin and polyethylenimine were purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.). Aprotinin (Trasylol) was obtained from Bayer, Belgium. Pirenzepine was a gift of Thomae (F.R.G.) and procyclidine (*5a*) was kindly provided by Deutsche Wellcome (F.R.G.). Methoctramine was kindly donated by Dr C. Melchiorre, University of Bologna, Italy. Arecaidine propargyl ester was synthesized in one of our laboratories (Mutschler & Hultzsch, 1973).

Demethyl-hexocyclium [1-cyclohexyl-1-phenyl-2-(1-methylpiperazin-4-yl)-1-ethanol, *1a*], demethyl-sila-hexocyclium [cyclohexyl((1-methylpiperazin-4-yl)methyl)phenylsilanol, *1b*], hexocyclium methyl sulphate [4-(2-cyclohexyl-2-hydroxy-2-phenylethyl)-1,1-dimethylpiperazinium methyl sulphate, *2a* $CH_3OSO_3^-$], sila-hexocyclium methyl sulphate [4-((cyclohexylhydroxyphenylsilyl)methyl)-1,1-dimethylpiperazinium methyl sulphate, *2b* $CH_3OSO_3^-$], hexahydro-diphenidol hydrochloride [1-cyclohexyl-1-phenyl-4-piperidino-1-butanol hydrochloride, *3a* ·HCl], hexahydro-sila-diphenidol hydrochloride [cyclohexylphenyl(3-piperidinopropyl)silanol hydrochloride, *3b* ·HCl], sila-procyclidine [cyclohexylphenyl(2-pyrrolidinoethyl)silanol, *5b*], and tricyclamol chloride [1-(3-cyclohexyl-3-hydroxy-3-phenylpropyl)-1-methylpyrrolidinium chloride, *6a* Cl^-] were prepared according to the literature: *1a* and *2a* $CH_3OSO_3^-$ (Zaugg *et al.*, 1958); *1b* and *2b* $CH_3OSO_3^-$ (Tacke *et al.*, 1989); *3a* ·HCl and *3b* ·HCl (Tacke *et al.*, 1985); *5b* (Tacke *et al.*, 1983; Tacke *et al.*, 1987a); *6a* Cl^- (Murfitt, 1956).

Synthetic chemistry *N*-methyl-hexahydro-diphenidol iodide [1-(4-cyclohexyl-4-hydroxy-4-phenylbutyl)-1-methylpiperidinium iodide, *4a* I^-], *N*-methyl-hexahydro-sila-diphenidol iodide [1-(3-(cyclohexylhydroxyphenylsilyl)propyl)-1-methylpiperidinium iodide, *4b* I^-], and sila-tricyclamol iodide [1-(2-((cyclohexylhydroxyphenyl)silyl)ethyl)-1-methylpyrrolidinium iodide, *6b* I^-] were prepared as follows: freshly distilled methyl iodide (18 mmol) was added under an atmosphere of dried nitrogen to a solution of *3a*, *3b* or *5b* (8 mmol) in dry ethanol (50 ml). After the reaction mixture was stirred for 3 h at 30°C, dry *n*-pentane (75 ml (*3a*, *3b*) and 150 ml (*5b*), respectively) was added, and the mixture was stirred for 1.5 h at 20°C. Thereafter, the precipitate was collected by filtration and then recrystallized from ethanol/*n*-pentane. (After dissolving the precipitate in dry ethanol, the fourfold (*3a*, *3b*) and twofold (*5b*) volume, respectively, of dry *n*-pentane was added dropwise to the solution). After drying the crystals *in vacuo* (because of their hygroscopic nature, the products *4b* and *6b* needed an especially

Table 1 Antagonist affinities (pK_i values) obtained in [^{3}H]-N-methylscopolamine ([^{3}H]-NMS) binding studies in NB-OK 1 (M_1 receptors), pancreas (glandular/smooth muscle receptors) and cardiac (cardiac receptors) homogenates

Antagonist	*NB-OK 1 cells*	*Pancreas*	*Heart*
1a Demethyl-hexocyclium	8.0	7.4	6.7
1b Demethyl-sila-hexocyclium	7.9	7.4	6.6
2a Hexocyclium	8.8	8.4	7.7
2b Sila-hexocyclium	8.9	8.4	7.6
3a Hexahydro-diphenidol	7.9	7.9	6.8
3b Hexahydro-sila-diphenidol	7.9	7.8	6.8
4a HHD methiodide	8.5	7.9	8.1
4b HHSiD methiodide	8.8	8.2	8.0
5a Procyclidine	8.1	7.6	7.1
5b Sila-procyclidine	8.5	7.9	7.4
6a Tricyclamol	8.9	8.0	7.8
6b Sila-tricyclamol	9.1	8.2	8.0
Atropine	9.5	9.4	9.0
Pirenzepine	8.2	6.8	6.5
Methoctramine	7.3	5.9	8.2

The numbers show the mean estimate of the value of $-\log$ inhibitor constant (pK_i) for $n = 3$ experiments. The standard deviation was approximately 0.15 log unit (see Methods).
HH(Si)D methiodide = N-methyl-hexahydro-(sila-) diphenidol iodide.

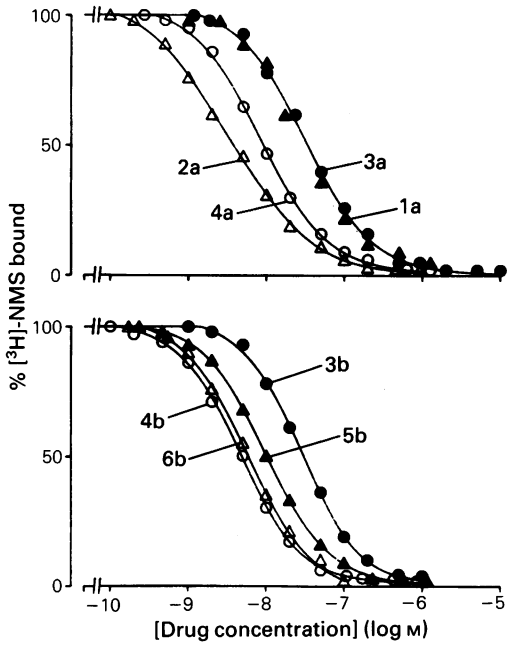


Figure 2 [^{3}H]-N-methylscopolamine ([^{3}H]-NMS) competition curves in human neuroblastoma NB-OK 1 cells. [^{3}H]-NMS binding to NB-OK 1 cells homogenates was measured in the absence or presence of 1a (▲), 2a (△), 3a (●), 4a (○) (top panel) or of the corresponding sila-analogues 3b (●), 4b (○), 5b (▲) or 6b (△) (bottom panel), as indicated in Methods. Average of 3 to 4 experiments performed in duplicate.

careful drying) analytically pure products were obtained [characterized by ^{1}H n.m.r., ^{13}C n.m.r. and FAB MS measurements (data not given) as well as by elemental analyses].

4a: $C_{22}H_{36}INO$ (457.4), yield 89%, m.p. 194°C; found: C, 57.8; H, 8.1; N, 3.1; calculated: C, 57.77; H, 7.93; N, 3.06%.

4b: $C_{21}H_{36}INOSi$ (473.5), yield 65%, m.p. 119–121°C; found: C, 53.3; H, 7.7; N, 3.1; calculated: C, 53.27; H, 7.66; N, 2.96%.

6b: $C_{19}H_{32}INOSi$ (445.5), yield 92%, m.p. 128–129°C; found: C, 51.6; H, 7.3; N, 3.1; calculated: C, 51.23; H, 7.24; N, 3.14%.

All antagonists studied were racemates.

Results

Radioligand binding studies in NB-OK 1, heart and pancreas homogenates

All compounds antagonized [^{3}H]-NMS binding to muscarinic receptors. Competition curves with the C/Si pairs *1a/1b–6a/6b* did not deviate significantly from results expected for competitive inhibition of tracer binding to a single receptor. Representative competition curves are shown in Figures 2 to 4 and pK_i values are given in Table 1.

Atropine, pirenzepine and the quaternary compounds *4a/4b* and *6a/6b* exhibited similar affinities for muscarinic receptors in pancreas and heart. At both receptors the following rank order of potencies was obtained: atropine > *4a/4b* = *6a/6b* > pirenzepine.

The affinities obtained for compounds *1a/1b*, *2a/2b*, *3a/3b* and *5a/5b* were significantly greater for receptors in pancreas as compared to receptors in heart, and the following rank orders of potencies were obtained; heart: *2a/2b* > *5a/5b* > *3a/3b* ≥ *1a/1b*; pancreas: *2a/2b* > *3a/3b* ≥ *5a/5b* ≥ *1a/1b*. In contrast, the pK_i value obtained for methoctramine was markedly lower in the pancreas as compared to heart.

Pirenzepine, compounds *1a/1b*, *2a/2b*, *4a/4b*, *5a/5b*, and *6a/6b* recognized preferentially the receptors expressed by NB-OK 1 cells, as compared to

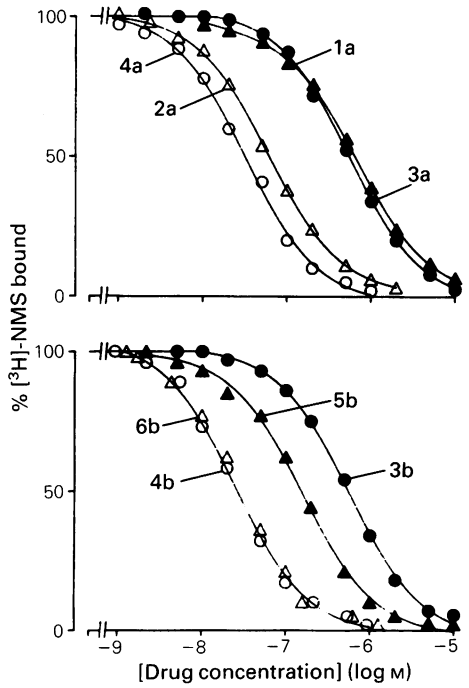


Figure 3 [^{3}H]-N-methylscopolamine ([^{3}H]-NMS) competition curves in rat heart. Details as in Figure 1, but with heart homogenates.

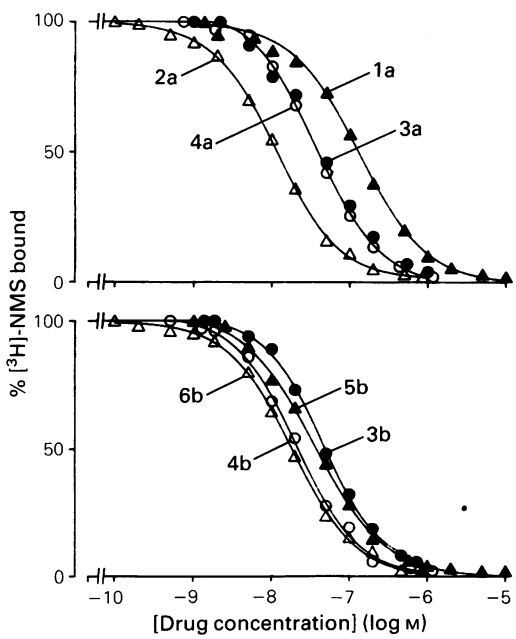


Figure 4 [^{3}H]-N-methylscopolamine ([^{3}H]-NMS) competition curves in rat pancreas. Details as in Figure 1, but with pancreas homogenates.

heart and pancreas muscarinic receptors. The following rank order of potencies was obtained: *6a/6b*, *2a/2b*, *4a/4b* ≥ pirenzepine = *5a/5b* ≥ *1a/1b*. The following rank order of M_1 selectivities (pK_i in NB-OK 1 cells vs pK_i in pancreas) was obtained: pirenzepine > *6a/6b* ≥ *4a/4b* ≥ *1a/1b* = *5a/5b* ≥ *2a/2b*.

Methoctramine and *3a/3b* presented a different selectivity pattern. Whereas methoctramine recognized the NB-OK 1 receptors with an affinity intermediate between its affinity for cardiac and pancreas receptors, compounds *3a/3b* exhibited the same high affinity for NB-OK 1 and pancreas receptors and a much lower affinity for the receptors in the heart.

Isolated tissue experiments on atria and ileum from guinea-pig

All compounds antagonized the negative inotropic effects in electrically stimulated atria and the contractions of the ileum induced by arecaidine propargyl ester or carbachol. There was a concentration-dependent parallel shift to the right of agonist concentration-response curves without either basal tension or maximum response being affected. In all experiments Schild plots were linear through the concentration range tested for each antagonist, and slopes were not significantly different from unity ($P > 0.05$). Thus, all compounds were apparently simple competitive antagonists in both atrial and ileal preparations. The pA_2 values are shown in Table 2.

Atropine, pirenzepine and the quaternary compounds *4a/4b* and *6a/6b* exhibited similar affinities for muscarinic receptors in ileum and atria. At both receptors the following rank order of potencies was obtained: atropine > *4a/4b* ≥ *6a/6b* > pirenzepine.

The affinities obtained for compounds *1a/1b*, *2a/2b*, *3a/3b*, and *5a/5b* were significantly greater at muscarinic receptors present in the ileum than at receptors in the atria, and the following rank orders of potencies were obtained; atria: *2a/2b* > *5a/5b* ≥ *3a/3b* ≥ *1a/1b*; ileum: *2a/2b* > *3a/3b* ≥ *5a/5b* ≥ *1a/1b*.

In contrast, the pA_2 value obtained for methoctramine was much lower in the ileum compared to the affinity obtained in the atria.

Discussion

This study has investigated the differences in affinity of a series of compounds structurally related to hexahydro-diphenidol (*3a*) and hexahydro-sila-diphenidol (*3b*) at muscarinic receptors. The principal conclusions we draw from these experiments are: (1) the differences in affinity values exhibited by atropine, pirenzepine, methoctramine and compounds *1a/1b*–*6a/6b* and the different rank orders of anti-

Table 2 Antagonist affinities (pA_2 values) at ileal and atrial muscarinic receptors[a]

Antagonist	*Ileum*	*Atria*
1a Demethyl-hexocyclium	7.63 ± 0.03	6.51 ± 0.08
1b Demethyl-sila-hexocyclium	7.87 ± 0.04	6.65 ± 0.04
2a Hexocyclium	8.49 ± 0.04	7.75 ± 0.03
2b Sila-hexocyclium	8.78 ± 0.05	7.57 ± 0.04
3a Hexahydro-diphenidol	7.98 ± 0.02	6.71 ± 0.04
3b Hexahydro-sila-diphenidol	7.96 ± 0.04[b]	6.53 ± 0.05[b]
4a HHD methiodide	8.00 ± 0.03	7.93 ± 0.04
4b HHSiD methiodide	8.18 ± 0.04	7.99 ± 0.02
5a Procyclidine	7.75 ± 0.04[a]	6.76 ± 0.04[a]
5b Sila-procyclidine	8.04 ± 0.04[a]	6.92 ± 0.04[a]
6a Tricyclamol	7.92 ± 0.06[a]	7.65 ± 0.03[a]
6b Sila-tricyclamol	8.22 ± 0.05[a]	7.88 ± 0.02[a]
Atropine	8.85 ± 0.03	9.21 ± 0.04
Pirenzepine	6.88 ± 0.04[b]	6.82 ± 0.03[b]
Methoctramine	6.17 ± 0.05	7.69 ± 0.03

The numbers show the mean estimate of the value of $-\log$ affinity constant (pA_2 ± s.e.), $n = 9$–16 experiments. [a] Arecaidine propargyl ester was used as the agonist in all cases, except carbachol. [b] Data previously published by Lambrecht *et al.*, (1988a,b)

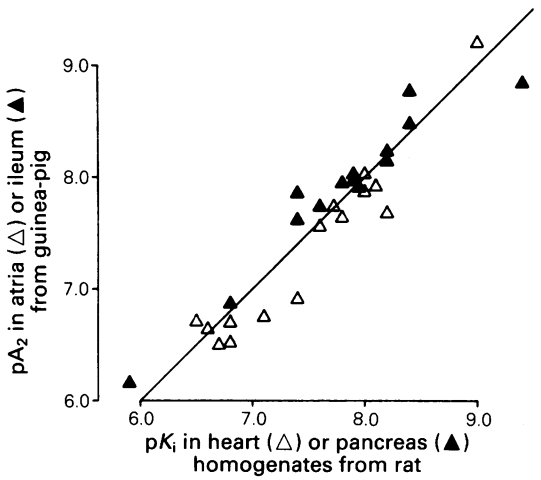


Figure 5 Comparison of pK_i and pA_2 values. pK_i values obtained in binding experiments are compared with pA_2 values obtained in pharmacological studies: data on rat cardiac homogenates are related to those on guinea-pig isolated atria (△), and rat pancreas homogenates with guinea-pig isolated ileum strips (▲) (data from Table 1 and 2).

muscarinic potencies confirm that NB-OK 1 cells, heart and pancreas possess different muscarinic receptors of the neuronal M_1, cardiac, and glandular/smooth muscle subtypes, respectively (Waelbroeck *et al.*, 1987a,b,c). (2) The rat heart and pancreas muscarinic binding sites are essentially similar to the muscarinic receptors mediating negative inotropic responses in guinea-pig atria and contractions in guina-pig ileum, respectively. (3) Structural variations in the hexahydro-diphenidol (*3a*) and hexahydro-sila-diphenidol (*3b*) molecules lead to new muscarinic antagonists which exhibit a different spectrum of selectivity from the parent compounds.

Comparison of binding and pharmacological experiments

Atropine, pirenzepine, methoctramine and compounds *1a/1b–6a/6b* showed quite wide variations in their affinities for the muscarinic receptors in pancreas, ileum and heart, their pK_i (Table 1) and pA_2 (Table 2) values differing by three orders of magnitude. The affinities of the antagonists for muscarinic receptors in rat pancreas and in guinea-pig ileum were very similar both individually and in rank order. A highly significant correlation was found between these values (Figure 5). The affinities for muscarinic receptors in rat heart and guinea-pig atria were also strikingly similar (Figure 5), but they were different from those obtained in pancreas and ileum for most antagonists. This implies that the structural demands for the antagonists made by the muscarinic binding sites in pancreas and heart, respectively, are very similar to those made by the receptors in ileum and atria, respectively.

Structure-selectivity relationships

In the course of structure-activity relationship studies of compounds of the diphenidol type, it was found that replacement of the central carbinol carbon atom by silicon increased the affinity to muscarinic receptors in guinea-pig ileum and atria up to one order of magnitude (Tacke & Zilch, 1986; Tacke & Becker, 1987). In contrast, sila-substitution of *1a/6a* (→*1b/6b*) had little effect on the affinity in binding studies as well as in pharmacological experiments (Tables 1 and 2). This might be explained by differences in electronic and stereochemical properties: compounds of the diphenidol type carry two phenyl rings at the central C/Si atom, whereas in the case of *1a/1b–6a/6b* one phenyl ring is hydrogenated to a cyclohexyl ring system.

The tertiary amines demethyl-hexocyclium (*1a*), procyclidine (*5a*) and their silicon analogues (*1b*, *5b*)

are muscarinic antagonists that possess a different selectivity profile from the parent compounds hexahydro-diphenidol (*3a*) and hexahydro-sila-diphenidol (*3b*). The following affinity rank orders at M_1, cardiac and glandular/smooth muscle receptors were obtained; *3a/3b*: M_1 = glandular/smooth muscle > cardiac; *1a/1b*, *5a/5b*: M_1 > glandular/smooth muscle > cardiac.

N-methylation of the tertiary amines *1a/1b*, *3a/3b* and *5a/5b* increased the affinity for cardiac as well as for NB-OK 1 cell M_1 receptors by up to one log unit (Tables 1 and 2). This also holds true for *1a/1b* (→*2a/2b*) at glandular/smooth muscle receptors in ileum and pancreas, whereas the affinity for glandular/smooth muscle receptors of *3a/3b* and *5a/5b* was not changed or only poorly increased by N-methylation. Thus, the selectivity profiles of the quaternary compounds hexocyclium (*2a*) and sila-hexocyclium (*2b*) are identical to that of the tertiary amines *1a/1b* (M_1 > glandular/smooth muscle > cardiac), the affinities of *2a/2b* being higher by about one order of magnitude than those of the tertiary compounds *1a/1b* at the three subtypes. In contrast, N-methylation of *3a/3b* (→*4a/4b*) and *5a/5b* (→*6a/6b*) changed the receptor selectivity patterns of the tertiary amines. The following affinity rank order was obtained; *4a/4b*, *6a/6b*: M_1 > glandular/smooth muscle ≥ cardiac. Thus, *4a/4b* and *6a/6b* show a selectivity profile that is qualitatively similar to that of pirenzepine.

In conclusion, the present study shows that on the basis of the binding profiles of pirenzepine, methoctramine and compounds *1a/1b–6a/6b* the muscarinic receptors in NB-OK 1 cells, heart and pancreas can be clearly subdivided into three subtypes: M_1, cardiac and glandular/smooth muscle receptors. We have provided evidence that the muscarinic binding sites in the rat heart and pancreas are very similar to guinea-pig atria and ileum receptors, respectively, but different from each other. We have shown that the antimuscarinic potency and subtype selectivity of hexahydro-diphenidol (*3a*) and hexahydro-sila-diphenidol (*3b*) and of the analogues *1a/1b*, *2a/2b* and *4a/4b–6a/6b* depend on different structural parameters (e.g. tertiary or quaternary nitrogen). The M_1, cardiac and glandular/smooth muscle muscarinic receptors make different structural demands for the antagonists thus different receptor selectivity profiles were obtained. Compounds *1a/1b–6a/6b* will facilitate further investigation of muscarinic receptor heterogeneity.

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Selectivity of bradykinin analogues for receptors mediating contraction and relaxation of the rat duodenum

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1 Bradykinin produces a biphasic response in the rat duodenum that consists of a relaxation ($pD_2 = 8.44$) followed by a contraction ($pD_2 = 6.91$).

2 The B_1 agonist des-Arg^9-bradykinin produced a contraction ($pD_2 = 7.16$) but no relaxation. Des-Arg^9-[Leu^8]-bradykinin, which is a B_1 antagonist in other systems produced contraction ($pD_2 = 7.65$) in the rat duodenum.

3 Four bradykinin analogues that are preferential B_2 agonists in other tissues had a biphasic effect with pD_2 values in the range 7.22–8.68 for relaxation and 6.26–6.91 for contraction.

4 [Thi^{5,8},D-Phe^7]-bradykinin, which is a B_2 antagonist in most other systems produced relaxation in the rat duodenum, with a pD_2 of 7.49.

5 It is concluded that the contractile component of the response to bradykinin in rat duodenum may be mediated by a subtype of the B_1 receptor and the relaxant component by a receptor of the B_2 subtype.

Introduction

The response of the rat duodenum to bradykinin is biphasic: low concentrations of the agonist (below 10 nM) produce relaxation, whereas higher concentrations cause a relaxation followed by contraction (Antonio, 1968; Faber & van der Meer, 1973). The differential effects of bradykinin potentiating peptides on the two components of the response, the finding that only the contractile component is subject to desensitization (tachyphylaxis), and the observation that des-Arg^9-bradykinin elicits only a contractile response, have led to the proposal that two different receptor types may mediate the two components of the biphasic effect (Camargo & Ferreira, 1971; Boschcov *et al.*, 1984). However, binding studies with plasma membrane preparations could not detect more than one class of binding sites and led to the suggestion that one type of bradykinin receptor is able to activate different effector mechanisms in the rat duodenum (Liebmann *et al.*, 1987).

Extensive structure-activity relationship studies employing synthetic peptide analogues, and carried out in numerous smooth muscle preparations, have led to the conclusion that the receptors for bradykinin may be classified into two types. B_1 receptors are characterized by their preferential activation by des-Arg^9-bradykinin, and are specifically inhibited by des-Arg^9-[Leu^8]-bradykinin (Regoli & Barabé, 1980). B_2 receptors are insensitive to des-Arg^9-[Leu^8]-bradykinin but are selectively inhibited by analogues such as [Thi^{5,8},D-Phe^7]-bradykinin that contain a substitution by D-phenylalanine in position 7 of the bradykinin amino acid sequence (Vavrek & Stewart, 1985; Stewart & Vavrek, 1986).

In the present paper we present the results of an analysis of the response of the rat duodenum to analogues of bradykinin that have been shown to be

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selective agonists or antagonists for either B_1 or B_2 receptors. Our results indicate that the relaxation and contraction components of the response to bradykinin are due to stimulation of the B_2 and B_1 receptor types, respectively.

Methods

The rat duodenum preparation was set up as previously described (Boschcov *et al.*, 1984). Briefly, the duodenum from rats of either sex (190–220 g body weight) was suspended in a 5 ml chamber with a salt solution of the following composition (mM): NaCl 137, KCl 2.7, $CaCl_2$ 1.36, $MgCl_2$ 0.49, NaH_2PO_4 0.36, $NaHCO_3$ 11.9, D-glucose 5.0. The bath solution was maintained at 37°C and bubbled with a mixture of CO_2 (5%) and O_2 (95%). The organs were submitted to a 1 g load and their isotonic contractions were recorded after a 30 min equilibration period.

The dose-response curves were obtained by administering the lower concentrations of the analogues (that produced only relaxation) at 10 min intervals and washing the preparation after a 90 s contact time. When higher concentrations were used, to elicit contractile responses, the interval between administrations was 30 min to avoid the tachyphylaxis that is observed for this component of the response. The relaxant component of the response was measured from the baseline to the deepest point of the relaxation and the contractile component from the baseline to the highest point of the response. The dose-response curves were analysed by linear regression of the double-reciprocal plot, from which ED_{50} values were obtained. The measurement of maximum responses relative to that of bradykinin was made by direct comparison of the effects of supramaximal concentrations.

All the peptides used in this study were synthetic products made in this laboratory, with the exception of [Thi^{5,8},D-Phe^7]-bradykinin which was kindly provided by Prof. J.M. Stewart, from the University of Colorado School of Medicine, Denver, CO, U.S.A.

Results

In our preparations, bradykinin induced a biphasic response; the ED_{50} was 3.6×10^{-9} M for the relaxation and 1.2×10^{-7} M for the contractile response.

The B_1 agonist des-Arg^9-bradykinin did not produce relaxation to any significant extent but was more potent than bradykinin in inducing contraction of the rat duodenum. The contractile response to this peptide also differed from that of bradykinin in that it was only very slowly reversed by washing of the preparation. In the duodenum contracted by bradykinin, baseline tension was fully restored within 5 min after washout, whereas the tonus caused by des-Arg^9-bradykinin usually had not returned to baseline 30 min after washout (Figure 1a). Interestingly in a tissue contracted by des-Arg^9-bradykinin, the administration of bradykinin elicited a biphasic response that was followed by fast relaxation after washing (Figure 1b). Tyr-bradykinin was even more effective than bradykinin in producing full relaxation of tissues previously contracted by des-Arg^9-bradykinin, after washout.

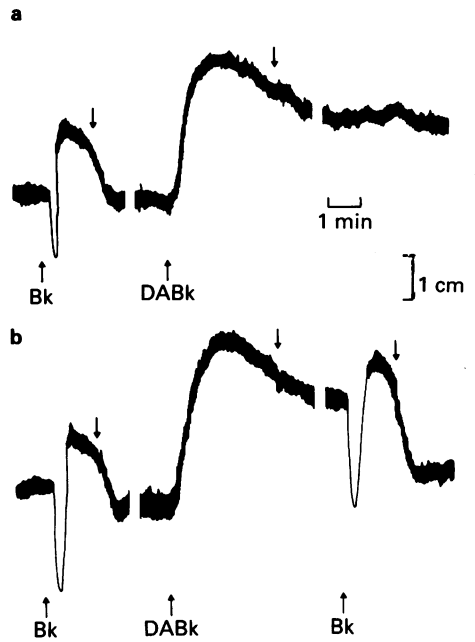


Figure 1 Responses of two rat duodenum preparations to 200 nM bradykinin (Bk) and to 200 nM des-Arg^9-bradykinin (DABk). Downward arrows indicate washings of the preparation with fresh medium. Interruptions of the tracings indicate a 30 min interval.

Cumulative dose-response curves for the contractile effect of des-Arg^9-bradykinin showed that this analogue is a full agonist, and yielded linear double-reciprocal plots from which an ED_{50} of 6.9×10^{-8} M ($pD_2 = 7.16 \pm 0.10$) was obtained.

Attempts to block the contractile effect of bradykinin with the B_1 antagonist des-Arg^9-[Leu^8]-bradykinin were compromised by the surprising finding that this compound was a powerful agonist for



Figure 2 Response of a rat duodenum preparation to increasing concentrations of des-Arg^9-[Leu^8]-bradykinin, ranging from 4 to 128 nM. Additions to the bath were made at 30 min intervals and the preparations were washed 1.5 min after each addition.

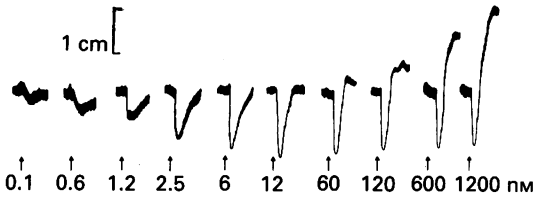


Figure 3 Responses of a rat duodenum preparation to increasing concentrations of Lys-Lys-bradykinin, ranging from 0.1 to 1200 nM. Additions to the bath were made at 10 min intervals up to 60 nM and at 30 min intervals thereafter. The preparations were washed 1.5 min after each addition.

producing contraction (Figure 2). This peptide produced no significant relaxant effect, and linear double-reciprocal plots for its contractile effect yielded an ED_{50} of 2.2×10^{-8} M ($pD_2 = 7.65 \pm 0.07$). However, the maximum response elicited by this analogue was only 40% of that caused by bradykinin, indicating that des-Arg^9-[Leu^8]-bradykinin is not a full agonist, although it is more potent than bradykinin when the respective ED_{50} values for producing contraction are compared.

Since no specific B_2 agonist is available, we studied the effects of several bradykinin analogues that are known to act at both types of receptor but with some preference for the B_2 type. Bradykinin itself has an apparent preference for B_2 receptors, as is indicated by pD_2 values of greater than 8 for B_2 receptor systems (such as the cat and guinea-pig ileum) and of the order of 6 for B_1 receptor systems (such as the rabbit aorta) (Regoli & Barabe, 1980; Regoli *et al.*, 1986). Besides bradykinin, we studied the responses of the rat duodenum to four synthetic bradykinin agonist analogues with modifications at the N-terminus (analogues 2–4, Table 1) and at the C-terminus (analogue 5). The four compounds elicited biphasic responses similar to those produced by bradykinin (Boschcov *et al.*, 1984), as is illustrated by the results obtained with Lys-Lys-bradykinin, shown in Figure 3. The four analogues were full agonists for both the relaxant and the contractile components of the response: pD_2 values for the relaxant effect were higher than those for the contractile effect (Table 1). The pD_2 values for the contractile effects showed little variation among the four analogues studied, ranging from 6.26 to 6.48; the efficacies of the analogues were between 22 and 37% relative to that of bradykinin.

A larger range of pD_2 values (from 7.22 to 8.68) was observed for the relaxant component of the responses, [Lys^9]-bradykinin showing the lowest activity (6% of that of bradykinin) whereas Lys-Lys-bradykinin was more potent than bradykinin (170% relative efficacy). The four analogues produced relaxation at concentrations 1–2 orders of magnitude lower than those needed for the contractile effects.

Our attempt to block the relaxant component of the bradykinin responses with the B_2 antagonist [Thi^{5,8},D-Phe^7]-bradykinin was frustrated by the finding that this compound itself behaved as an agonist for producing relaxation. Figure 4 shows that [Thi^{5,8},D-Phe^7]-bradykinin produced a dose-dependent effect in which only the relaxation component of the bradykinin response was observed.

Table 1 pD_2 values of preferential B_2 agonists on the rat isolated duodenum

Agonist	*Relaxant effect*	*Contractile effect*
1 Bradykinin	8.44 ± 0.08 (28)	6.91 ± 0.05 (16)
2 Lys-Lys-bradykinin	8.68 ± 0.07 (4)	6.38 ± 0.07 (10)
3 Tyr-bradykinin	8.04 ± 0.05 (4)	6.48 ± 0.07 (4)
4 Ile-Ser-bradykinin	7.35 ± 0.23 (4)	6.26 ± 0.09 (9)
5 [Lys^9]-bradykinin	7.22 ± 0.19 (4)	6.38 ± 0.08 (4)

pD_2 values were obtained from linear regressions of double reciprocal plots and the s.e.mean are indicated. The number of experiments is indicated in parentheses.

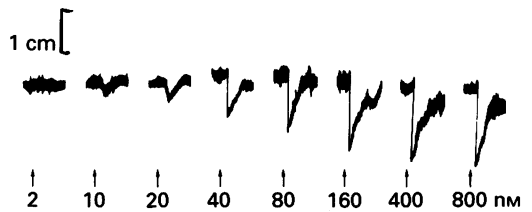


Figure 4 Responses of a rat duodenum preparation to increasing concentrations of [Thi^{5,8},D-Phe^7]-bradykinin, ranging from 2 to 800 nM. Intervals between additions were 10 min, and the preparation was washed 1.5 min after each addition.

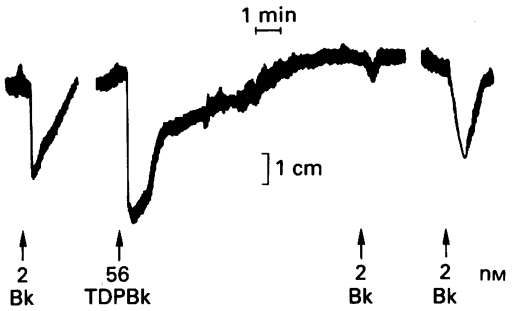


Figure 5 Inhibition of the relaxant response to bradykinin (Bk) in the presence of previously added [Thi^{5,8}, D-Phe^{7}]-bradykinin (TDPBk). The additions of the two compounds, at the concentrations shown, are indicated by the arrows. Interruptions of the tracing indicate washing of the preparation followed by a 30 min resting period.

The analogue behaved as a full agonist with an ED_{50} of 3.2×10^{-8} M ($pD_2 = 7.49 \pm 0.08$, $n = 4$).

Since the relaxant effect of [Thi^{5,8},D-Phe^{7}]-bradykinin is transient, we attempted to determine whether it might possess antagonist activity towards bradykinin by adding bradykinin to the bath after the relaxant effect of the analogue had subsided. Figure 5 shows that the relaxant effect of bradykinin was inhibited in the presence of [Thi^{5,8},D-Phe^{7}]-bradykinin. A study of the effect of different concentrations of this compound on bradykinin dose-response curves by the method of Arunlakshana & Schild (1959), yielded a pA_2 value of 7.50. Interestingly, this value is the same as that for the pD_2 for [Thi^{5,8},D-Phe^{7}]-bradykinin, indicating that the inhibition of the responses to bradykinin shown in Figure 5 may represent true competition between two agonists.

Discussion

The two components of the biphasic response of the rat duodenum to bradykinin have been attributed to different mechanisms. The relaxant effect was shown to be due to a direct action on the smooth muscle (Antonio, 1968; Ufkes & Van der Meer, 1975), and the response appears to be mediated by stimulation of adenylate cyclase (Liebmann *et al.*, 1987). Although two different types of receptors have been postulated for the relaxant and contractile effects (Camargo & Ferreira, 1971; Boschcov *et al.*, 1984), receptor binding studies with rat duodenum membrane preparations revealed a single binding site with an equilibrium dissociation constant of 1.0×10^{-9} M (Liebmann *et al.*, 1987). Our present results, however, clearly indicate that different types of bradykinin receptor are involved in the two components of the response.

The finding that the selective B_1 agonist des-Arg^{9}-bradykinin has a marked ability to produce contraction but not relaxation is strong evidence that the contractile component of the response to bradykinin is due to its interaction with receptors of the B_1 type. The contractile effect of des-Arg^{9}-bradykinin in the rat duodenum is characterized by its persistence after washout of the preparation, which appears to be due to a slow dissociation from the receptors.

Des-Arg^{9}-[Leu^{8}]-bradykinin, which has been shown to be an antagonist in other B_1 receptor systems, did not produce relaxation but possessed a high degree of efficacy for producing contraction of the rat duodenum and was the most potent of the analogues studied in this respect. To our knowledge this is the first description of an agonist action of des-Arg^{9}-[Leu^{8}]-bradykinin, and this observation characterizes the receptor mediating contraction of the rat duodenum as belonging to a subtype of B_1 receptors that, besides binding des-Arg^{9}-[Leu^{8}]-bradykinin, is also activated by this analogue. It must be pointed out, however, that this analogue is not as efficacious as bradykinin in giving rise to a stimulus when it binds to the B_1 receptor, i.e. des-Arg^{9}-[Leu^{8}]-bradykinin is a partial agonist.

With regard to the relaxant component of the bradykinin response, the data presented in Table 1 indicate that it is due to an interaction of the agonists with receptors of the B_2 type. Also in this case, we have found that the analogue [Thi^{5,8},D-Phe^{7}]-bradykinin, which is an antagonist of the B_2 receptor in other tissues, behaves as an agonist in the rat duodenum. Its relaxing effect, like that of bradykinin, is transient, and this permitted the determination of its effect on subsequent responses to bradykinin. The inhibition of responses to bradykinin in the presence of [Thi^{5,8},D-Phe^{7}]-bradykinin (Figure 5) was similar to the inhibition observed in the presence of a previous dose of bradykinin itself. This indicates that, although the relaxant response to either compound had subsided, the receptors may still have been occupied. The inhibition of the response to bradykinin by [Thi^{5,8},D-Phe^{7}]-bradykinin, cannot be attributed to an antagonist action of the latter compound, however, since it may only reflect some degree of refractoriness of the effector system. This finding suggests that it will be important not to neglect a possible transient agonistic effect in studies of supposed antagonists.

Although [Thi^{5,8},D-Phe^{7}]-bradykinin has been shown to be an antagonist of bradykinin in most of

the B_2 receptor systems that have been studied (Regoli *et al.*, 1986), there are published reports of agonist effects of that compound in vas deferens (Llona *et al.*, 1987) and in neuroblastoma cells (Braas *et al.*, 1988). It appears that in these two systems, as well as in the rat duodenum, there are receptors of a B_2 subtype that are characterized by an agonist response to [Thi^{5,8},D-Phe^7]-bradykinin. Indeed the evidence points to the existence of multiple bradykinin receptors in different tissues, as suggested by other studies of structure-activity relationships (Braas *et al.*, 1988).

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Presynaptic β-adrenoceptors in rat atria: evidence for the presence of stereoselective β_1-adrenoceptors

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1 Presynaptic β-adrenoceptor activity was studied in rat isolated atria, previously loaded with [^{3}H]-noradrenaline. The stimulation-induced release of ^{3}H transmitter was measured in the presence of cocaine, and adrenaline was used as a facilitatory β-adrenoceptor agonist.

2 Adrenaline (0.1 and 2 nM) increased, by about 50%, the evoked efflux of tritium. With phenoxybenzamine present, the same activity was shown with 10 nM adrenaline.

3 The β_2-selective adrenoceptor blocking drugs: IPS 339 and ICI 118 551 caused a concentration-dependent decrease in the activity of adrenaline. Cardioselective β-blocking drugs: acebutolol, betaxolol, nebivolol and its isomers (R 67 138 and R 67 145) also reduced dose-dependently the agonistic action of adrenaline. The order of potency for nebivolol and its isomers was R 67 138 > nebivolol > R 67 145. The activity of pindolol was not concentration-dependent. The inhibitory effect of acebutolol was also observed in the presence of blockade of α-adrenoceptors.

4 The postsynaptic β-adrenoceptor blocking activity of nebivolol and its isomers was studied in pithed rats. They reduced isoprenaline-induced tachycardia without altering hypotensive responses. The order of potency was: R 67 138 > nebivolol > R 67 145.

5 It is concluded that in rat isolated atria, presynaptic β_2- and β_1-adrenoceptors coexist and that facilitatory β_1-adrenoceptors are stereospecific.

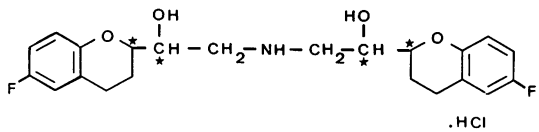
Introduction

The existence of presynaptic β-adrenoceptors was first proposed by Adler-Graschinsky & Langer (1975), who demonstrated the facilitatory effect of the β-agonist, isoprenaline, on the impulse-evoked release of noradrenaline. Further support for the existence of these receptors was obtained from a number of mainly *in vitro* experiments, in various tissues including guinea-pig and rat atria (Majewski *et al.*, 1980; 1981), rat portal vein (Dahlöf *et al.*, 1978; Westfall *et al.*, 1979), cat spleen (Celuch *et al.*, 1978), and human blood vessels (Stjärne & Brundin, 1976). Their presence was shown by the increase in release of noradrenaline during low frequency nerve stimulation by, most frequently, isoprenaline and adrenaline, with inhibition of neuronal uptake by cocaine or desipramine. The facilitatory mechanism mediated by these receptors was inhibited by β-adrenoceptor blocking drugs.

Attempts have been made to characterize the presynaptic β-adrenoceptors in terms of β_1 or β_2 subtypes, by use of selective agonists and antagonists. The results are somewhat controversial, possibly because the selectivity of drugs used is only relative and/or the facilitatory β-adrenoceptors differ with tissue or species.

Finally, the stereospecific nature of presynaptic β-adrenoceptors has been little studied. Celuch *et al.* (1978) in the cat isolated perfused spleen showed an increase in [^{3}H]-noradrenaline overflow induced by (−)-isoprenaline but not by the (+)-isomer. Using stereoselective β antagonists, Misu *et al.* (1981, 1983) demonstrated the greater antagonistic activity of (−)-propranolol and (−)-metoprolol on the effects of salbutamol in guinea-pig pulmonary artery. On the other hand, Kalsner (1980) did not find differences between (+)- and (−)-propranolol activities in guinea-pig atria, using isoprenaline as a β-agonist. More recently, Chang *et al.* (1986) demonstrated the stereoselectivity of presynaptic β_1-adrenoceptors in the rat hypothalamus.

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R 67138	S	R	R	R
R 67145	R	S	S	S

Figure 1 Structure of nebivolol showing the four asymmetric carbons. Nebivolol is the racemic mixture of the R 67 138 (**SRRR**)- and R 67 145 (**RSSS**)-enantiomers.

The aim of the present study was to investigate the agonistic activity of very low concentrations of adrenaline, the putative endogenous mediator for these receptors, and the antagonistic activity of some β-adrenoceptor antagonists. By use of selective β_1-blockers (acebutolol, betaxolol and nebivolol), selective β_2-blockers (IPS 339 and ICI 118 551), non-selective β-blocking drugs with a partial agonist activity (practolol and pindolol) and two enantiomers of nebivolol: R 67 138 and R 67 145 (Figure 1), it was hoped to characterize the presynaptic β-adrenoceptor subtype.

Preliminary results have been presented at the French Congress of Pharmacology in Nice (Davy *et al.*, 1987) and in Lausanne (Davy *et al.*, 1989).

Methods

Rat isolated atria

Male Sprague-Dawley rats, weighing 250–300 g were used (Charles River France).

Atria from untreated rats were dissected out and set up in an organ bath containing 3 ml of Krebs-Henseleit solution with disodium edetate and ascorbic acid (Majewski *et al.*, 1980). The solutions in the organ bath and in the reservoir were gassed with carbogen (95% O_2, 5% CO_2) and maintained at 37°C. A pair of parallel platinum electrodes was placed on either side of atria for electrical field stimulation.

After 20 min of equilibration, the atria were incubated with [^{3}H]-noradrenaline (37 kBq ml^{-1}, 0.4 μmol) for 20 min, and then washed for 60 min with Krebs-Henseleit solution (7 ml min^{-1}). Cocaine (10 μmol) was added before the end of the washing procedure and then remained present for the duration of the experiment.

The Krebs-Henseleit solution bathing the atria (3 ml) was collected after 3 min periods of contact with the atria. Five consecutive collections were made, the last three of them being used to measure radioactivity by liquid scintillation counting.

The sympathetic nerves of the atria were stimulated at a frequency of 3 Hz for 15 s with 1 ms square pulses, at 10 V. The Krebs-Henseleit solution was collected after a period of 3 min in which the stimulation was applied, for radioactivity counting (PS_1).

The resting noradrenaline efflux was taken as the mean of radioactivity content of the three samples collected immediately before the stimulation (E_1). The stimulation-induced radioactive efflux was calculated by substracting the resting efflux from the radioactive content of the sample collected in the 3 min period in which the stimulation was applied ($S_1 = PS_1 - E_1$).

In the first series of experiments, following the first period of stimulation, the preparation was washed for 20 min with Krebs-Henseleit + cocaine solution, then five consecutive collections before a second period of stimulation were carried out as previously. The same radioactivity counting procedures and calculations were applied ($S_2 = PS_2 - E_2$). In a second series of experiments, a third period of stimulation was applied, after the same successive sequences of washing and collections of Krebs solution, and the same calculation procedures applied ($S_3 = PS_3 - E_3$).

In a third series of experiments, following the incubation with [^{3}H]-noradrenaline, phenoxybenzamine (10 μM) was added to the Krebs solution before the end of washing, at the same time as cocaine (20 μM). The same stimulation procedures (S_1 and S_2) were applied as in the first series.

Presynaptic β-adrenoceptor activation

When adrenaline was used as a β-adrenoceptor stimulating agent, it was added to the Krebs solution immediately after the first period of stimulation, and remained present up to the end of the experiment. When a β-adrenoceptor blocking drug was assayed, it was added to the Krebs solution at the same time as cocaine.

The stimulation efflux for the second period was calculated as the percentage of the corresponding efflux for the first period (S_2/S_1%). In the experiments with three periods of stimulation, the same calculation was applied for the third stimulation (S_3/S_1%), after stimulation-induced tritium efflux had been checked as constant in a control group.

Radioactivity counting

The 3 ml Krebs-Henseleit solution samples were added to 10 ml scintillation fluid (Picofluor). Radioactivity was counted in a Packard Tricarb 4530 liquid scintillation counter.

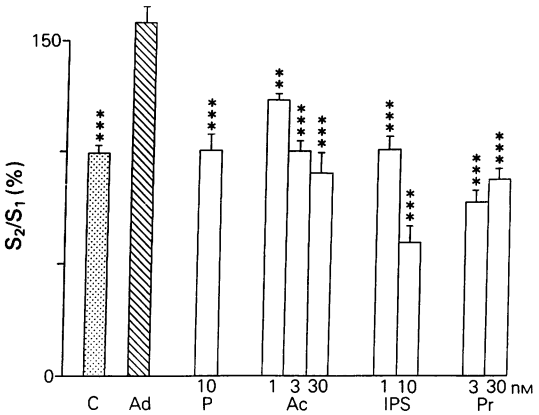


Figure 2 The effect of the β-adrenoceptor blocking drugs: propranolol (P), acebutolol (Ac), IPS 339 (IPS) and practolol (Pr), on adrenaline (2 nM, Ad)-induced increase in stimulation-evoked efflux of tritium from rat isolated atria (C = control group). There were two periods of stimulation (3 Hz, 15 s). The efflux of tritium in the second period was expressed as a percentage of that in the first (S_2/S_1 %). The vertical bars represent s.e.means. $^{**}P < 0.01$; $^{***}P < 0.001$ vs adrenaline alone.

Pithed rats

Three groups of rats were pithed (Shipley & Tilden, 1947) after pentobarbitone anaesthesia. They were artificially respired (Harvard rodent respirator, 1 ml 100 g^{-1}, 50 strokes min^{-1}) and mean blood pressure recorded from the left carotid artery (Narco physiograph MK III). Heart rate was derived from the arterial pulse signal (Narco biotachometer). The pudendal vein was cannulated for intravenous administration of drugs.

The maximal tachycardia and hypotensive effect produced by isoprenaline (1 $\mu g\,kg^{-1}$) were measured before and 5, 10 and 20 min after the i.v. injection of a β-adrenoceptor antagonist.

Drugs

The following drugs were used: cocaine hydrochloride (Coopération pharmaceutique française), [^{3}H]-noradrenaline (C.E.A., 1480 kBq $mmol^{-1}$), noradrenaline bitartrate (Aguettant), adrenaline hydrochloride (Sigma), isoprenaline chlorhydrate (Isuprel, Winthrop), phenoxybenzamine (S, K & F), acebutolol hydrochloride (Rhône-Poulenc), *t*-butyl-amino-3-ol-2 propyl)oximino-9 fluorene hydrochloride (IPS 339) (gift from Dr Wermuth), betaxolol (Synthélabo), pindolol (Sandoz), propranolol hydrochloride, practolol and (±)-erythro-3-isopropylamino-1-(7-methyl-4-indanyloxy)-2-butanol hydrochloride (ICI 118 551) (ICI Pharma), nebivolol, R 67 138 and R 67 145 (Janssen).

Statistical analysis

Results are expressed as mean ± s.e.mean. *n* is the number of assays. Significance tests were performed by Student's *t* test.

Results

Rat isolated atria

Stimulation-induced efflux of tritium in control experiments with two or three periods of stimulation In those experiments with two periods of stimulation, the ratio $(S_2/S_1) \times 100$ was 90 ± 5.7 ($n = 11$).

When three successive periods of stimulation were applied, the ratios $(S_2/S_1) \times 100$ and $(S_3/S_1) \times 100$ were 91 ± 4.4 and 97 ± 8 respectively ($n = 7$). As these ratios were not significantly different, the mean value of all the data was used for subsequent calculations: 94 ± 4.3 ($n = 14$).

In control experiments with phenoxybenzamine, the stimulation-induced release of tritium was increased 3.3 fold as compared with the overflow in the absence of α-adrenoceptor blockade. The ratio $(S_2/S_1) \times 100$ was 83 ± 7 ($n = 5$).

Effect of adrenaline on the stimulation-induced efflux of [^{3}H]-noradrenaline In the first series of experiments, 0.05 nM adrenaline did not enhance [^{3}H]-noradrenaline released by field stimulation: $(S_2/S_1) \times 100 = 108 \pm 10.7$ ($n = 6$). By contrast, 0.1 and 2 nM significantly increased it: $(S_2/S_1) \times 100 = 135 \pm 7.6$ ($n = 5$) and 150 ± 6.3 ($n = 6$) respectively ($P < 0.001$ versus control assay).

The same enhancement was observed in the second series of experiments with a lower adrenaline concentration (0.1 nM) and three periods of stimulation. The ratios $(S_2/S_1) \times 100$ and $(S_3/S_1) \times 100$ were 141 ± 5.7 and 154 ± 15 ($n = 7$). These data did not differ in value, thus in further calculations where the effect of a drug was being compared with the adrenaline group, the mean value of the response elicited by the second and the third period of stimulation was used: 147 ± 8 ($n = 14$). It differed significantly from control: 94 ± 4.3 ($P < 0.001$).

Three adrenaline concentrations were investigated in the presence of α-adrenoceptor blockade: 0.1 nM, 1 nM and 10 nM. In these conditions the ratios $(S_2/S_1) \times 100$ were respectively: 85 ± 8 ($n = 7$), 91 ± 3.8 ($n = 5$) and 121 ± 7.8 ($n = 5$). This last increase was significant ($P < 0.01$ versus control assay: 83 ± 7).

Effect of β-adrenoceptor blocking drugs on adrenaline-induced enhancement of [^{3}H]-noradrenaline

efflux In the first series of experiments, the following compounds were added to the Krebs solution as previously described: propranolol (10 nM), acebutolol (1, 3 and 30 nM), IPS 339 (1 and 10 nM), practolol (3 and 30 nM). Adrenaline concentration was 2 nM.

Each of the β-blocking drugs induced a reduction in the stimulating effect of adrenaline (Figure 2). A dose-dependent activity was observed with both the selective β_1-blocking drug, acebutolol, and the β_2-selective blocking agent IPS 339. A 100% inhibition of the action of adrenaline was achieved with acebutolol, 3 nM, and IPS 339, 1 nM. By contrast, 3 and 30 nM practolol caused the same antagonistic activity, exceeding 100%.

In the second series of experiments, six β-adrenoceptor blocking drugs sharing different specificities were assayed: pindolol (0.03, 0.1, and 3 nM), ICI 118 551 (0.1, 0.3, 1, 3 and 10 nM), betaxolol (0.3, 1, 3 and 10 nM), nebivolol and its isomers, R 67 138 and R 67 145 (0.5, 2 and 10 nM). As shown in Figure 3 each drug tested reduced the enhancement by adrenaline of tritium efflux. This inhibitory effect was dose-dependent except for that of pindolol.

Pindolol and ICI 118 551 exhibited significant activity from 0.1 nM (85 and 50% inhibition respectively) and betaxolol from 1 nM (70% inhibition). The order of potency for nebivolol and isomers was R 67 138 > nebivolol > R 67 145. R 67 138 caused significant inhibition from 0.5 nM (69%). With 10 nM the reduction in tritium efflux was 100%, 74% and 54% respectively for the three compounds.

Effect of acebutolol on adrenaline-induced enhancement of [^{3}H]-noradrenaline efflux in the presence of phenoxybenzamine The selective β_1-blocking drug, acebutolol (3 nM) reduced the facilitatory effect of adrenaline on ^{3}H efflux by about 20% ($n = 3$, data not shown). A higher concentration of acebutolol (30 nM) almost abolished it: $(S_2/S_1) \times 100 = 94 \pm 3.1$ ($n = 6$) ($P < 0.05$ versus 10 nM adrenaline group).

Pithed rats

The initial hypotensive responses to isoprenaline were similar in the three groups of rats (-11 ± 1 mmHg) as were the associated tachycardias (144 ± 6; 143 ± 2 and 132 ± 5 beats min).

Nebivolol and its isomers (125 μg kg^{-1}) did not significantly reduce heart rate during 20 min following administration. During the same time, blood pressure was not significantly modified by nebivolol and R 67 138 but it was slightly enhanced by R 67 145 (+10 mmHg).

As shown in Figure 4, none of the three β-adrenoceptor blocking drugs inhibited the hypotensive effect of isoprenaline. By contrast, inhibition of tachycardia appeared 5 min after injection and developed throughout the following 20 min. The order of potency was R 67 138 > nebivolol > R 67 145. Twenty min after injection the inhibition reached 77%, 65% and 50%, respectively.

Discussion

The present experiments have demonstrated a marked facilitation, in rat isolated atria, of the evoked release of [^{3}H]-noradrenaline by adrenaline when used in very low concentrations: 2 nM and

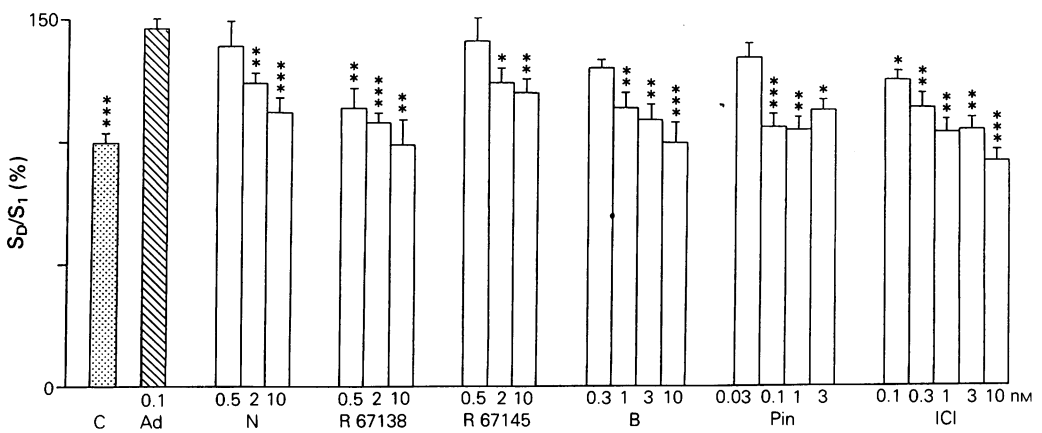


Figure 3 The effect of the β-adrenoceptor blocking drugs: nebivolol (N), R67 138, R 67 145, betaxolol (B), pindolol (Pin) and ICI 118 551 (ICI) on adrenaline (0.1 nM, Ad)-induced increase in stimulation-evoked efflux of tritium from rat isolated atria (C = control group). There were three periods of stimulation (3 Hz, 15 s): S_1 as a control, S_2 and S_3 with drugs added. Adrenaline resulted in identical S_2/S_1 and S_3/S_1 ratios, so the effects of various drugs added in S_2 or in S_3 were plotted (S_D). The vertical bars represent s.e.means. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ vs adrenaline alone.

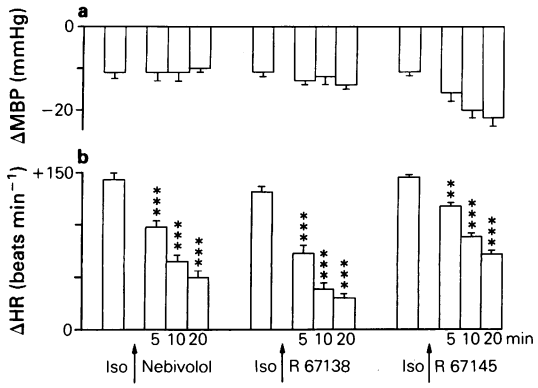


Figure 4 The effect of nebivolol and its isomers R 67 138 and R 67 145 (125 $\mu g\,kg^{-1}$ i.v.) on (a) the hypotension and (b) the tachycardia induced by isoprenaline (Iso) (1 $\mu g\,kg^{-1}$) in pithed rats. The vertical bars represent s.e.means. $^{**}P < 0.01$; $^{***}P < 0.001$ vs isoprenaline alone.

0.1 nM. Both concentrations induced about the same effect, the enhancement attaining 50% to 60% of the initial value. A lower concentration: 0.05 nM has been found to be inactive.

Similar investigations on rat atria have shown an enhancement of the stimulation-induced efflux of radioactivity with a higher concentration of adrenaline (10 nM) (Majewski *et al.*, 1981; 1982). These authors demonstrated that, depending on concentration, adrenaline had either no effect on (1 nM), or inhibited (1 μM), noradrenaline release. The latter higher dose of adrenaline enhanced efflux of radioactivity in the presence of phenoxybenzamine, suggesting it could also activate prejunctional α-adrenoceptors.

We, successfully, used lower concentrations of adrenaline to increase the evoked release of [^{3}H]-noradrenaline. Moreover, in our control groups the ratio S_2/S_1 (efflux of radioactivity in the second period of stimulation as a percentage of that in the first) was nearly 100% while it was about 50% in the other studies cited. This may be due to our conditions of stimulation: the size of electrodes, their distance and the potency of the resultant field. In any case, our experimental conditions allowed very low adrenaline concentrations to activate presynaptic β-adrenoceptors, and these results afford support for the hypothesis that circulating adrenaline may be the natural agonist for these receptors (Langer & Armstrong, 1984).

With phenoxybenzamine present, the large increase observed in evoked [^{3}H]-noradrenaline overflow confirms previous *in vitro* findings (Dahlöf *et al.*, 1978) and results of a recent *in vivo* study (Remie *et al.*, 1988). However, the activation of facilitatory prejunctional β-adrenoceptors cannot be achieved using the same weak concentrations as in the absence of α-blockade.

When α- and β-adrenoceptor presynaptic feedback is operative, the adrenaline-induced increase in noradrenaline overflow quickly reaches a maximum. With larger concentrations of adrenaline, α-adrenoceptor-mediated inhibition occurs and this overrides the facilitation of noradrenaline release. However, when α-adrenoceptors are blocked, the physiological balance between the two mechanisms is broken, resulting in a strongly elevated concentration of noradrenaline in the junctional cleft, which could compete with adrenaline for presynaptic β-adrenoceptors. This hypothesis is in agreement with our findings that presynaptic β_1-adrenoceptors are effective in the preparation we used.

In these conditions, all the β-adrenoceptor blocking drugs we studied reduced the effect of adrenaline on tritium efflux. These drugs differ by their selectivity: acebutolol and betaxolol are known as cardioselective β-blockers, nebivolol is a new compound still under investigation with a high affinity for the β_1-adrenoceptor, as demonstrated by binding assays (affinity $\beta_1/\beta_2 = 47$) and *in vitro* studies (IC_{50} in guinea-pig isolated trachea is 288 times higher than in atria) (Janssen preclinical report). Two selective β_2 adrenoceptor blocking agents were assayed: IPS 339 (Imbs *et al.*, 1977) and ICI 118 551 (O'Donnell & Wanstall, 1980) and finally, two β-adrenoceptor blocking drugs possessing partial agonist activity, practolol and pindolol, were also studied.

The selective β_1- or β_2-adrenoceptor blockers exhibited an inhibitory effect which was concentration-dependent. The selective β_1-blocking drug acebutolol has been assayed both in the absence and in the presence of a α-adrenoceptor blockade. Its antagonistic effect was shown in both cases, but was larger when presynaptic α-adrenoceptors were effective. This was probably due to the lower active concentration of the β-agonist, adrenaline, in the first experiments.

IPS 339, usually considered to be a selective β_2-adrenoceptor blocking drug, at 10 nM was able to reduce S_2/S_1 to levels significantly less than control. It should be noted that a similar finding was not observed with the other β_2-adrenoceptor blocking drug ICI 118 551. This unexpected finding could result from an effect of the compound on another kind of receptor. This compound has not been extensively studied and its selectivity and affinity for neuronal receptors is not known. It is possible that at a concentration of 10 nM it activates prejunctional inhibitory receptors. The inhibitory effect induced by practolol was identical for the two concentrations studied, both perhaps being too high and resulting in a maximal effect. By contrast, the inhibitory effect of

pindolol was not dose-related. The largest concentration used (3 nM) did not result in a 100% inhibition. This can be attributed to the important intrinsic sympathomimetic activity of this drug. It has been demonstrated that the stimulant effects of pindolol differ considerably from one tissue to another (Clark *et al.*, 1982), depending on the subtype of β-adrenoceptor involved. More recently, Aellig & Clark (1987) suggested that although pindolol is a non-selective β-adrenoceptor blocking agent, it selectively stimulates β_2-adrenoceptors. Thus, in the present experiments, it is possible that pindolol had some agonist activity on presynaptic β_2-adrenoceptors.

As discussed above, nebivolol is a highly selective β_1-adrenoceptor blocking agent. In the pithed rat, we further observed its antagonistic action on the tachycardia produced by isoprenaline, with no modification of its vascular effects. The β-adrenoceptor blocking activities of its isomers were qualitatively similar, i.e. cardioselective, but their potencies differed quantitatively, R 67 138 was the most active and R 67 145 the least active. It is interesting to note that the relative potencies of the three drugs were the same on presynaptic and postsynaptic receptors.

A number of authors have demonstrated the existence of β_2-adrenoceptors on presynaptic sympathetic fibres (Westfall *et al.*, 1979; Langer, 1980; Dahlöf, 1981; Majewski, 1983; Göthert, 1986; Misu & Kubo, 1986). Moreover, most earlier workers concluded that presynaptic β-adrenoceptors are exclusively of the β_2 subtype, due to the failure of various β_1-agonists and antagonists to stimulate or inhibit [^{3}H]-noradrenaline release in *in vitro* experiments (Westfall *et al.*, 1979; Dahlöf *et al.*, 1980). In contrast, our findings support the hypothesis of the presence of presynaptic β_1- as well as β_2-adrenoceptors, in the rat atria. Their activity could be shown by the use of more physiological conditions, i.e. low adrenaline concentration as β-agonist, in the absence of α-adrenoceptor blockade. Moreover, the present study demonstrates their stereoselectivity. A similar observation has been made by Chang *et al.* (1986) in rat hypothalamic slices, using (+)- and (−)-acebutolol. Finally, our results support the hypothesis of Misu & Kubo (1986), that presynaptic β_1- and β_2-adrenoceptors coexist in several tissues and therefore the antihypertensive action of β-antagonists may, in part, be due to blockade of presynaptic β-adrenoceptors.

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Reversal of haemorrhagic shock in rats by cholinomimetic drugs

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1 In an experimental model of haemorrhagic shock resulting in the death of all rats within 20–30 min, the intravenous (i.v.) injection of the tertiary amine cholinesterase inhibitor physostigmine (17–70 $\mu g\,kg^{-1}$) induced a prompt, sustained and dose-dependent improvement of cardiovascular and respiratory function, with marked increase in the volume of circulating blood and survival of all treated animals, at least for the 2 h of observation.

2 Similar results were obtained with the i.v. injection of the cholinoceptor agonist oxotremorine (5–25 $\mu g\,kg^{-1}$), while neostigmine (54 or 70 $\mu g\,kg^{-1}$), a quaternary cholinesterase inhibitor which cannot cross the blood-brain barrier, had negligible effects.

3 The anti-shock activities of oxotremorine and physostigmine were blocked by the intracerebroventricular injection of either of the combined nicotinic and M_2-muscarinic receptor antagonists gallamine and pancuronium, or of the nicotinic antagonist mecamylamine. They were also blocked by intraperitoneal injection of the adrenergic neurone blocking agent guanethidine, but they were not antagonized by either the combined M_1- and M_2-muscarinic receptor antagonist atropine, the M_1-muscarinic receptor antagonist pirenzepine, or the M_2-muscarinic receptor antagonist 4-diphenylacetoxy-N-methylpiperidine methobromide.

4 It is concluded that cholinomimetic drugs can reverse hypovolaemic shock through central activation (seemingly mediated by nicotinic receptors) of sympathetic tone, with mobilization and redistribution of the residual blood.

Introduction

There is strong evidence that acetylcholine plays a role in central cardiovascular regulation (Brezenoff & Giuliano, 1982). Pharmacological activation of brain cholinoceptors results in marked pressor responses in several vertebrate species, including man (Dirnhuber & Cullumbine, 1955; Varagić, 1955; Medacović & Varagić, 1957; Brezenoff & Jenden, 1969; Brezenoff, 1972; Janowsky *et al.*, 1986). This has been observed for centrally-acting cholinomimetic drugs—including the cholinesterase inhibitor, physostigmine, and the cholinoceptor agonists, carbachol, arecoline and acetylcholine—as well as for centrally-administered cholinomimetics which do not normally pass the blood-brain barrier. Peripherally-administered cholinoceptor agonists and cholinesterase inhibitors that fail to pass the blood-brain barrier, such as neostigmine, either have no pressor effect or decrease blood pressure.

On the other hand, atropine sulphate (but not its quaternary ammonium derivative, atropine methylbromide, which does not pass the blood-brain barrier) almost completely prevents the increase in blood pressure and pulse amplitude induced in rats by the intravenous (i.v.) injection of adrenocorticotropin (ACTH), in an experimental model of haemorrhagic shock (Guarini *et al.*, 1986). This further supports the suggestion that central cholinergic neurones play an important role in blood pressure homeostasis and may indicate that a decrease in brain cholinergic tone is involved in the complex pathophysiology of cardiovascular shock. If so, centrally-acting cholinomimetic drugs should be beneficial in shock conditions. We have verified this possibility by studying the effect of physostigmine and oxotremorine, two prototypic centrally-acting cholinomimetic drugs, in comparison with neostigmine, a peripherally-acting cholinomimetic drug, in an experimental model of haemorrhagic shock resulting in the death of all rats within 20–30 min.

Methods

Animals and surgery

After induction of anaesthesia with ethylurethane (1.25 $g\,kg^{-1}$, i.p.; Fluka AG, Buchs, Switzerland) and

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heparin treatment (600 i.u. kg^{-1} i.v.; Prodotti Gianni, Milan, Italy), adult Wistar rats of either sex (250–300 g) had a common carotid artery and an iliac vein cannulated. Systemic arterial pressure and pulse amplitude were continuously recorded from the common carotid artery by means of a pressure transducer (Statham P23Db) connected to a polygraph (Battaglia-Rangoni, Bologna, Italy). The respiratory rate was recorded by means of three electrodes subcutaneously implanted on the chest of the animal and connected through a preamplifier (Ari A380) to the same polygraph. Severe haemorrhagic shock was produced by intermittent bleeding through the venous catheter until the mean arterial pressure (MAP = diastolic pressure plus one third of pulse pressure) fell to, and stabilized at, 16–26 mmHg. A total volume of 2.19 ± 0.02 ($n = 202$; mean ± s.e.mean) ml of blood per 100 g body wt was withdrawn over a period of 25–30 min, so that the volume removed approximated 50% of the estimated total blood volume (Collins *et al.*, 1969).

Drugs and treatments

After 5 min of stabilization, the rats were given an i.v. bolus injection of either physostigmine salicylate (BDH Chemicals Ltd, Poole, U.K.), oxotremorine (Janssen, Beerse, Belgium), neostigmine methylsulphate (Roche, Milan, Italy), or an equal volume of saline (1 ml kg^{-1}). As appropriate, atropine sulphate, atropine methylbromide, gallamine triethiodide, mecamylamine hydrochloride (Sigma Chemicals Co., St. Louis, MO, U.S.A.), pirenzepine dihydrochloride (Istituto De Angeli, Milan, Italy), pancuronium bromide (Organon B.V., Oss, Holland), guanethidine sulphate (Ciba-Geigy, Basel, Switzerland), or 4-diphenylacetoxy-N-methylpiperidine methobromide (4-DAMP) were administered 2 min before the start of bleeding. Drug doses are given as those of the salt. The injection of oxotremorine was always preceded by i.p. pretreatment with atropine methylbromide (2 mg kg^{-1}) in order to prevent its peripheral muscarinic effects.

Intracerebroventricular (i.c.v.) injection

Some rats were prepared for i.c.v. treatment 5–7 days before the experiment by stereotaxically implanting, under pentobarbitone anaesthesia (70 mg kg^{-1} i.p.; Carlo Erba, Milan, Italy), stainless-steel cannulae (Plastic Products Co., Roanoke, VA., U.S.A.) into a brain lateral ventricle (Pellegrino *et al.*, 1979). Correct placement was verified by injecting 4 μl of toluidine blue dye through the cannula at the end of the experiment, followed by decapitation and dissection of the brain.

Blood volume determination

The volume of residual circulating blood was measured either by bleeding the previously shocked animal to death through the arterial catheter, 5–10 min after treatment with saline or physostigmine, or using the dilution principle according to Wang (1959). Briefly, 0.1 ml of a 0.451% solution of Evans blue (BDH Chemicals Ltd, Poole, U.K.) was injected into the rat 2–3 min after treatment and blood sampling was carried out 5 min thereafter.

Statistical methods

MAP and pulse pressure of all groups were first compared with each other by means of an analysis of variance, separate comparisons being made for data obtained before bleeding, after bleeding, 5–10 min and 120 min after treatment. In the case of after-treatment data, analysis of variance was followed by Dunnett's test for multiple comparisons with a control; for this purpose MAP and pulse pressure values of dead rats were considered = 0 (Dunnett, 1964). The respiratory rate was analysed by Student's t test for paired data; the volume of residual circulating blood and the haematological parameters by Student's t test for unpaired data. Survival rates were analysed by comparison between ratios, as assessed by a χ^2 test (Lison, 1979).

A probability of less than 0.05 was taken throughout to indicate significance.

Results

As summarized in Table 1, the degree of hypovolaemia was invariably incompatible with survival and all control rats died within 25 ± 4 min after saline injection. In such extreme conditions, physostigmine and oxotremorine caused a prompt and sustained, dose-dependent increase in blood and pulse pressures. At the maximum effective dose of physostigmine (70 μg kg^{-1}) blood pressure started to rise 10–40 s after treatment, reaching the pre-bleeding value within 5–10 min. This was followed by a slow, gradual decrease. However, at the end of the recording period (2 h after treatment), the MAP was still +161% of the post-bleeding, pretreatment value.

The respiratory rate (115 ± 3 breaths min^{-1} before bleeding, and 41 ± 1 breaths min^{-1} after bleeding) was significantly increased (63 ± 2 breaths min^{-1} 10 min after treatment; $P < 0.001$; Student's t test for paired data).

On administration of oxotremorine 15 μg kg^{-1}, blood pressure started to rise 5–8 s after treatment, reaching the pre-bleeding value within 5–10 min.

Table 1 Effects of oxotremorine, physostigmine and neostigmine in an experimental model of haemorrhagic shock in rats

Treatment ($\mu g\,kg^{-1}$ *after bleeding*)	$\frac{MAP}{PP}$ (mmHg) *Before bleeding*	*After bleeding*	*5–10 min after treatment*	*120 min after treatment*	*No. of deaths 120 min after treatment*
Saline, i.v. (15)	$\frac{92 \pm 4}{47 \pm 4}$	$\frac{23 \pm 1}{13 \pm 1}$	$\frac{26 \pm 3}{15 \pm 2}$	$\frac{0}{0}$	15
Physostigmine 17 i.v. (6)	$\frac{93 \pm 2}{42 \pm 2}$	$\frac{22 \pm 1}{12 \pm 2}$	$\frac{36 \pm 3^*}{35 \pm 3^*}$	$\frac{30 \pm 4^*}{31 \pm 3^*}$	0***
35 i.v. (6)	$\frac{82 \pm 5}{48 \pm 3}$	$\frac{23 \pm 1}{16 \pm 1}$	$\frac{58 \pm 6^*}{35 \pm 2^*}$	$\frac{45 \pm 5^*}{33 \pm 3^*}$	0***
70 i.v. (10)	$\frac{89 \pm 5}{46 \pm 4}$	$\frac{23 \pm 1}{11 \pm 1}$	$\frac{90 \pm 6^*}{41 \pm 2^*}$	$\frac{60 \pm 7^*}{40 \pm 4^*}$	0***
Neostigmine 54 i.v. (10)	$\frac{91 \pm 6}{54 \pm 4}$	$\frac{24 \pm 2}{16 \pm 2}$	$\frac{32 \pm 4}{25 \pm 6^*}$	$\frac{35 \pm 6}{30 \pm 5}$	6**
70 i.v. (6)	$\frac{74 \pm 3}{58 \pm 4}$	$\frac{24 \pm 2}{15 \pm 2}$	$\frac{32 \pm 7}{21 \pm 6}$	$\frac{36 \pm 6}{22 \pm 5}$	3**
Oxotremorine 5 i.v. (8)	$\frac{102 \pm 4}{46 \pm 3}$	$\frac{23 \pm 1}{11 \pm 1}$	$\frac{52 \pm 2^*}{29 \pm 4^*}$	$\frac{38 \pm 4^*}{22 \pm 3^*}$	0***
10 i.v. (8)	$\frac{92 \pm 3}{38 \pm 4}$	$\frac{20 \pm 1}{9 \pm 1}$	$\frac{84 \pm 5^*}{36 \pm 4^*}$	$\frac{50 \pm 5^*}{36 \pm 3^*}$	0***
15 i.v. (8)	$\frac{86 \pm 6}{43 \pm 3}$	$\frac{21 \pm 1}{10 \pm 1}$	$\frac{95 \pm 6^*}{34 \pm 2^*}$	$\frac{58 \pm 6^*}{34 \pm 4^*}$	0***
25 i.v. (8)	$\frac{93 \pm 1}{39 \pm 4}$	$\frac{21 \pm 1}{11 \pm 1}$	$\frac{113 \pm 4^*}{33 \pm 2^*}$	$\frac{73 \pm 5^*}{35 \pm 4^*}$	0***

MAP = mean arterial pressure; PP = pulse pressure; i.p. = intraperitoneally; i.v. = intravenously; i.c.v. = into a brain lateral ventricle. Values are means ± s.e. Numbers in parentheses indicate the sample size. Analysis of variance showed P values for between groups variation as follows: MAP before bleeding, $P > 0.05$; PP before bleeding, $P > 0.05$; MAP after bleeding, $P > 0.05$; PP after bleeding, $P > 0.05$; MAP 5–10 min after treatment, $P < 0.001$; PP 5–10 min after treatment, $P < 0.001$; MAP 120 min after treatment, $P < 0.001$; PP 120 min after treatment, $P < 0.001$. *$P < 0.05$, at least, versus the corresponding value of saline-treated rats (Dunnett's test). **$P < 0.05$ and ***$P < 0.001$ versus the corresponding value of saline-treated rats (χ^2 test).

This was followed by a slow and gradual decrease; at the end of the observation period (2 h after treatment) MAP was, however, +176% of the post-bleeding value. Respiratory rate, which had fallen to 42 ± 4 breaths min^{-1} compared to the basal, pre-bleeding rate of 112 ± 2, was increased to 66 ± 4 breaths min^{-1}, 10 min after treatment ($P < 0.001$; Student's t test for paired data). A higher dose of oxotremorine ($25\,\mu g\,kg^{-1}$) produced, even in these severely bled animals, a hypertensive response, MAP peaking 20 mmHg above the pre-bleeding value. None of the animals died within the 2 h observation period after receiving any of the doses of either physostigmine or oxotremorine used.

Neostigmine, at either of the doses used (54 and $70\,\mu g\,kg^{-1}$), failed to increase significantly either arterial or pulse pressure. However, in some animals it kept them within ranges compatible with survival and, indeed, 40–50% of the treated rats were still surviving 2 h after treatment.

Figure 1 shows some representative recordings and it clearly shows the abrupt effect of oxotremorine, compared with the slower blood pressure increase induced by physostigmine.

In agreement with previous findings (Brezenoff, 1973), in normal, non-bled rats, $70\,\mu g\,kg^{-1}$ physostigmine induced a short-lived hypertensive response: starting from a pretreatment value of 99 ± 4 mmHg ($n = 5$), MAP rose to a peak value of 125 ± 3 mmHg within 5–6 min, but this response lasted only 15–30 min. A similar effect was obtained with oxotremorine at a dose of $15\,\mu g\,kg^{-1}$: starting

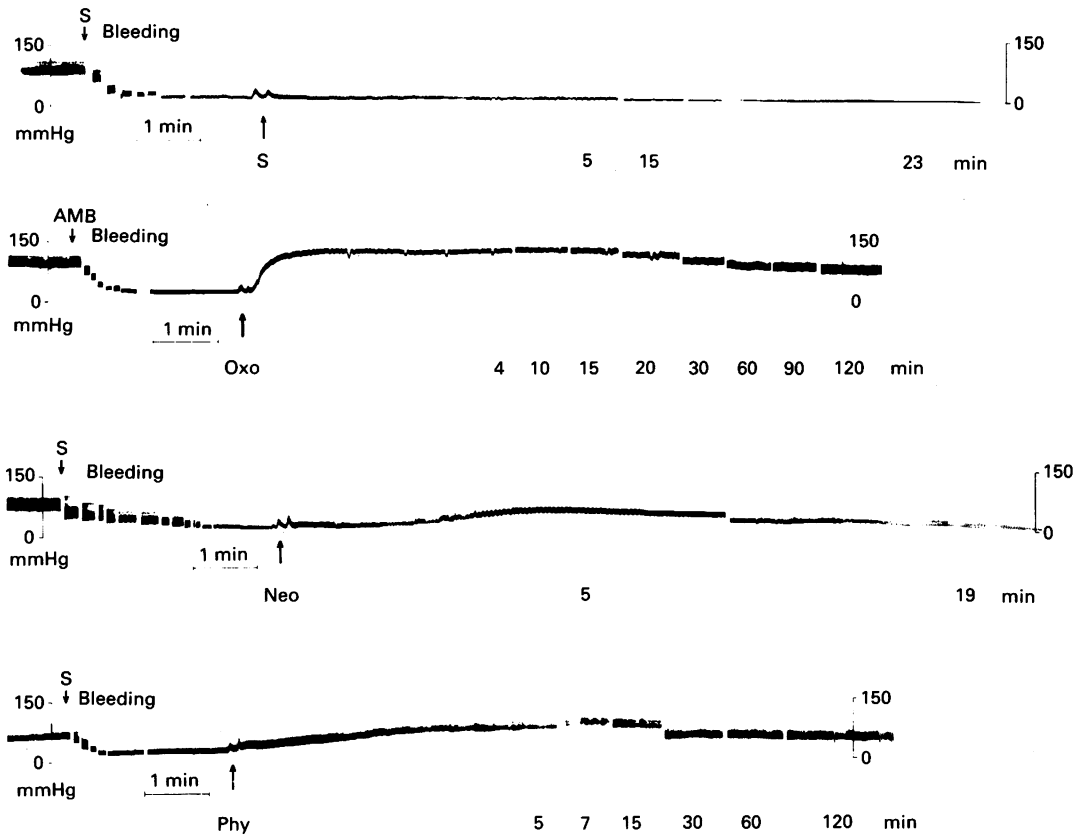


Figure 1 Representative recordings showing the effect of saline, oxotremorine, neostigmine and physostigmine on arterial blood pressure and pulse amplitude after hypovolaemic shock, induced by bleeding in rats pretreated i.p. with saline or atropine methylbromide. S: saline, $1\,ml\,kg^{-1}$ i.p. or i.v.; Oxo: oxotremorine, $25\,\mu g\,kg^{-1}$ i.v.; Neo: neostigmine, $54\,\mu g\,kg^{-1}$ i.v.; Phy: physostigmine, $70\,\mu g\,kg^{-1}$ i.v.; AMB: atropine methylbromide, $2\,mg\,kg^{-1}$ i.p.

from a pretreatment value of 97 ± 3 mmHg ($n = 4$), MAP peaked at 128 ± 5 mmHg within 3–5 min, and then decreased to basal values within 35–40 min.

Table 2 shows that the cardiovascular effects of physostigmine ($70\,\mu g\,kg^{-1}$) and oxotremorine ($10\,\mu g\,kg^{-1}$) in bled rats were not affected by either atropine methylbromide $2\,mg\,kg^{-1}$ i.p. (a peripheral M_1- and M_2-muscarinic antagonist) or atropine sulphate 2 or $8\,mg\,kg^{-1}$ i.p. (an M_1- and M_2-muscarinic antagonist which freely crosses the blood-brain barrier). Likewise, both the more selective M_1-muscarinic antagonist pirenzepine (tested only against physostigmine) when given either i.v. ($4\,mg\,kg^{-1}$) or i.c.v. ($160\,\mu g\,kg^{-1}$), and the selective M_2-muscarinic antagonist 4-DAMP (tested against both physostigmine and oxotremorine), injected i.c.v. ($80\,\mu g\,kg^{-1}$), were ineffective. On the other hand, the mixed nicotinic and M_2-muscarinic receptor antagonists gallamine and pancuronium, injected i.c.v. at a dose of 180 and $35\,\mu g\,kg^{-1}$, respectively, almost completely prevented the effect of physostigmine: there was only a partial and transient recovery of blood pressure and all rats died within the 2 h of observation. The same effect was obtained, against both physostigmine and oxotremorine, with the nicotinic antagonist mecamylamine, injected i.c.v. at a dose of $80\,\mu g\,kg^{-1}$. The effect of physostigmine was also prevented by the adrenergic neurone-blocking drug guanethidine ($10\,mg\,kg^{-1}$ i.p.), 5 out of 6 treated rats dying within the 2 h observation period (Table 2).

The volume of residual circulating blood, measured 5–10 min after treatment by bleeding the animal to death through the arterial catheter, was $1.66 \pm 0.10\,ml\,100\,g^{-1}$ body wt in rats treated with physostigmine ($70\,\mu g\,kg^{-1}$; $n = 6$), and $0.77 \pm 0.12\,ml\,100\,g^{-1}$ body wt in rats treated with saline ($n = 6$; $P < 0.001$; Student's t test). Compar-

Table 2 Reversal of haemorrhagic shock induced by centrally-acting cholinomimetic drugs: effect of cholinoceptor antagonists and of guanethidine

Pretreatment (2 min before starting bleeding)	*Treatment* ($\mu g\,kg^{-1}$ after bleeding)	$\frac{MAP}{PP}$ (mmHg) *Before bleeding*	*After bleeding*	*5–10 min after treatment*	*120 min after treatment*	*No. of deaths 120 min after treatment*
Saline 1 ml kg^{-1} i.p. (15)	Saline, i.v.	$\frac{92 \pm 4}{47 \pm 4}$	$\frac{23 \pm 1}{13 \pm 1}$	$\frac{26 \pm 3}{15 \pm 2}$	$\frac{0}{0}$	15
Atropine methylbromide 2 mg kg^{-1} i.p. (8)	Oxotremorine, 10 i.v.	$\frac{92 \pm 3}{38 \pm 4}$	$\frac{20 \pm 1}{9 \pm 1}$	$\frac{84 \pm 5^*}{36 \pm 4^*}$	$\frac{50 \pm 5^*}{36 \pm 3^*}$	0**
2 mg kg^{-1} i.p. (4)	Physostigmine, 70 i.v.	$\frac{82 \pm 6}{44 \pm 5}$	$\frac{23 \pm 1}{13 \pm 1}$	$\frac{86 \pm 5^*}{40 \pm 3^*}$	$\frac{59 \pm 5^*}{40 \pm 4^*}$	0**
Atropine sulphate 2 mg kg^{-1} i.p. (8)	Oxotremorine, 10 i.v.	$\frac{87 \pm 5}{40 \pm 4}$	$\frac{22 \pm 1}{8 \pm 2}$	$\frac{84 \pm 6^*}{39 \pm 5^*}$	$\frac{55 \pm 4^*}{40 \pm 5^*}$	0**
2 mg kg^{-1} i.p. (10)	Physostigmine, 70 i.v.	$\frac{89 \pm 5}{43 \pm 3}$	$\frac{22 \pm 1}{9 \pm 1}$	$\frac{80 \pm 5^*}{36 \pm 4^*}$	$\frac{59 \pm 4^*}{38 \pm 3^*}$	0**
8 mg kg^{-1} i.p. (4)	Oxotremorine, 10 i.v.	$\frac{90 \pm 4}{41 \pm 3}$	$\frac{21 \pm 2}{10 \pm 1}$	$\frac{86 \pm 5^*}{38 \pm 3^*}$	$\frac{54 \pm 3^*}{37 \pm 4^*}$	0**
8 mg kg^{-1} i.p. (4)	Physostigmine, 70 i.v.	$\frac{77 \pm 5}{42 \pm 3}$	$\frac{20 \pm 2}{11 \pm 1}$	$\frac{72 \pm 4^*}{37 \pm 3^*}$	$\frac{58 \pm 4^*}{37 \pm 5^*}$	0**
Pirenzepine 4 mg kg^{-1} i.v. (6)	Physostigmine, 70 i.v.	$\frac{102 \pm 4}{55 \pm 3}$	$\frac{22 \pm 1}{15 \pm 2}$	$\frac{87 \pm 4^*}{52 \pm 3^*}$	$\frac{60 \pm 5^*}{50 \pm 4^*}$	0**
160 $\mu g\,kg^{-1}$ i.c.v. (6)	Physostigmine, 70 i.v.	$\frac{99 \pm 6}{53 \pm 4}$	$\frac{22 \pm 1}{15 \pm 2}$	$\frac{83 \pm 8^*}{48 \pm 8^*}$	$\frac{61 \pm 6^*}{45 \pm 5^*}$	0**
4-DAMP 80 $\mu g\,kg^{-1}$ i.c.v. (6)	Oxotremorine, 10 i.v.	$\frac{98 \pm 4}{32 \pm 5}$	$\frac{21 \pm 2}{8 \pm 2}$	$\frac{98 \pm 3^*}{28 \pm 6^*}$	$\frac{60 \pm 4^*}{30 \pm 4^*}$	0**
80 $\mu g\,kg^{-1}$ i.c.v. (6)	Physostigmine, 70 i.v.	$\frac{99 \pm 6}{52 \pm 5}$	$\frac{21 \pm 1}{9 \pm 2}$	$\frac{100 \pm 7^*}{39 \pm 6^*}$	$\frac{64 \pm 5^*}{41 \pm 4^*}$	0**
Gallamine 180 $\mu g\,kg^{-1}$ i.c.v. (6)	Physostigmine, 70 i.v.	$\frac{105 \pm 7}{50 \pm 6}$	$\frac{21 \pm 1}{11 \pm 1}$	$\frac{41 \pm 4^*}{19 \pm 3}$	$\frac{0}{0}$	6
Pancuronium 35 $\mu g\,kg^{-1}$ i.c.v. (8)	Physostigmine, 70 i.v.	$\frac{101 \pm 6}{43 \pm 4}$	$\frac{22 \pm 1}{10 \pm 1}$	$\frac{39 \pm 4^*}{22 \pm 2}$	$\frac{0}{0}$	8
Mecamylamine 80 $\mu g\,kg^{-1}$ i.c.v. (10)	Oxotremorine, 10 i.v.	$\frac{86 \pm 6}{39 \pm 2}$	$\frac{22 \pm 1}{10 \pm 1}$	$\frac{37 \pm 2}{13 \pm 1}$	$\frac{0}{0}$	10
80 $\mu g\,kg^{-1}$ i.c.v. (10)	Physostigmine, 70 i.v.	$\frac{93 \pm 4}{42 \pm 3}$	$\frac{21 \pm 1}{9 \pm 1}$	$\frac{41 \pm 2^*}{17 \pm 2}$	$\frac{0}{0}$	10
Guanethidine 10 mg kg^{-1} i.p. (6)	Physostigmine, 70 i.v.	$\frac{85 \pm 9}{50 \pm 3}$	$\frac{24 \pm 1}{11 \pm 2}$	$\frac{30 \pm 6}{23 \pm 9}$	$\frac{29}{26}$	5

MAP = mean arterial pressure; PP = pulse pressure; i.p. = intraperitoneally; i.v. = intravenously; i.c.v. = into a brain lateral ventricle. Values are means ± s.e. Numbers in parentheses indicate the sample size. Analysis of variance showed P values for between groups variation as follows: MAP before bleeding, $P > 0.05$; PP before bleeding, $P > 0.05$; MAP after bleeding, $P > 0.05$; PP after bleeding, $P > 0.05$; MAP 5–10 min after treatment, $P < 0.001$; PP 5–10 min after treatment, $P < 0.001$; MAP 120 min after treatment, $P < 0.001$; PP 120 min after treatment, $P < 0.001$. *$P < 0.05$, at least, versus the corresponding value of saline-treated rats (Dunnett's test). **$P < 0.001$ versus the corresponding value of saline-treated rats (χ^2 test).

able results were obtained with the dye dilution method: 2.83 ± 0.21 ml $100\,g^{-1}$ body wt in rats treated with physostigmine ($70\,\mu g\,kg^{-1}$; $n = 6$), and 1.42 ± 0.18 ml $100\,g^{-1}$ body wt in rats treated with saline ($n = 5$; $P < 0.001$; Student's t test). It has been repeatedly demonstrated (Lawson, 1943; 1944) that the volume obtained by bleeding the animal to circulatory standstill consistently represents about one-half the actual blood volume.

The red-cell count ($5.61 \pm 0.22 \times 10^6\,mm^{-3}$), haematocrit ($33.4 \pm 1.7\%$) and haemoglobin content ($11.0 \pm 0.6\,g\%$) in physostigmine ($70\,\mu g\,kg^{-1}$)-treated rats ($n = 6$) were not different from the control values ($5.90 \pm 0.38 \times 10^6\,mm^{-3}$; $32.80 \pm 2.05\%$; $11.40 \pm 0.74\,g\%$, respectively; $n = 6$), while the number of leukocytes was significantly higher in treated than in control rats ($9.20 \pm 1.59 \times 10^3\,mm^{-3}$ and $4.90 \pm 0.48 \times 10^3\,mm^{-3}$, respectively; $P < 0.001$, Student's t test).

Discussion

These data show, firstly, that centrally-acting cholinomimetic drugs induce a prompt and sustained reversal of an otherwise rapidly-fatal haemorrhagic shock; secondly, that such an effect is evoked through central activation (that is likely to be mediated by nicotinic receptors) of peripheral sympathetic mechanisms, since it is prevented by i.c.v. gallamine, pancuronium or mecamylamine and by i.p. guanethidine (which cannot cross the blood-brain barrier); and, thirdly, that such a reversal of haemorrhagic shock is associated with a marked increase in the volume of circulating blood, probably as a result of mobilization from peripheral pooling.

In the normotensive, non-bled animal, the hypertensive response to cholinomimetics also occurs through the activation of peripheral sympathetic mechanisms and is blocked by guanethidine (Dirnhuber & Cullumbine, 1955; Varagić, 1955; Medacović & Varagić, 1957; Brezenoff & Giuliano, 1982). However, in the normotensive animal, the hypertensive response to cholinomimetics involves central muscarinic mechanisms and is in fact abolished by atropine sulphate and scopolamine but not by atropine methylbromide or methylscopolamine (which do not pass the blood-brain barrier). In contrast, in our extremely hypotensive animals the pressor effects of cholinomimetics seem to involve central nicotinic mechanisms. They are prevented by the i.c.v. injection of gallamine or pancuronium, which are also antagonists at M_2-muscarinic receptors (Birdsall & Hulme, 1983; Mitchelson, 1984; Ellis & Seidenberg, 1987; Swanson & Phillis, 1988) and by the i.c.v. injection of mecamylamine, which is a selective antagonist at nicotinic receptors (Brezenoff & Caputi, 1980; Matta *et al.*, 1987). Whereas atropine (an M_1- and M_2-muscarinic antagonist), pirenzepine, a more selective M_1-muscarinic antagonist (Brown *et al.*, 1980; Hammer *et al.*, 1980; Doods *et al.*, 1987), and 4-DAMP, a selective M_2-muscarinic antagonist (Barlow & Shepherd, 1985; Brezenoff *et al.*, 1988) did not inhibit them. We have no obvious explanation for such a discrepancy, or for the finding that in our experimental conditions, oxotremorine seems to act through the activation of nicotinic receptors. Another difference between the hypertensive effect of cholinomimetics in normotensive animals and the reversal of the shock condition they can bring about in bled animals is the duration of the effect: in the normotensive animals the increase in blood pressure lasts 15–30 min, while in the hypovolaemic rats it is still highly significant 2 h after treatment.

In our experimental model of severe hypovolaemic shock, other pressor agents (e.g. angiotensin II, $50\,\mu g\,kg^{-1}$ i.v., and vasopressin, $1\,\mu g\,kg^{-1}$ i.v.) induced only transient (3–4 min) and moderate increases in blood pressure, all treated rats dying within 40–50 min.

Our present results further support the hypothesis that central cholinergic circuits play an important role in blood pressure regulation and suggest that, in conditions of cardiovascular disaster, there may be a functional depression of such circuits with the pharmacological restoration of central cholinergic tone apparently being able to reverse the situation.

If these findings are confirmed in other species, including primates and man, a new therapeutic approach to the immediate management of emergencies leading to acute and severe hypovolaemia could be envisaged. All the more so when it is borne in mind that centrally-acting cholinomimetic drugs have potent analgesic activity (George *et al.*, 1962; Pleuvry & Tobias, 1971; Bartolini *et al.*, 1980), which could be of some additional advantage in many types of shock.

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2-Chloroadenosine attenuates kainic acid-induced toxicity within the rat striatum: relationship to release of glutamate and Ca^{2+} influx

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1 The mechanism by which 2-chloroadenosine (2-chloroado) exerts a neuroprotective action against the excitotoxic effect of kainic acid (KA) when injected into the rat striatum was investigated.

2 Histological examination two weeks after a single injection of KA (2.2 nmol) into rat striatum revealed widespread neuronal damage. Co-injection of 2-chloroado (6–25 nmol) with the neurotoxin afforded dose-dependent neuroprotection. This effect was reversed by administration of an equimolar concentration of the adenosine receptor antagonist theophylline.

3 Both K^+ (30 mM) and KA (1 mM) enhanced the release of endogenous glutamate from guinea-pig purified cerebrocortical synaptosomes in a predominantly (approximately 70%) Ca^{2+}-dependent manner. 2-Chloroado (10 nM–1 μM) inhibited the release of glutamate evoked by both KA and K^+. These effects were partially reversed by the selective A_1-adenosine receptor antagonist 8-cyclopentyltheophylline (CPT) (1 μM).

4 Crude rat cortical synaptosomes were loaded with the fluorescent calcium indicator quin-2 and Ca^{2+} influx monitored following two successive depolarising stimuli (30 mM K^+; 'S1' and 'S2'). 2-Chloroado (10 nM–1 μM) produced a dose-dependent reduction in the S2:S1 ratio when added before the S2 period of stimulation. This effect was reversed by 1 μM theophylline. However, KA (1 mM) failed to enhance Ca^{2+} influx in the same preparation.

5 These results suggest that the anti-excitotoxic action of 2-chloroado is mediated primarily through a specific presynaptic receptor mechanism involving reduction of transmitter glutamate release, possibly occurring through an inhibition of Ca^{2+} influx.

Introduction

It is now well established that the ubiquitously distributed amino acids L-glutamate and L-aspartate serve as major excitatory transmitters within the mammalian central nervous system (CNS) (Robinson & Coyle, 1987). At present there appear to be at least three separate postsynaptic receptors that mediate the actions of these excitatory amino acid (EAA) transmitters. These are selectively activated by the exogenous excitants N-methyl-D-aspartic acid (NMDA), kainic acid (KA) and quisqualic acid (QA) (Evans & Watkins, 1981). Currently, much attention is being focussed on EAAs and their suggested involvement in neurological disorders such as Huntington's disease, epilepsy and ischaemic cell loss (Engelson, 1986; Robinson & Coyle, 1987; Bradford & Peterson, 1987). Hence, there is considerable interest in the potential therapeutic use of antagonists of the postsynaptic actions of glutamate and aspartate (Schwarcz & Meldrum, 1985).

Kainate (KA) has proved to be a valuable research tool in the investigation of the possible *in vivo* mechanisms by which excitatory amino acids induce neuronal death (Coyle, 1987). When injected into the rat striatum, KA elicits a profound neuronal loss (Coyle & Schwarcz, 1976; McGeer & McGeer, 1976), which appears to be critically dependent upon an intact cortico-striatal afferent (glutamatergic) input (Bizière & Coyle, 1978; McGeer *et al.*, 1978). It was suggested by Ferkany *et al.* (1982) that KA

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acting at a presynaptic receptor can induce release of glutamate in a Ca^{2+}-dependent manner. This released glutamate would then interact with postsynaptic EAA receptors, while KA simultaneously and in cohort with glutamate acts at its own postsynaptic receptor to induce toxicity (Coyle, 1983). However, there is still much confusion as to the presynaptic actions of KA. Some studies have suggested that it directly causes a Ca^{2+}-dependent release of glutamate (Poli *et al.*, 1985; Pastuszko *et al.*, 1984), while others have proposed that KA enhances synaptic levels of glutamate by virtue of its ability to inhibit (albeit rather weakly) the glutamate carrier (Pocock *et al.*, 1988).

Adenosine and its analogues such as 2-chloroadenosine (2-chloroado) are potent inhibitors of the release of a wide range of transmitters (Phillis & Wu, 1981) including endogenous (Corradetti *et al.*, 1984) and exogenous (Fastbom & Fredholm, 1985) glutamate. Several studies have indicated that those actions of adenosine may be mediated through an inhibition of voltage-dependent Ca^{2+} influxes (Ribeiro *et al.*, 1979; Wu *et al.*, 1982; Dolphin & Prestwich 1985). In the present study we have shown that 2-chloroado has a profound protective action against KA-induced toxicity in the rat striatum and that anti-excitotoxic properties of 2-chloroado are linked to its ability to inhibit endogenous glutamate release. This latter action is apparently mediated, at least in part, through an inhibition of presynaptic Ca^{2+} influx. A preliminary account of these findings was communicated to the British Pharmacological Society (Arvin *et al.*, 1987).

Histology

Male adult Wistar rats (200 g) were anaesthetised with pentobarbitone (60 mg kg^{-1}) intraperitoneally and placed in a David Kopf small animal stereotaxic apparatus. A Hamilton needle was inserted into the striatum through a burr hole in the calvarium (coordinates 8.4 A; 2.2 L; 0.6 V). All drugs were dissolved in phosphate buffered saline (PBS, 1 μl, pH adjusted to 7.4) and injected slowly over 5 min. The needle was left in the brain for a further 5 min to ensure the full absorption of the drug. After the operation, the hole was filled with sterile bone wax. The animals were allowed to recover for two weeks at which time they were perfused with 100 ml of 0.9% saline followed by 300 ml of 4% *para*-formaldehyde dissolved in 0.1 M sodium phosphate buffer (pH = 7.4). The brain was removed and left in fixative for a further 2 h and then transferred to a solution of 30% sucrose/1 mM $MgCl_2$ and left for two days. Coronal sections, 40 μm, were cut on a freezing microtome and maintained free floating in phosphate buffered saline (PBS) until stained. As well as the routine use of cresyl fast violet, histochemical staining was performed as below.

NADPH-diaphorase The slices were incubated free floating in nicotinamide adenine dinucleotide phosphate (NADPH)-diaphorase staining solution (0.1 M Tris HCl pH = 8.0 containing 1 mM NADPH, 0.2 mM nitroblue tetrazolium (NBT), 15 mM sodium malate, 0.4% Tween 80) for 40–60 min at 37°C.

Acetylcholinesterase The slices were incubated with the staining solution (5 mg acetylthiocholine iodide, 6.5 ml 0.2 M Tris malate buffer pH = 5.5, 0.5 ml 0.1 M sodium citrate, 1 ml 0.03 M cupric sulphate, 1 ml 0.005 M potassium ferrocyanide, 1 ml distilled water), made up in the order indicated. Five hours before perfusion, the animals were pretreated with 20 mg kg^{-1} of the irreversible anticholinesterase DFP, dissolved in arachis oil and injected i.m.

Each slice was subsequently mounted on a gelatine-coated slide, air dried and dehydrated through a range of alcohol concentrations.

Release studies

Preparation of synaptosomes This was performed according to a modification of the method of Nicholls (1978). Briefly, large Dunkin-Hartley guinea-pigs (of either sex) were killed by cervical dislocation and exsanguination and the brain rapidly removed. The cerebral cortices were dissected on ice and homogenised in 10 volumes of ice-cold 0.32 M sucrose (pH = 7.4) with 8–10 strokes of a loose fitting teflon/glass homogeniser at 1500 r.p.m. The homogenate was centrifuged at 1000 *g* for 10 min, and the resulting supernatant then centrifuged at 17500 *g* to yield the crude synaptosomal pellet. This was resuspended in 36 ml of ice-cold 0.32 M sucrose (pH 7.4) and 6 ml of this suspension was gently layered onto 6 separate discontinuous Ficoll in sucrose (FiS) density gradients, each comprising 4 ml of 12% FiS, 1 ml of 9% FiS and 4 ml of 5% FiS. The gradients were adjusted with 0.32 M sucrose and centrifuged at 100,000 *g* for 60 min. The resultant double synaptosomal bands formed at the 12%/9% and 9%/5% interfaces were pooled and divided equally amongst eight centrifuge tubes containing 35 ml of ice-cold 0.32 M sucrose and centrifuged at 50,000 *g* for 30 min to yield a purified synaptosomal pellet. These pellets were maintained on ice until required. Each pellet was resuspended in 3.2 ml of previously oxygenated ice-cold Krebs medium (composition (mM): NaCl 120, KCl 3.1, KH_2PO_4 1.2, $MgSO_4$ 1.2, glucose 20, $NaHCO_3$ 25 and $CaCl_2$ 2.6) to yield a protein concentration of approximately 0.6 mg ml^{-1}.

Fluorometric assay for glutamate Immediately following resuspension, 1.5 ml synaptosomal aliquots

were placed in a quartz glass cuvette with a small magnetic stirring bar (to prevent synaptosomal clumping) and transferred to a temperature-controlled cell holder, of a Perkin Elmer LS-5B Luminescence Spectrometer, maintained at 30°C. Following the attainment of a steady fluorescence reading, the concentration of extrasynaptosomal glutamate within the incubation medium was assessed after the addition of 50 μl GDH (60 units) and 1 mM NADP. After the peak response was reached, the machine was zeroed and depolarising concentrations of either 30 mM K^+ or 1 mM KA added to the synaptosomes and the subsequent increase in fluorescence monitored by use of excitation and emission wavelengths of 340 and 460 nm, respectively. Increases in fluorescence were monitored over a 5 min period. The system was calibrated by adding known amounts of glutamate to 1.5 ml Krebs buffer, in the presence of the same concentrations of NADP and GDH used in the *in situ* assay described above. The addition of up to 6 μM glutamate produced a linear increase in fluorescence.

Quin-2 experiments Male Wistar rats (300 g), were killed by cervical dislocation (rats were used in preference to guinea-pigs, as guinea-pig synaptosomes have been found to leak markedly quin-2 (Meldolesi *et al.*, 1984)), the brains removed and the cerebral cortices dissected free. The tissue was then homogenised in 10 volumes of ice-cold buffered 0.32 M sucrose (pH = 7.4), by a teflon/glass homogeniser at low speed for approximately 15 strokes. The homogenate was centrifuged at 1000 *g* for 10 min in a Beckman J2-21 centrifuge at 4°C. The supernatant was then removed and slowly diluted with the same volume of ice-cold Krebs-bicarbonate medium. Following centrifugation at 12,000 *g* for a further 20 min, the resulting pellet was resuspended in medium (1 ml g^{-1} original wet tissue) and incubated on ice for 45 min.

The crude synaptosomal suspension was diluted in 6.7 volumes of Krebs medium at room temperature and incubated at 37°C for 20 min with 50 μM (final concentration) quin-2 ester, after which it was diluted with 10 volumes of medium and further incubated for 40 min at the same temperature, with constant shaking throughout. The preparation was then centrifuged at 9000 *g* for 5 min at 4°C. The pellet was resuspended in medium at room temperature (50 ml g^{-1} original wet wt.). Three ml of membranes were then pelleted at 13,000 *g* for 20 s in a bench microfuge, resuspended in fresh medium, transferred to a quartz cuvette and placed in the luminescence spectrometer. The synaptosomes were exposed to two successive additions of 30 mM K^+, at 5 min intervals and the increase in fluorescence monitored at 339 nm excitation, 492 nm emission. The effects of drugs were usually tested during the second K^+ stimulus (S2) and compared to the first stimulus (S1), using control S1/S2 ratios.

Materials

Kainic acid (KA), 2-chloroadenosine (2-chloroado) and common reagents were obtained from Sigma Chemicals (Poole). Glutamate dehydrogenase (GDH) was obtained from Boehringer Mannheim and NADP and NADPH from Park Scientific Ltd.

Results

Histology

A single injection of KA (2.2 nmol) into the striatum two weeks before perfusion and staining caused a marked loss of NADPH-diaphorase (Figure 1a), and acetylcholinesterase (Figure 2a) positive neurones, as well as a considerable increase in the number of glial elements as seen with Nissl staining (Figure 3a). Cell counts were taken to quantify this neuronal loss (Figure 4a and b). Co-injection of 2-chloroado (25 nmol) with KA (2.2 nmol), resulted in a pronounced neuroprotection, with clear preservation of both NADPH-diaphorase (Figure 1b), and acetylcholinesterase (Figure 2b) neurones and a considerable reduction in reactive glial elements (Figure 3b).

The effect of 2-chloroado (3–50 nmol) was quantitatively assessed with cell counts for both diaphorase and acetylcholinesterase neurones (Figure 4a and b); the neuroprotective effect of 2-chloroado was dose-dependent, with maximal protection occurring with 25 nmol and none being seen with 3 nmol. The protection by 2-chloroado (25 nmol) appeared to be partially reversed by the adenosine antagonist theophylline (Figures 3c, 4a and b), although this did not reach statistical signifcance.

Glutamate release

K^+ (30 mM) and KA (1 mM) released glutamate from guinea-pig purified cortical synaptosomes to approximately the same extent and in a predominantly (70%) calcium-dependent fashion (1.3 nmol mg^{-1} protein 5 min^{-1} with calcium, and 0.40 nmol mg^{-1} protein 5 min^{-1} in the absence of calcium) (Table 1). 2-Chloroado (1 μM) produced highly significant reductions in both the K^+- and KA-evoked release of glutamate in an apparently dose-dependent manner. A clear effect of 2-chloroado was still demonstrable at a concentration as little as 0.01 μM, at least in the case of K^+-stimulated release. As well as clear dose-dependency, the inhibitory effect of 2-chloroado on glutamate release was found to be

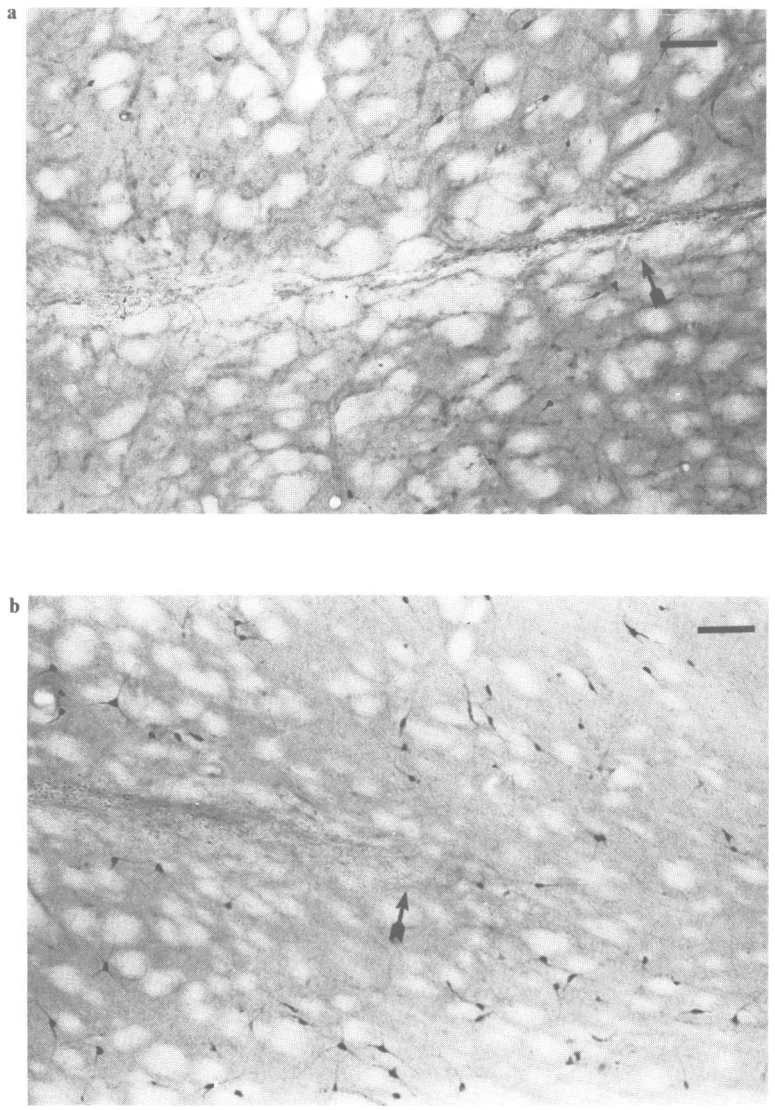


Figure 1 A photomicrograph of a 40 μm thick section through rat striatum stained for NADPH-diaphorase. Rats were injected with 1 μl of a solution of 2.2 nmol kainic acid (KA) (a) or the same volume of 2.2 nmol KA + 25 nmol 2-chloroadenosine (b), pH adjusted to 7.4, two weeks before sectioning and staining. The solution was injected over 5 min with the needle left in place for a further 5 min. Arrows indicate the site of injection. Calibration bar = 11.5 μm.

reversed by the selective A_1-adenosine receptor antagonist 8-cyclopentyltheophylline (CPT) (with a change in the rate of glutamate release from 0.85 nmol mg^{-1} protein 5 min^{-1} with 2-chloroado in the absence of CPT to the rate of 1.15 nmol mg^{-1} protein 5 min^{-1} in the presence of CPT and 2-chloroado) (Table 1).

Intracellular free calcium

Subjecting rat crude cortical synaptosomes, that had been pre-loaded with quin-2, to two successive depolarising pulses of 30 mM K^+ produced sharp increases in fluorescence, indicative of calcium influx into the synaptosomes (S1 and S2). With control

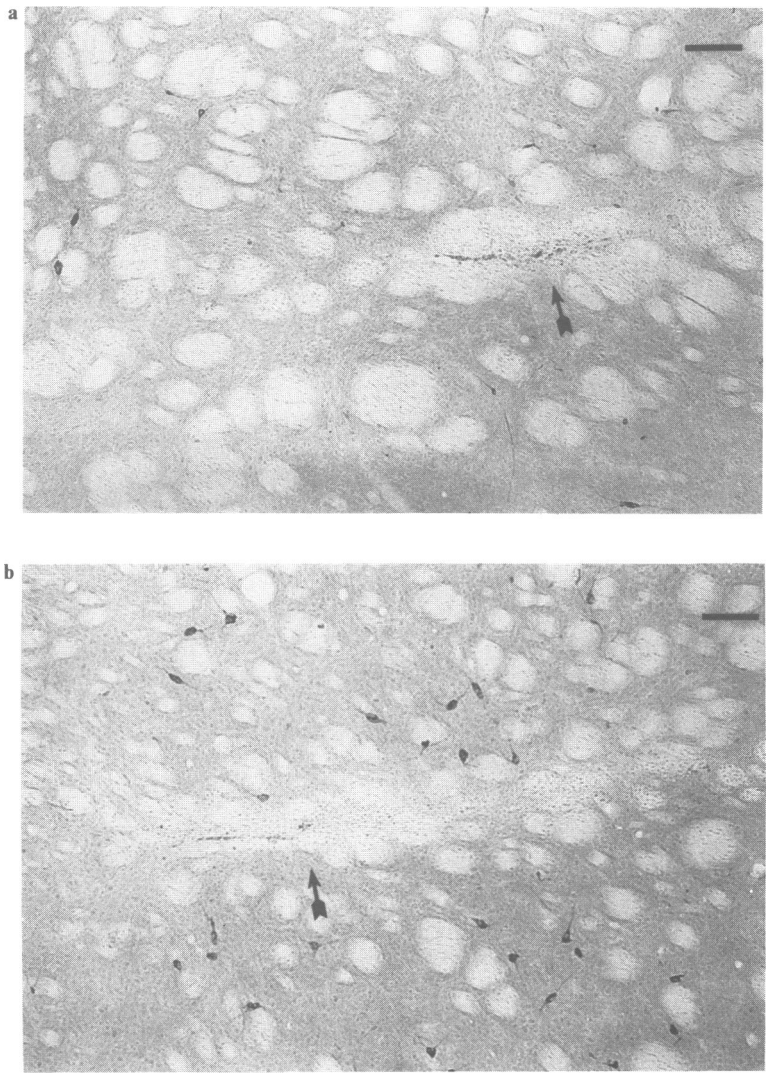
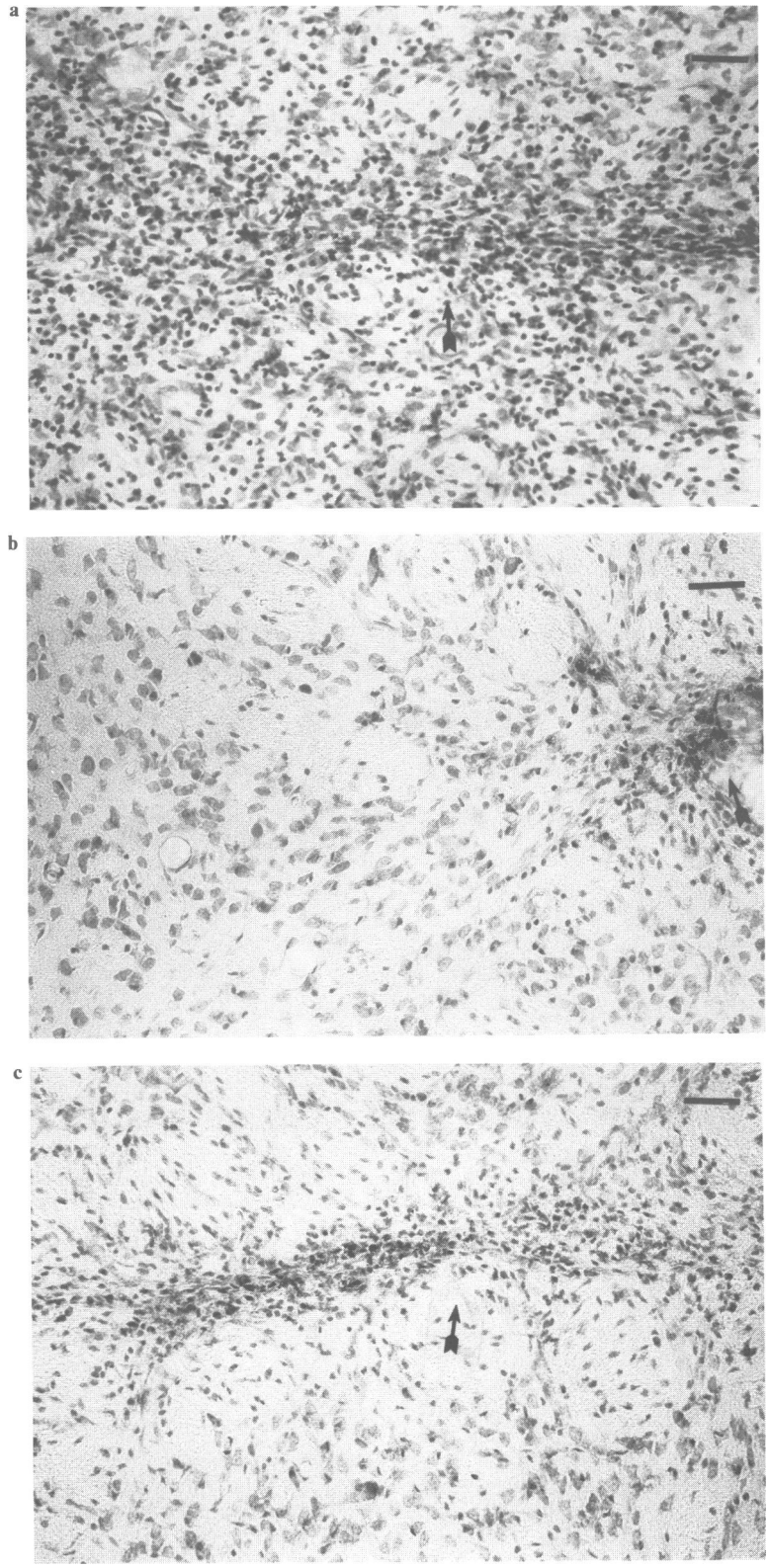


Figure 2 A 40 μm thick section through rat striatum stained for acetylcholinesterase. The procedure was as for Figure 1. (a) The effect of an injection of 2.2 nmol kainic acid (KA). (b) The protective effect of 2-chloroadenosine (25 nmol) injected with 2.2 nmol KA. Arrows indicate the injection site. Calibration bar = 11.5 μm.

synaptosomes, S2/S1 ratios of approximately one were seen (Figure 5). Pre-incubation of synaptosomes with 2-chloroado (1–0.01 μM) two minutes before the S2 K^+ stimulation, produced a marked reduction in the S2/S1 ratios. This effect was dose-dependent, with 1 μM 2-chloroado a 50% reduction in S2/S1 ratios was observed (compared with the effect of 1 μM CLAD on glutamate release), whereas 0.01 μM 2-chloroado caused no change. The 50% reduction in S2/S1 ratios seen with 1 μM 2-chloroado was reversed with 1 μM theophylline (S2/S1 = 1.1) (Figure 5; $P < 0.01$ by Student's t test).

Discussion

In the present study, we have provided histological evidence that the stable adenosine analogue 2-chloroado can attenuate the neurotoxic damage elicited by KA, when co-injected into the rat striatum.


a
b
c

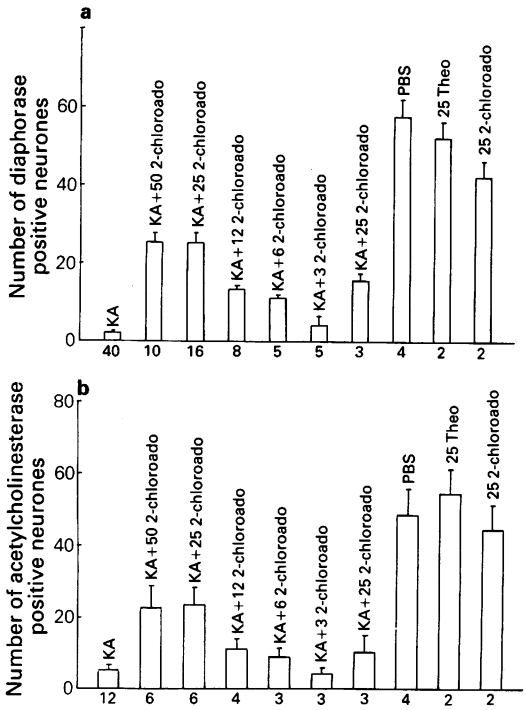


Figure 4 Cell counts carried out for NADPH-diaphorase (a) and acetylcholinesterase (b), positive neurones. These were carried out as follows: the needle tract was located and centred under the light microscope using a fixed magnification of $\times 70$. Positively stained neurones were counted 80 μm rostral to, 80 μm caudal to and at the point of injection. The three values were averaged for each side of the striatum and are shown as mean; horizontal bars indicate s.e.mean. The n value (number below column) indicates the number of striata used for each cell count. KA = kainic acid 2.2 nmol; 2-chloroado = 2-chloroadenosine; Theo = theophylline. Doses shown are in nmol.

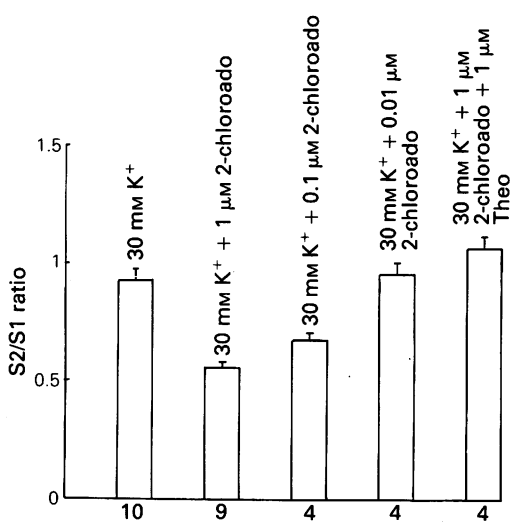


Figure 5 Measurements of intrasynaptosomal free calcium. Freshly prepared crude rat cortical synaptosomes were loaded with the calcium indicator dye quin-2. The synaptosomes were subjected to two successive depolarising pulses of 30 mM K^+ (S1 and S2), at 5 min intervals (see Methods). The effect of 2-chloroadenosine (2-chloroado) with or without theophylline (Theo) on calcium influx was tested by their addition 2 min before the second K^+ stimulus (S2). S2/S1 ratios of about one were observed for control experiments and the effect of the adenosine analogues on S2/S1 ratios are shown. Results are mean of n (the value below the column) number of experiments; vertical bars show s.e.mean.

We have used three histochemical parameters to evaluate the effect of KA (alone or with adenosine analogues) within the striatum, namely, staining for NADPH-diaphorase, acetylcholinesterase and Nissl substance. The first two are markers for somatostatin/neuropeptide Y (NPY)- and acetylcholine-containing neurones in the striatum respectively, while Nissl staining provides a global assessment of the effect of kainic acid within the striatum. The neuroprotective (anti-excitotoxic) action of 2-chloroado appeared to be a specific receptor-mediated event, since it was both dose-dependent and reversed by an equimolar dose of the specific adenosine receptor antagonist, theophylline.

Many studies have indicated the importance of an intact excitatory glutamatergic afferent innervation for the development of KA-induced neurodegeneration. Areas receiving a glutamatergic innervation and which are highly sensitive to KA include the striatum (Bizière & Coyle 1978; McGeer *et al.*, 1978), hippocampus (Nadler & Cuthbertson, 1980), superior colliculus (Fosse & Fonnum 1986), and the avian optic tectum (Streit *et al.*, 1980). However, this requirement for a glutamatergic input may not be mandatory, since it appears that the destruction of other excitatory pathways such as the 5-

Figure 3 A 40 μm thick section through rat striatum stained for Nissl substance with Cresyl fast violet. The procedure was the same as in Figures 1 and 2. (a) The effect of an injection of 2.2 nmol kainic acid (KA) alone. (b) The protective effect of 25 nmol 2-chloroadenosine (2-chloroado) injected with 2.2 nmol KA. (c) Shows that this protection could be reversed by co-application of 20 nmol theophylline with 25 nmol 2-chloroado and 2.2 nmol KA. Arrows indicate the site of injection. Calibration bar = 4 μm.

Table 1 K^+ and kainate (KA)-induced release of glutamate (Glu) from purified guinea-pig cortical synaptosomes: effect of adenosine analogues

Treatment	*Glu release* (nmol mg^{-1} protein $5\,min^{-1}$)	
30 mM K^+	1.30 ± 0.05 ($n = 10$)	
30 mM K^+ + 1 μM 2-chloroado	0.85 ± 0.05*** ($n = 6$)	[a]
30 mM K^+ + 0.1 μM 2-chloroado	0.85 ± 0.05*** ($n = 6$)	[a]
30 mM K^+ + 0.01 μM 2-chloroado	1.00 ± 0.15** ($n = 6$)	[a]
30 mM K^+ + 1 μM 2-chloroado + 1 μM CPT	1.15 ± 0.15 NS ($n = 3$)	[a]
1 mM KA	1.20 ± 0.05 ($n = 11$)	
1 mM KA + 1 μM 2-chloroado	0.85 ± 0.05*** ($n = 6$)	[b]
1 mM KA + 0.1 μM 2-chloroado	0.80 ± 0.15** ($n = 6$)	[b]
1 mM KA + 0.01 μM 2-chloroado	1.05 ± 0.15 NS ($n = 6$)	[b]
1 mM KA + 1 μM 2-chloroado + 1 μM CPT	1.10 ± 0.05* ($n = 3$)	[c]

Depolarisation-induced release of glutamate from freshly resuspended synaptosomes by 30 mM K^+ and 1 mM KA. Following the assessment of extra-synaptosomal levels of glutamate (see Methods), 30 mM K^+ or 1 mM KA was added to the synaptosomes and the increase in fluorescence monitored for 5 min. In all experiments the Ca^{2+}-independent release was assessed by repeating the assay in a Ca^{2+}-free Krebs buffer containing 1 mM EGTA. The Ca^{2+}-dependent release was estimated to be about 70% of the total release of glutamate for both K^+ and KA under these conditions. The effect of 2-chloroadenosine (2-chloroado) on both K^+- and KA-induced release was investigated by adding 2-chloroado to the synaptosomes for 5 min following the assessment of extra-synaptosomal glutamate, before the addition of 30 mM K^+ or 1 mM KA. 8-Cyclopentyltheophylline (CPT) was added in conjunction with 2-chloroado for 5 min before the depolarising stimulus.

[a] Effect of 2-chloroado compared with release evoked by 30 mM K^+ alone.

[b] Effect of 2-chloroado compared with release evoked by 1 mM kainate alone.

[c] Reversal of effects of 2-chloroado by CPT, compared with effect of 1 μM 2-chloroado alone, on release evoked by 1 mM kainate.

Results are all shown as means ± s.e.mean; n = number of experiments.

***$P < 0.001$; **$P < 0.01$; *$P < 0.05$; NS, not significant by Student's t test.

hydroxytryptaminergic input to the striatum (Berger *et al.*, 1982) or a cholinergic input to the hippocampus (Nadler & Cuthbertson, 1980; Köhler *et al.*, 1978), also prevents KA neurotoxicity.

Several studies have indicated that KA exerts a presynaptic action to enhance synaptic levels of glutamate, which may then serve to trigger subsequent neurotoxic events postsynaptically. However, there is still much debate as to whether it does this by enhancing transmitter glutamate release via a Ca^{2+}-dependent (neuronally-derived) or Ca^{2+}-independent (probably derived from both neuronal and non-neuronal elements) mechanism, or whether the increase in the extracellular concentration of glutamate is mediated merely through the ability of KA to inhibit the high affinity neuronal and/or glial uptake of glutamate (Johnston *et al.*, 1979).

Some *in vitro* experiments have shown that KA enhances the release of endogenous glutamate or its 'false transmitter' counterpart D-[^{3}H]-aspartate in a predominantly Ca^{2+}-dependent manner (Nicklas *et al.*, 1980; Ferkany & Coyle, 1983; Pastuszko *et al.*, 1984; Poli *et al.*, 1985; Connick & Stone, 1986; Gallo *et al.*, 1987). This is wholly consistent with a major part of the action of KA being on synaptic transmitter pools of glutamate. Some of these studies also confirmed the additional ability of KA to inhibit the high affinity uptake of glutamate. In contrast, however, other *in vitro* release studies suggested that KA increases synaptic glutamate concentrations by a calcium-independent release mechanism involving non-neuronal elements (Nicklas *et al.*, 1980; Krespan *et al.*, 1982) possibly coupled with inhibition of the high affinity (neuronal and/or glial) uptake process (Notman *et al.*, 1984). These contradictions in the data accumulated from *in vitro* studies are also evident in *in vivo* attempts to elucidate the precise mechanism(s) of action of KA in releasing glutamate (Butcher *et al.*, 1987; Young *et al.*, 1988; Connick & Stone, 1988).

Adenosine is now widely considered to act as a major inhibitory neuromodulator within the mammalian CNS, through a possible action on neurotransmitter release and/or through its potent vasodilatator effects (Phillis & Wu, 1981). Indeed, these properties have led to the suggestion that adenosine may act as an endogenous anti-convulsant (Dragunow *et al.*, 1985). Stable adenosine analogues have been shown to exert potent depressant effects *in vitro* on endogenous (Corradetti *et al.*, 1984) and exogenous (Dolphin & Archer, 1983; Dolphin & Prestwich, 1985; Fastbom & Fredholm, 1985) glutamate release. Similarly, 2-chloroado was found to inhibit the tityustoxin-induced release of glutamate

in vivo (Young & Bradford, 1986). These actions of adenosine and its analogues on transmitter release are thus likely to involve directly a presynaptic receptor site, and present a possible means for investigating presynaptic involvement in the neurotoxic actions of KA.

Using the newly developed technique of continuous fluorometry to monitor the Ca^{2+}-dependent and independent release of glutamate from purified synaptosomes (Nicholls *et al.*, 1987), we have observed that KA (1 mM) induces an immediate and predominantly Ca^{2+}-dependent release of glutamate from freshly polarised synaptosomes. This KA-induced release is markedly attenuated by prior exposure to 2-chloroado (10 nM–1 μM), an effect which was prevented by the selective and potent adenosine antagonist CPT (1 μM). Identical effects of 2-chloroado on the release of glutamate were observed when 30 mM K^+ was used as the depolarising agent. These data are, however, not in agreement with the recent findings of Pocock *et al.* (1988) who suggested that the enhancement of extracellular glutamate concentrations was due to the ability of KA to inhibit the high affinity uptake of glutamate with a resultant 'leakage' of glutamate from a Ca^{2+}-independent, cytosolic source. These contrasting observations are possibly attributable to the different methodologies used in our experiments (Neville, Arvin & Roberts, in preparation).

We have further attempted to elucidate the ionic mechanism underlying the potent inhibitory actions of 2-chloroado on glutamate release, by monitoring Ca^{2+} influx into crude synaptosomes loaded with the calcium indicator quin-2. Our data indicate that 2-chloroado, at similar concentrations to those that are effective in the endogenous release studies, potently inhibited K^+-stimulated Ca^{2+} influx. This effect was antagonised by theophylline. However, we were unable to observe any significant KA-stimulated Ca^{2+} influx in the same preparation. Although many studies have indicated a stimulatory effect of KA on pre- and post-synaptic $^{45}Ca^{2+}$ uptake using a variety of different preparations both *in vivo* (Lazarewicz *et al.*, 1987; Butcher *et al.*, 1987; Young *et al.*, 1988) and *in vitro* (Wroblewski *et al.*, 1985; Riveros & Orrego, 1986; Crowder *et al.*, 1987), some workers have disputed these findings (Retz & Coyle, 1984; O'Shaughnessy *et al.*, 1986). Further studies using Ca^{2+} indicator dyes as an index of Ca^{2+} fluxes (similar to the present study) have illustrated either negligible (MacDermott *et al.*, 1986; Kudo & Ogura, 1986; Burgoyne *et al.*, 1988) or marked effects of KA on Ca^{2+} (Murphy *et al.*, 1987). It is unclear why these major discrepancies exist and certainly an effect of KA on Ca^{2+} influx might be expected from the observed calcium-dependent nature of the KA-stimulated release of glutamate from purified synaptosomes.

Hence, despite the apparent lack of efficacy of KA in our experiments in promoting synaptosomal Ca^{2+} influx, we cautiously propose the following mechanism to account for the potent anti-neurotoxic effects of 2-chloroado. 2-Chloroado may act presynaptically on specific receptors to inhibit the voltage-dependent release of glutamate, thus reducing its concentration within the synaptic cleft, and thereby removing a critical component of the mechanism for KA-induced neurotoxicity. This action of 2-chloroado is possibly mediated exclusively through an inhibition of Ca^{2+} influx. Several studies have illustrated a parallel distribution of adenosine 'A_1'-receptors with glutamatergic afferents (Wojcik & Neff, 1983; Goodman *et al.*, 1983), consistent with likely involvement of this receptor type in the above scheme.

The precise mechanisms by which 2-chloroado exerts its potent anti-excitotoxic effects against KA have still to be resolved. However, it is evident from this study, that adenosine-like compounds may provide a new pharmacological strategy for preventing or alleviating many excitatory amino acid linked neurological disorders. Indeed, recent studies have illustrated that adenosine-like compounds are effective in the prevention of ischaemic cell loss in animal models (Evans *et al.*, 1987) and tissue cultures (Goldberg *et al.*, 1988).

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Opposite central and peripheral control by endogenous opioids of intestinal motility in fed rats

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1 The effects of the inhibitors of endopeptidase EC 24.11, thiorphan and phosphoramidon administered i.c.v. (40 $\mu g\,kg^{-1}$) i.p. (400 $\mu g\,kg^{-1}$), or orally (400 $\mu g\,kg^{-1}$), on intestinal motor activity in fed rats was compared to the effects of similar doses of the angiotensin converting enzyme inhibitor, captopril and the synthetic enkephalin analogue [D-Ala^2 Met^5] enkephalinamide (Dalamide). Drugs were administered alone or after pretreatment with naloxone or N-methyl levallorphan (300 $\mu g\,kg^{-1}$, i.p.) given 10 min prior to gavage with a standard meal.

2 In control conditions, in the duodenum, the disruption of the migrating myoelectric complex (MMC) by gavage with a standard meal lasted between 105.6 and 119.1 min. This duration was significantly decreased by thiorphan (60.3 ± 15.0 min), phosphoramidon (67.9 ± 7.3 min), captopril (26.3 ± 10.2 min) and Dalamide (42.4 ± 9.6 min), administered i.c.v.

3 In contrast, after the i.p. administration of thiorphan, phosphoramidon and Dalamide the delay in the return of the MMC pattern was increased. Such an effect was also seen after the oral administration of phosphoramidon or Dalamide. Neither i.p. nor oral captopril administration altered the duration of postprandial pattern.

4 A prior treatment with naloxone i.p. (300 $\mu g\,kg^{-1}$) that had no effect *per se*, antagonized the effect produced by i.c.v. administration of thiorphan, phosphoramidon or Dalamide, but failed to reverse the effect of captopril. In contrast, i.p. administration of N-methyl levallorphan (300 $\mu g\,kg^{-1}$) did not affect the response induced by central administration of thiorphan, phosphoramidon, captopril or Dalamide, but was able to prevent that of thiorphan, phosphoramidon or Dalamide when they were administered i.p. or orally.

5 These data strongly support the hypothesis of a dual control by endogenous opioids of intestinal motility in the rat: a central component that favours, and a peripheral control that delays the occurrence of the MMC profile in fed rats.

Introduction

Various preparations and extracts of the opium poppy were in use for their antidiarrhoeal effects some time before the exploitation of the plant for its analgesic properties (see Mannara & Bianchetti, 1985). The pioneering work by Margolin (1954) suggested that this constipating effect could be related to a slowing of intestinal transit in mice. More recently, it was shown that morphine also delays gastric emptying in rats (Stewart & Curd, 1984), and that this antipropulsive action on the gastrointestinal tract is associated with a decrease in contractile activity (Weisbrodt *et al.*, 1980; Galligan & Burks, 1983). Furthermore, because these effects occur after direct administration of small amounts to the brain as well as by administration via a peripheral route, a central origin for the action of morphine on the gut has been suggested (Buéno & Ruckebusch, 1978; Stewart *et al.*, 1979). The many inconsistencies that appear in the literature concerning the possible site of action of opioids in affecting gut motility can be explained on the basis of differences in experimental conditions, routes of administration, opioid agonist used, species or gastrointestinal segment considered (see for reviews: Mannara & Bianchetti, 1985; Buéno & Fioramonti, 1988). Numerous works (Galambos *et al.*, 1976; Schultz *et al.*, 1979; Buéno *et al.*, 1985; Fioramonti *et al.*, 1987) suggest a possible direct action of opiates on the gut which is in agreement with the previous identification of opioid receptors (Kosterlitz *et al.*, 1972) and

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enkephalins (Polak *et al.*, 1977) in the digestive tract. Despite numerous studies using exogenous administrations of stable long acting analogues of enkephalins, only a few studies have addressed the possible physiological participation of endogenous opioid peptides in the long term regulation of intestinal functions.

An understanding of the enzymatic breakdown of the enkephalins has led to the synthesis of potent inhibitors of 'enkephalinase', endopeptidase EC 24.11. Such an inhibitor, thiorphan (Roques *et al.*, 1980), does not cross the blood brain barrier (BBB) (Schwartz *et al.*, 1985), and thus allows the possibility of preventing selectively the degradation of endogenous opioid peptides at central or peripheral sites. Using thiorphan or its precursor (acetorphan), the possible involvement of a central control by enkephalins of intestinal water and ion transport has been demonstrated in the dog (Primi, 1986). Likewise, the existence of both a central inhibitory and a peripheral stimulatory control by endogenous opioids of gastric emptying of a nutritive fat meal in the mouse has recently been proposed (Liberge *et al.*, 1988).

The present study was performed in fed rats to investigate the site of action of endogenous opioid peptides that may be involved in the control of intestinal motility by use of a chronic electromyographic technique. The effects on intestinal motor activity in fed rats of intracerebroventricular (i.c.v.), intraperitoneal (i.p.), and oral (p.o.) administration of thiorphan have been compared with those of similar treatments by phosphoramidon, another inhibitor of endopeptidase EC 24.11 (Schwartz *et al.*, 1985), by captopril, selective inhibitor of angiotensin converting enzyme (ACE) (Schwartz *et al.*, 1981) and by [D-Ala^2-Met^5] enkephalinamide (Dalamide), a long acting synthetic analogue of enkephalins. These drugs were administered either alone or following a prior treatment with the opioid antagonists naloxone or N-methyl levallorphan.

Methods

Animal preparation

Twelve male Wistar rats weighing 300–400 g, housed individually and maintained at an ambient temperature of 20–21°C, were used in the study. Under halothane anaesthesia, animals were prepared for a long term electromyographic recording of intestinal motility (Ruckebusch & Fioramonti, 1975) by implantation in the intestinal wall of 4 groups of 3 nichrome wires (80 μm in diameter) placed on the small intestine at 5, 10, 20 and 25 cm from the pylorus. The electrode wires (60 cm in length) were exteriorized on the back of the neck. In addition, a small polyethylene catheter was inserted into each lateral ventricle of the brain (Stewart *et al.*, 1978).

Recordings

Recordings were started 5 days after surgery. The electrical activity of the intestine was recorded each day from 09 h 00 min to 19 h 00 min with an electroencephalograph (Reega VIII, Alvar, Paris) having a paper speed of 2.4 cm min^{-1}. The identification of the intestinal motor pattern was facilitated by the summation of the spiking activity every 20 s; this gave an integrated record of the myoelectrical activity which was plotted continuously through each 24 h period on the y axis of a potentiometric recorder with a paper speed of 5 cm h^{-1} (Latour, 1973).

Experimental procedure

The experiments were performed each day at 13 h 00 min, following 5 h of food deprivation.

In a first series of experiments, the rats received either 5 μl (i.c.v.), 0.2 ml (i.p.) or 0.2 ml (p.o.) of sterile isotonic saline alone (vehicle) or containing thiorphan (Laboratoire Bioprojet, Paris), phosphoramidon (Sigma, France), captopril (Squibb, Paris) or Dalamide (Sigma, France) at doses of 40 μg kg^{-1}, i.c.v., and 400 μg kg^{-1} i.p. or p.o. Either treatment was followed 10 min later by gavage with a 3 ml nutritive meal of hydrated cow's milk powder (sugars 0.5 g; fats 0.5 g; proteins 0.5 g).

In a second series of experiments, the same experimental model and procedure were used to compare the effects of two opiate antagonists: naloxone (Endo laboratories, Garden City, New York) that is active at central and peripheral sites after parenteral administration, and a quaternary narcotic antagonist, N-methyl levallorphan (SR 58002 C, Sanofi-Recherche, Montpellier, France) that penetrates to the brain poorly (Bianchetti *et al.*, 1985). Each of these was administered at different doses i.p. (50 to 2000 μg kg^{-1}) in order to determine the threshold for alteration of postprandial motility.

In a third series of experiments, the highest dose of naloxone or of N-methyl levallorphan that had no effect by itself (300 μg kg^{-1}, i.p.) was administered 10 min prior to the i.c.v., i.p. or p.o. administration of thiorphan, phosphoramidon, captopril, and Dalamide.

The effect of the different treatments was evaluated by measuring the duration of the disruption of the postprandial MMC in the duodenum and the jejunum. At the end of the experiment, after the return of the fasted motility pattern, the animals were fed with 8 g of pelleted laboratory chow for

rodents (Usine d'Alimentation Rationnelle, Epinay, France).

Statistical analysis

Data were analysed using the non-parametric Wilcoxon-test that allows paired comparisons and are given as mean ± s.d.

Results

The continuous integrated (summation of the spikes every 20 s) recording of small intestinal myoelectrical activity in conscious rats shows (Figure 1) two distinct intestinal motor activity profiles (Ruckebusch & Fioramonti, 1975). In fasted animals, the motility is characterized by the occurrence of a Migrating Myoelectrical Complex (MMC) the mean duration of which is 12 to 18 min and which migrates at a speed of 2 to 3 cm per min. The MMC is organised in three different phases: a period of quiescence (phase I) followed sequentially by periods of irregular (ISA or phase II) and regular spiking activity (RSA or phase III). After feeding, this profile is replaced by a continuous ISA.

Experiment 1

The gavage with a standard meal was followed by a disruption of the MMC pattern in both duodenum and jejunum, which was not affected by the administration of the vehicle, irrespective of the route used (i.c.v., i.v. or i.p.). The duration of the postprandial pattern was significantly decreased by the i.c.v. administration of thiorphan, phosphoramidon (Figure 1), captopril or Dalamide (respectively 60.3 ± 15.0 min; 67.9 ± 7.3 min; 26.3 ± 10.2 min and 42.4 ± 9.6 min in the duodenum, $n = 12$, Figure 2). The opposite effects were observed after i.p. administration of thiorphan, phosphoramidon or Dalamide where the recovery of the MMC pattern was delayed and the period during which ISA was found was increased by at least 18%. This last effect was reproduced by oral administration of phosphoramidon or

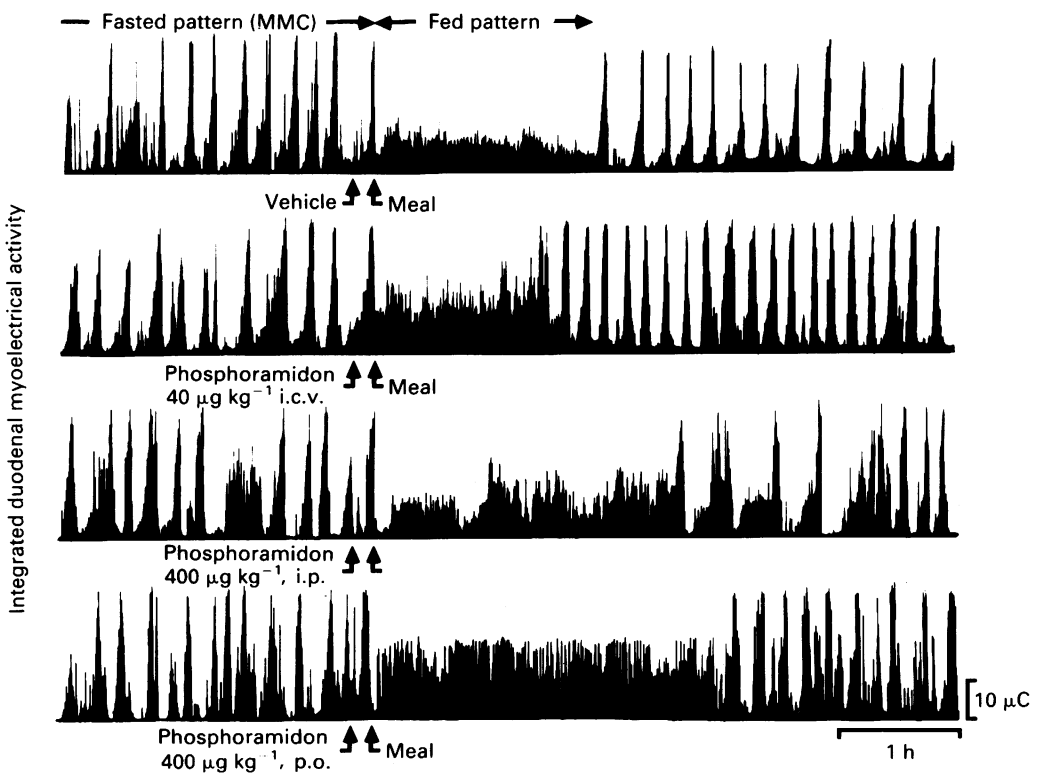


Figure 1 Effects of i.c.v. (40 μg kg^{-1}), i.p. (400 μg kg^{-1}) and p.o. (400 μg kg^{-1}) administration of phosphoramidon on duodenal myoelectrical activity (integrated record) of rats fed by gavage with a test meal; i.c.v. treatment shortens while i.p. and p.o. administration delay the return of the fasted pattern (MMC). μC = microcoulomb.

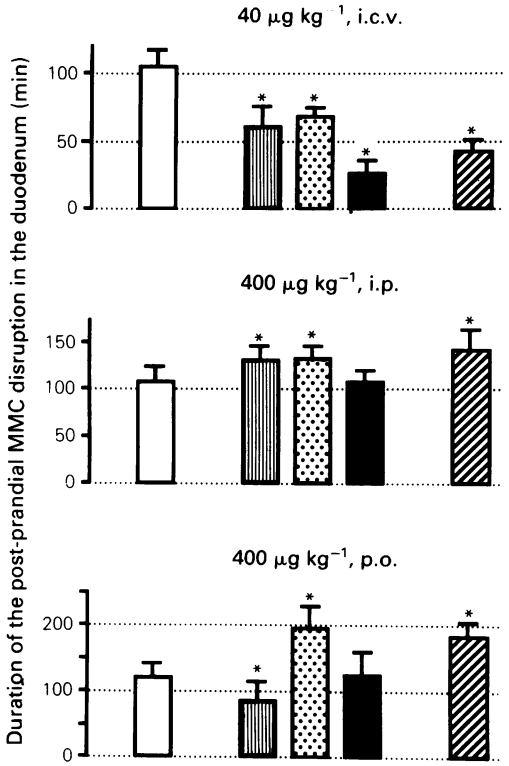


Figure 2 Effects of i.c.v. (40 $\mu g\,kg^{-1}$), i.p. (400 $\mu g\,kg^{-1}$) and p.o. (400 $\mu g\,kg^{-1}$) administration of thiorphan, phosphoramidon, captopril and Dalamide on the duration of the postprandial pattern on duodenum following gavage with a test meal in rats; columns show mean values ($n = 12$) and vertical bars indicate s.d. Open columns, vehicle; vertically lined columns, thiorphan; stippled columns, phosphoramidon; solid columns, captopril; hatched columns, Dalamide. * $P < 0.05$, W-test.

Dalamide but not by thiorphan which induced an early return of the MMC profile. Neither the i.p. nor p.o. administration of captopril altered the duration of the postprandial pattern (Table 1).

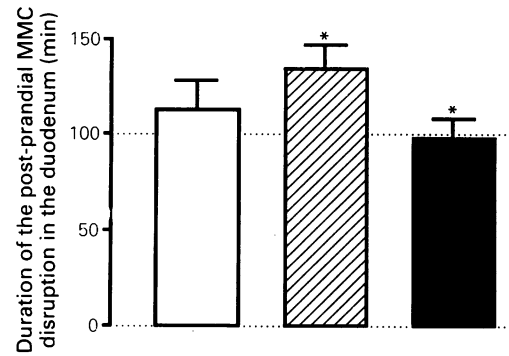


Figure 3 Effects of i.p. administration of naloxone (1 $mg\,kg^{-1}$) and N-methyl levallorphan (2 $mg\,kg^{-1}$) on the duodenal fed pattern following gavage with a test meal in rats; columns show mean values ($n = 12$) and vertical bars indicate s.d. Open column, vehicle; hatched column, naloxone; solid column, N-methyl levallorphan.

Experiment 2

Intraperitoneal administration of naloxone (1 $mg\,kg^{-1}$) and N-methyl levallorphan (2 $mg\,kg^{-1}$) alone produced opposite effects, increasing (+19%) and decreasing (−12%) respectively the duration of the postprandial pattern at both duodenal (Figure 3) and jejunal levels. However, they failed to alter the pattern when given at a lower dose (300 $\mu g\,kg^{-1}$, i.p.).

Experiment 3

Previous i.p. treatment by a lower dose of naloxone of 300 $\mu g\,kg^{-1}$ that had no effect by itself antagonized the effect of i.c.v. administration of thiorphan, phosphoramidon or Dalamide, but failed to reverse that of captopril (Table 2). In contrast, N-methyl levallorphan (300 $\mu g\,kg^{-1}$) did not affect significantly ($P \geqslant 0.05$) the response to i.c.v. administration of thiorphan, phosphoramidon, captopril or Dalamide but suppressed the action of thiorphan, phos-

Table 1 Action of i.c.v., i.p. and p.o. administration of thiorphan, phosphoramidon, captopril and Dalamide on the inhibition of the jejunal migrating myoelectric complex (MMC) pattern observed following gavage with a test meal in rats

	Postprandial disruption of the jejunal MMC pattern (min)				
	Vehicle	*Thiorphan*	*Phosphoramidon*	*Dalamide*	*Captopril*
i.c.v.	94.3 ± 10.7	55.6 ± 15.6*	60.7 ± 7.9*	39.3 ± 8.2*	20.1 ± 9.1*
i.p.	102.4 ± 15.9	123.8 ± 14.0*	125.7 ± 12.2*	128.5 ± 23.0*	95.3 ± 15.0
p.o.	105.1 ± 17.1	71.5 ± 24.2*	182.3 ± 26.5*	170.8 ± 20.6*	100.5 ± 23.4

* Significantly different from vehicle values (W-test, $P \leqslant 0.05$, $n = 12$).

Table 2 Influence of previous i.p. (300 $\mu g\,kg^{-1}$) administration of naloxone or N-methyl levallorphan (NML) on the effects of i.c.v., i.p. and p.o. administration of thiorphan, phosphoramidon, captopril and Dalamide on the inhibition of the duodenal migrating myoelectric complex (MMC) pattern induced by gavage with a test meal in rats

		Postprandial disruption of the duodenal MMC pattern (min)		
	Route	*Control*	*Naloxone*, i.p.	*NML*, i.p.
Vehicle	i.c.v.	105.6 ± 11.8	110.2 ± 16.4	113.1 ± 18.8
	i.p.	111.4 ± 17.8	106.8 ± 9.8	118.1 ± 17.4
	p.o.	119.1 ± 19.0	107.3 ± 14.5	116.5 ± 11.2
Thiorphan	i.c.v.	60.3 ± 15.0*	107.0 ± 10.8†	71.4 ± 17.0*
	i.p.	131.8 ± 15.3*	117.2 ± 15.1†	109.2 ± 10.7†
	p.o.	84.8 ± 28.0*	112.4 ± 13.5†	104.0 ± 13.0†
Phosphoramidon	i.c.v.	67.9 ± 7.3*	112.9 ± 13.7†	58.1 ± 12.5*
	i.p.	132.8 ± 14.0*	102.7 ± 10.3†	112.3 ± 14.4†
	p.o.	194.1 ± 34.1*	109.0 ± 12.6†	103.7 ± 12.1†
Dalamide	i.c.v.	42.4 ± 9.6*	117.3 ± 16.7†	49.3 ± 7.0*
	i.p.	142.6 ± 22.9*	103.5 ± 12.1†	111.6 ± 15.2†
	p.o.	182.4 ± 22.0*	106.1 ± 17.7†	100.7 ± 9.4†
Captopril	i.c.v.	26.3 ± 10.2*	30.1 ± 8.5*	24.9 ± 11.2*

* Significantly different from vehicle values; † significantly different from control values (W-test, $P \leqslant 0.05$, $n = 12$).

phoramidon or Dalamide injected i.p. or administered orally.

Discussion

Previous studies have shown that inhibitors of endopeptidase EC 24.11 affect intestinal absorption processes in the dog (Primi, 1986) and gastric emptying in mice (Liberge *et al.*, 1988), and the present study demonstrates that the administration of thiorphan alters small intestinal motility in fed rats. The ability of Dalamide or phosphoramidon to reproduce the effects of thiorphan by central or peripheral administration strongly supports the idea that such effects are mediated by endogenous opioid peptides.

Nevertheless, it has been reported that endopeptidase EC 24.11 may be involved in the enzymatic breakdown of other neuropeptides that are involved in the control of small intestinal motility at central and/or peripheral sites. The central administration of thiorphan potentiates the hypothermic effect of neurotensin in mice (Coquerel *et al.*, 1986) and the i.c.v. administration of neurotensin restores a fasted pattern of intestinal motility in fed rats (Buéno *et al.*, 1983), suggesting that neurotensin could be involved in the central action of thiorphan. However, while the potentiation of neurotensin-induced hypothermia by thiorphan is not reversed by naloxone (Coquerel *et al.*, 1986), in our study naloxone is found to be able to abolish the effects of thiorphan on gut motility. Endopeptidase EC 24.11 may also be involved in the degradation of cholecystokinin (CCK) (Deschodt-Lanckman & Strosberg, 1983), a neuropeptide that is reported to increase the period of the postprandial MMC disruption by a central action (Duc, 1988). However, the latter effect is the opposite of that of thiorphan given i.c.v., suggesting that the central action of thiorphan is not mediated by CCK. Similarly, endopeptidase EC 24.11 may be the major degrading enzyme for atrial natriuretic factor (ANF) (Sonnenberg *et al.*, 1988). Although ANF has a relaxant action on the vascular smooth muscle (Garcia *et al.*, 1985), there is no evidence for an action of ANF on intestinal smooth muscle. Finally the lack of blockade by naloxone of the central effects of captopril on gut motility suggests the possible involvement of a non-opioid mechanism, despite the possibility of breakdown of enkephalins *in vitro* by ACE (Schwartz *et al.*, 1981).

The poor ability of thiorphan (Schwartz *et al.*, 1985) and Dalamide (Rapoport *et al.*, 1980) to cross the BBB suggests that they are acting at the sites of their administration. This last finding is confirmed by the fact that a previous i.p. treatment with naloxone blocks the effects of i.c.v. administration of thiorphan and Dalamide, while N-methyl levallorphan, which does not reach the CNS, is ineffective. In contrast, both antagonists block the effects of the i.p. administration of thiorphan or Dalamide. These data also suggest that phosphoramidon does not penetrate to the CNS to any significant degree.

Our experiments suggest the existence of an opposite central and peripheral control by endogenous opioid peptides of small intestinal motility in fed rats. This observation and its possible physiological relevance are reinforced by the opposite effects

that are produced by the i.p. administration of high doses of naloxone or N-methyl levallorphan given alone. At a central level, enkephalins or related peptides may act to shorten the postprandial pattern that is represented by a permanent irregular spiking activity (ISA), and associated with the highest rate of intestinal transit (Buéno *et al.*, 1975). During the postprandial period of prolonged ISA there may be inhibition of both gastric peristalsis and gastric emptying (Thomas *et al.*, 1934) and the present results are consistent with previous experiments suggesting an inhibition of gastric emptying in mice by a central opioid mechanism (Liberge *et al.*, 1988) and with the central antipropulsive effects of morphine in rats (Galligan & Burks, 1983; Stewart & Curd, 1984). Conversely, by a direct action at a peripheral level, endogenous opioid peptides appear to act to delay the occurrence of the MMC which is in agreement with the peripheral stimulation of gastric emptying by enkephalins that was observed in mice (Liberge *et al.*, 1988).

In conclusion, using inhibitors of endopeptidase EC 24.11 we have demonstrated the existence in fed rats of central reorganizing and a peripheral disrupting physiological control by endogenous opioid mechanisms of the MMC pattern.

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Accumulation of extracellular calcium at the endplate of mouse diaphragm after ecothiopate *in vitro*

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1 Experiments were carried out to investigate the accumulation from the extracellular medium of $^{45}Ca^{2+}$ by the endplate region of skeletal muscle.

2 Mouse diaphragm muscle was incubated in physiological saline labelled with ^{45}Ca at 37°C for periods of up to 1.5 h.

3 The muscle was divided into junctional and non-junctional portions and the Ca from the extracellular fluid accumulated at the endplate determined from the ^{45}Ca content of the portions.

4 The accumulation of extracellular Ca at the endplate region of muscles incubated in physiological saline alone was nil, but there was accumulation in the presence of the anticholinesterase ecothiopate iodide 0.5×10^{-6} M (ECO). Stimulation of the phrenic nerve at 0.02 Hz caused no further increase in accumulation, but reduced the amount of spontaneous fasciculation. In tetrodotoxin (TTX) 10^{-6} M, the accumulation was halved, and in 3.5 mM Mg^{2+} the accumulation was nil. Carbachol 10^{-4} M resulted in an accumulation of Ca similar to that in ECO.

5 It is concluded that there was an accumulation of extracellular Ca following excitation of the nerve by stimulation at a low frequency and during the spontaneous fasciculations, and about half of the accumulation of extracellular Ca after ECO in the experiments was due to the postsynaptic action of ACh released non-quantally from the nerve terminals.

Introduction

There is much evidence that anticholinesterases or cholinoceptor agonists provoke structural damage at the endplate region of skeletal muscle (see Salpeter *et al.*, 1982 for review) with the possibility that overloading with Ca may be a causative mechanism. As cholinoceptor agonists permit entry of Ca^{2+} through open endplate channels, it is possible that the necrotizing effect of anticholinesterases may be due to a prolongation and intensification of the postsynaptic action of acetylcholine (ACh) released quantally from the nerve terminals either spontaneously or evoked by action potentials following stimulation of the nerve and by that released non-quantally. Another contribution to an overloading of the endplate region with Ca may derive from the excitation of the nerve terminals during anticholinesterase-induced fasciculation. Thus it remains to be determined what is the contribution made by various events induced or modified by anticholinesterases to the loading of the endplate with Ca^{2+} and perhaps to the genesis of localized necrosis.

It has already been established by Evans (1974) that carbachol caused uptake of Ca into the muscle at the endplate, as did physostigmine, but that stimulation of the nerve in the absence or in the presence of physostigmine caused no additional accumulation. This apparent lack of effect of stimulation is not in accord with the results of Ariens *et al.* (1969) that myopathy after anticholinesterases was enhanced by neural activity.

The following is an account of experiments undertaken to investigate the entry of Ca at the endplate after ecothiopate under conditions of low frequency stimulation similar to those in which prolonged localized contractions associated with prolonged endplate current (Burd & Ferry, 1987), fasciculations and enhanced synaptic currents have been observed, and to determine which of the various processes is important for the induction of myopathy. It is concluded that there was substantial accumulation of

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Ca at the endplate even in the absence of excitation of the nerve and attributable to spontaneous events and that there was an additional accumulation associated with excitation of the nerve.

Methods

Preparations of the phrenic nerve and diaphragm muscle were dissected from male albino mice of about 20 g weight after the animals were killed by a blow to the head and section of the cervical spinal cord. The preparations were maintained at 37 ± 0.2°C in a physiological saline of the following composition (mM): NaCl 137, NaH_2CO_3 12, $CaCl_2$ 2, $MgCl_2$ 1, KCl 5, NaH_2PO_4 1, dextrose 25 and gassed with 5% CO_2 in O_2. In some experiments extra $MgCl_2$ was added without any other change.

Electrophysiological methods

Diaphragms were pinned horizontally to the Sylgard bottom of a Perspex bath and records were made of biolectric potentials at the endplate region with a KCl-filled micropipette.

Determination of the accumulation of Ca

The method used to determine the Ca content of muscle was based on that of Evans (1974). The whole mouse diaphragm was pinned to Sylgard in the bottom of a petri dish and immersed in 25 ml of the physiological saline containing $^{45}CaCl_2$ made up from a stock solution of 0.8–1 mCi mmol (Radiochemical Centre, Amersham), in amounts to give 3–8 $\times 10^5$ c.p.m. ml^{-1} of physiological saline. The incubation medium thus contained 2 mM $CaCl_2$ plus about 0.5 μM $^{45}CaCl_2$ stock and is referred to as ^{45}Ca-saline.

In some experiments, the phrenic nerve of one hemidiaphragm was stimulated at 0.02 Hz via Ag wire electrodes, the other half of the diaphragm being unstimulated but still attached by the central tendon to the contracting hemidiaphragm.

The preparations were incubated under the various conditions described in Results and were rinsed in ^{45}Ca-free physiological saline, usually for 30 min and were then dehydrated in two changes of acetone over a minimum of 2 h. The diaphragm was cut into hemidiaphragms, the ribs removed and the muscle cut transversely about 1 mm on either side of the intramuscular branches of the phrenic nerve into a central junctional portion and two peripheral nonjunctional portions (Hebb *et al.*, 1964). Immersion of the muscle in xylene made the nerve more visible.

The junctional portion and the nonjunctional portions from each hemidiaphragm were placed in different polythene scintillation vials which were loosely capped and placed for 30 min in a water bath at 60°C to drive off the xylene. The junctional portion and the nonjunctional portions were then weighed with a torsion balance (usually each was 1–2 mg). They were digested with 0.5 ml of concentrated HCl for 30 min at 60°C or overnight at room temperature. Absolute ethanol (2 ml) and 10 ml of either Toluene fluor (PPO 5 g, POPOP 0.3 g, toluene to 1 l) or Bray's fluor (PPO 4 g, POPOP 0.2 g, naphthalene 100 g, methanol 100 μl; 1,4-dioxan to 1 l) were added to each vial and the radioactivity of the ^{45}Ca was determined by liquid scintillation counting at an average efficiency of 30%. Standards consisted of 0.1 ml of the ^{45}Ca-saline and were counted in the same way as the samples of muscle to allow calculation of Ca accumulation.

In order to calculate the accumulation of Ca at the endplate, it is necessary to take account of any Ca accumulated by the non-synaptic part of the muscle cell. It was assumed that such non-synaptic accumulation was evenly distributed along the length of the cell, and the amount of Ca in the non-synaptic part of the excised junctional portion was estimated as the product of the weight of the junctional portion ($J_{wt.}$) and the accumulation of Ca per unit weight of the nonjunctional portion (NJ_{Ca}) of the same hemidiaphragm. Thus the accumulation of Ca at the endplate was calculated as the amount of Ca in the junctional portion ($J_{Ca} \times J_{wt.}$) less the amount of Ca in the same weight of nonjunctional portion of the hemidiaphragm ($NJ_{Ca} \times J_{wt.}$). The following equations describe the calculation of the accumulation of Ca at the endplate:

$$J_{Ca} = \frac{J_{c.p.m.}}{J_{wt.}} \times \frac{Std_{Ca}}{Std_{c.p.m.}} \text{ nmol mg}^{-1} \text{ dry wt.}$$

$$NJ_{Ca} = \frac{NJ_{c.p.m.}}{NJ_{wt.}} \times \frac{Std_{Ca}}{Std_{c.p.m.}} \text{ nmol mg}^{-1} \text{ dry wt.}$$

$$EP_{Ca} = \frac{J_{wt.}(J_{Ca} - NJ_{Ca})}{J_{wt.} + NJ_{wt.}} \text{ nmol mg}^{-1} \text{ total dry wt.}$$

where

J_{Ca} = accumulation of Ca in junctional portion (nmol mg^{-1}).
NJ_{Ca} = accumulation of Ca in nonjunctional portion (nmol mg^{-1}).
$J_{c.p.m.}$ = counts min^{-1} of junctional portion.
$NJ_{c.p.m.}$ = counts min^{-1} of nonjunctional portion.
$J_{wt.}$ = weight of junctional portion in mg.
$NJ_{wt.}$ = weight of nonjunctional portion in mg.
Std_{Ca} = nmol of Ca^{2+} in saline containing ^{45}Ca which was the standard with counts min^{-1} of $Std_{c.p.m.}$

EP_{Ca} = accumulation of Ca at the endplate (nmol mg^{-1} total wt).

In preliminary experiments, as no difference was found in the accumulation of Ca in left and right hemidiaphragms, they were taken as equivalent.

Statistical methods

Usually each experiment was carried out on a group of hemidiaphragms and the mean and standard deviation (s.d.) of each group are reported in the text. Non-parametric two-tailed tests were used to estimate the probability of identicality of groups of results, and when $P < 0.05$, a significant difference was reported. Unpaired data were subjected to the Mann-Whitney test and paired data to the Wilcoxon test.

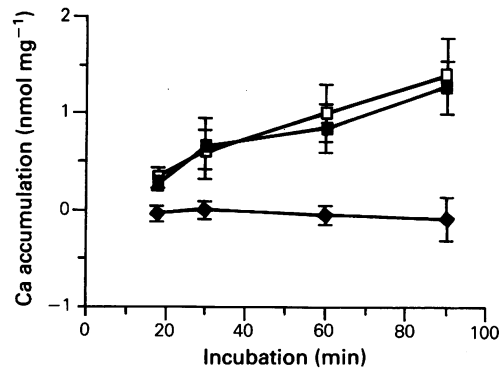


Figure 1 Graph showing the progress of accumulation of Ca in portions of hemidiaphragms incubated in ^{45}Ca-saline, values are mean with s.d. shown by vertical bars (see Table 1). Note time-dependent accumulation in (■) junctional portions and in (□) non-junctional portions with no (◆) accumulation at the endplate.

Materials

The anticholinesterase ecothiopate iodide was made up from the commercially available Ecothiopate Eyedrops 0.25%, B.N.F., (Phospholine Eyedrops, Ayerst) which also contained potassium acetate to buffer the stock solution. The increase in C_K of the physiological saline due to this buffer was 60 nM which was considered negligible.

The ingredients of the physiological saline were BDH AR grade, and drugs were purchased from Sigma.

Results

The progress of accumulation of Ca

The accumulation of Ca in ^{45}Ca-saline alone The progress of accumulation of the ^{45}Ca in the junctional and nonjunctional portions and at the endplate was examined by incubating diaphragms in ^{45}Ca-saline for periods of 18, 30, 60 or 90 min before processing them as described in Methods. There was considerable variation between hemidiaphragms in the values of the accumulation of Ca in the junctional or in the nonjunctional portions (J_{Ca} and NJ_{Ca}), the coefficient of variation of these quantities in the group of 9 hemidiaphragms being 0.3–0.5. The values for the accumulation of Ca in the junctional portions (J_{Ca}) at various times were significantly different from each other except between 30 and 60 min, and for the nonjunctional portions (NJ_{Ca}) between 60 and 90 min. These results indicate a progression accumulation of Ca with increasing time of incubation in ^{45}Ca for up to 60 min (Figure 1).

The calculation of the accumulation of Ca at the endplate of individual hemidiaphragms gave values for EP_{Ca} at the various times which were not significantly different from one another (Figures 1, 2). The mean values of EP_{Ca} are small and at 18, 30 and 60 min are due to small but just significant differences between J_{Ca} and NJ_{Ca} shown by the Wilcoxon test.

It is concluded that although there was a progressively increasing accumulation of Ca along the length of the muscle cells, there was no additional accumulation at the endplate.

The effect of ecothiopate on the accumulation of Ca

In this experiment, diaphragms were immersed at zero time in ^{45}Ca-saline containing ecothiopate (ECO) 0.5 μM. Diaphragms exposed to ECO fasciculated, and had miniature endplate potentials (m.e.p.ps) of increased duration and amplitude. After incubation in ECO for 15, 30, 60 or 90 min, diaphragms were washed in ^{45}Ca-free physiological saline for 30 min and then prepared for assay of ^{45}Ca. Such diaphragms usually appeared swollen at the endplate region. At each time, the accumulation of Ca in the junctional portions (J_{Ca}) was significantly greater than that in the nonjunctional portions (NJ_{Ca}) of diaphragms. The accumulation of Ca

in the junctional portions (J_{Ca}) and in the non-junctional portions (NJ_{Ca}) increased throughout the 90 min. The values of J_{Ca} at each time were different from those at all other times, and the same for NJ_{Ca}.

Statistical testing of the nonjunctional accumulation of Ca in the presence and the absence of ECO showed no difference with incubation for up to 60 min, but at 90 min, the nonjunctional accumulation of Ca in the presence of ECO was greater than in its absence. This indicates that the early part of the progress of nonjunctional accumulation was independent of ECO. The junctional accumulations (J_{Ca}) at each time in ECO were significantly different from those in the absence of ECO.

The values of the accumulation of Ca at the endplate (EP_{Ca}) at 18, 30 and 60 min were significantly different from each other, and that at 90 min was not different from the values at 30 or 60 min indicating a progressive accumulation of Ca at the endplate until a plateau was reached at 60 min. With incubation in ECO, the EP_{Ca} at 18, 30, 60 and 90 min was significantly greater than that found at similar times in the absence of ECO. The results are shown in Table 1 and in Figure 2.

It is concluded that in the presence of ECO there was a progressive accumulation of Ca for up to 60 min in the nonsynaptic part of the cell which was not different from that found in the absence of ECO, and a progressive accumulation of Ca at the endplate which was different from the lack of accumulation in the absence of ECO (Figure 2).

In another experiment, 6 hemidiaphragms were incubated in ^{45}Ca-saline and ECO for 30 min, then rinsed in physiological saline for a few seconds to remove any film or droplets of the incubation medium, then dehydrated in acetone. These diaphragms had a NJ_{Ca} of 2.99 ± 0.724 nmol mg^{-1} and a J_{Ca} of 3.95 ± 0.624 nmol mg^{-1} which were greater than those of preparations washed in ^{45}Ca-free saline for 30 min (Table 1). The EP_{Ca} was 0.334 ± 0.075, which was not different from that of the washed preparations. It is concluded that the Ca which had accumulated at the endplate was not lost by washing for 30 min.

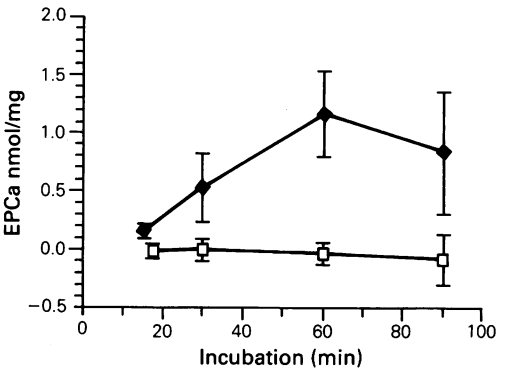


Figure 2 Graph showing progress of accumulation of Ca at the endplate EP_{Ca} in ^{45}Ca-saline alone (□) and in that containing ecothiopate (ECO) 0.5 μM (◆). Values are mean and s.d. Note progression of accumulation of EP_{Ca} over 60 min in ECO. Values of EP_{Ca} in the absence of ECO are from Figure 1.

These experiments on the progress of accumulation of Ca have shown that the endplate accumulation (EP_{Ca}), which is the quantity of interest in this investigation, and which is derived from the difference between the junctional and nonjunctional accumulation is independent of the absolute Ca content of these portions.

The effect of nerve stimulation in ecothiopate on the accumulation of Ca

In previous experiments (Burd & Ferry, 1987), it was found that stimulation of the nerve at 0.02 Hz resulted in the appearance for about 15 min of prolonged contractions localized at the endplate and associated with prolonged endplate currents. To find

Table 1 The effect of the duration of incubation in $^{45}Ca^{2+}$ and ecothiopate (ECO) on the accumulation of Ca at the endplate (EP_{Ca}) and by the nonjunctional portion (NJ_{Ca}) and junctional portion (J_{Ca})

Incub (min)	EP_{Ca} (nmol mg^{-1})	NJ_{Ca} (nmol mg^{-1})	J_{Ca} (nmol mg^{-1})	n
15	0.162 ± 0.046	0.328 ± 0.081	0.657 ± 0.144	8
30	*0.531 ± 0.29	0.851 ± 0.418	1.86 ± 0.532	15
60	†1.17 ± 0.38	1.17 ± 0.23	*3.06 ± 0.546	7
90	*†0.836 ± 0.521	2.79 ± 0.41	*4.18 ± 1.07	7

Values are mean and s.d. of *n* hemidiaphragms. Mean values marked †, * are not different from other values so marked in the same column; unmarked values are significantly different from each other value ($P < 0.05$).

out if these prolonged localized contractions were associated with an increased accumulation of Ca, experiments were done on paired hemidiaphragms from the same mouse, one stimulated at 0.02 Hz, the other unstimulated, the duration of the exposure to ECO being 15 min.

In this experiment, diaphragms were incubated in ^{45}Ca for 15 min so that the tracer could permeate through the extracellular space before the addition of ECO.

Whole diaphragms were immersed in ^{45}Ca-saline at time −15 min and at −10 min, indirect stimulation at 0.02 Hz of one hemidiaphragm was begun, the other hemidiaphragm was not stimulated. At time 0 min, ECO was added and after incubation for a further 15 min, the diaphragms were placed unstimulated in unlabelled physiological saline to wash for 30 min, and then prepared for assay of ^{45}Ca. With the stimulated or unstimulated paired hemidiaphragms the Wilcoxon test showed the accumulation of Ca in the junctional portions (J_{Ca}) to be significantly greater than that in the nonjunctional portions (NJ_{Ca}). The accumulation of Ca at the endplate (EP_{Ca}) of stimulated hemidiaphragms was 0.334 ± 0.075 nmol mg^{-1}, and when compared with the 0.213 ± 0.016 nmol mg^{-1} ($n = 6$) of unstimulated hemidiaphragms (Figure 3), the variability of the results was such that these groups were not significantly different.

It is concluded that the results did not support the hypothesis that stimulation of the nerve at 0.02 Hz in the presence of ECO increased the accumulation of Ca by the diaphragm above that found in the presence of ECO without stimulation. Even though there was no significant effect of stimulation, because of the variability of the small number of observations and the difference between the mean values, perhaps the stimulated EP_{Ca} may not be the same as the unstimulated EP_{Ca}.

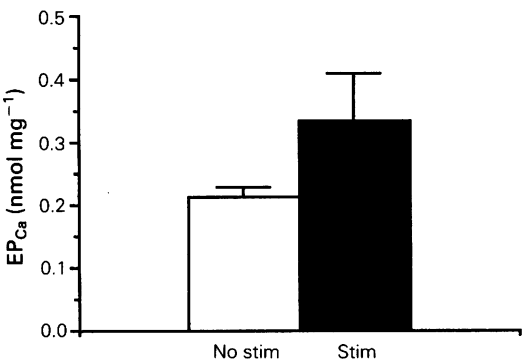


Figure 3 Histogram showing the mean (columns) and s.d. (vertical bars) of the accumulation of Ca at the endplate (EP_{Ca}) in hemidiaphragms incubated in ^{45}Ca-saline with ecothiopate (ECO) for 15 min and those stimulated in ECO. For further details see text.

However, the results are that the accumulation of Ca^{2+} at the endplate after stimulation was not different from that in the absence of stimulation. It is possible that this accumulation is not caused by action potentials in the nerve, or that the accumulation depends on the aggregate of evoked and spontaneous neural activity. Perhaps in these experiments the aggregate was similar in stimulated and unstimulated preparations because stimulation had changed the amount of spontaneous activity (see below). Therefore, to distinguish between the accumulation of Ca consequent upon spontaneous excitation of the nerves and that due to other mechanisms of the spontaneous release of transmitter, experiments were done in which action potentials in nerve and muscle were prevented by treatment with tetrodotoxin.

The effect of tetrodotoxin on the accumulation of Ca after ecothiopate

In this experiment three groups each of three diaphragms were used and incubated for 15 min in ^{45}Ca-saline to load the extracellular space and for a further 30 min in ^{45}Ca-saline only, or with added ECO, or in ^{45}Ca-saline and 10^{-6} M tetrodotoxin (TTX) throughout with ECO added for the final 30 min.

Those diaphragms incubated in ^{45}Ca-saline or in ^{45}Ca-saline with TTX and ECO did not fasciculate. Those incubated in ECO fasciculated and had a swollen endplate region at the end of the incubation. Some of the diaphragms in TTX and ECO were swollen at the endplate, but most seemed normal to the naked eye.

It was found that the accumulation at the endplate (EP_{Ca}) in ^{45}Ca-saline only was -0.147 ± 0.103 nmol mg^{-1}, in ^{45}Ca-saline and ECO was 0.361 ± 0.157 nmol mg^{-1} and in ^{45}Ca-saline, ECO and TTX was 0.13 ± 0.061 nmol mg^{-1} ($n = 6$). Each group was significantly different from each of the others, see Figure 4. The value obtained with incubation in ECO for 30 min was not different from that found in the experiments on the progress of accumulation and shown in Table 1.

It is concluded that TTX prevented about half the accumulation of Ca at the endplate and also prevented the fasciculation. Thus it seems that the fasciculation after ECO due to spontaneous excitation of the nerves (Ferry, 1988) may have been responsible for about half of the accumulation of Ca.

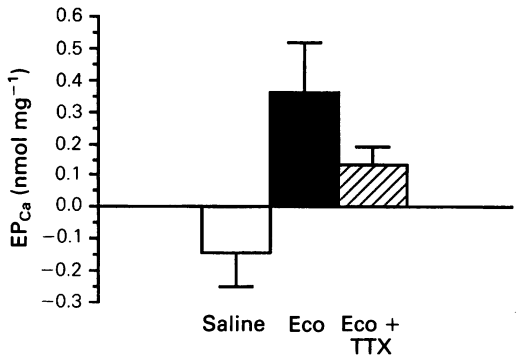


Figure 4 Histogram showing the mean (columns) and s.d. (vertical bars) of the accumulation of Ca at the endplate (EP_{Ca}) incubated for 30 min in ^{45}Ca-saline only, with added ecothiopate (ECO) and with ECO and tetrodotoxin (TTX). For further details see text.

Another mechanism which may have contributed to the accumulation at the endplate is the postsynaptic action of ACh released spontaneously either quantally or nonquantally.

The effect of carbachol on the accumulation of Ca

Although Evans (1974) reported an accumulation of Ca at the endplate after incubation with carbachol it was decided to repeat these experiments under the conditions in this laboratory. In these experiments, unstimulated diaphragms were incubated for 30 min in ^{45}Ca-saline containing carbachol 10^{-4} M. They were then washed in ^{45}Ca-free saline and prepared for assay of ^{45}Ca. These diaphragms were swollen in the endplate region. In five hemidiaphragms there was an accumulation of Ca at the endplate of 0.649 ± 0.142 nmol mg^{-1} which was significantly different from that found after incubation in physiological saline alone, and was not different from that found after incubation in ECO for the same time and without stimulation of the nerve (Table 1).

It is concluded that carbachol caused an accumulation of Ca at the endplate. This was about one third of that reported by Evans (1974) in his experiments with 5 mM $[Ca^{2+}]_o$. In our experiments the $[Ca^{2+}]_o$ was 2 mM. Evans showed that in 1 mM ACh, the accumulation of Ca at the endplate in 2 mM $[Ca^{2+}]$ was 0.36 of that in 5 mM. Thus our results are consistent with those published by Evans.

In another experiment with carbachol, ECO was included in the solution used to wash the diaphragms. The accumulation of Ca at the endplate in this experiment was 0.664 ± 0.337 nmol mg^{-1} and was not different from that found when the washing solution did not include ECO.

It is concluded that as ECO did not affect the EP_{Ca} of preparations preloaded by incubation in carbachol, it did not affect the efflux of Ca^{2+}. Thus the accumulation of ^{45}Ca in ECO was not due to prevention of efflux by ECO.

The effect of indirect stimulation on fasciculations after ecothiopate

In the experiments with ECO and with TTX, the accumulation of Ca at the endplate could be fractionated into that part due to a TTX-labile fasciculation and that due to a TTX-resistant process, possibly the action of spontaneously released m.e.p.ps and non-quantal ACh. One explanation of the finding that there was no difference in the accumulation of Ca at the endplate between stimulated and unstimulated preparations might be that in both preparations a similar spontaneous release of ACh caused a similar accumulation, and that in the stimulated preparation the aggregate of the fasciculations and nerve stimulation caused accumulation no different from the unstimulated preparation due to the fasciculations only. This might happen if stimulation inhibited fasciculation so that the reduction of accumulation due to this inhibition was equivalent to the gain attributable to stimulation of the nerve, resulting in no net change in the component of EP_{Ca} due to excitation of the nerve.

An experiment was done to investigate the effect on the fasciculations of stimulation of the nerve in three preparations. In these preparations, contractions at 0.1 Hz were recorded, the output of the force transducer being stored on an analogue storage oscilloscope with a 10 s sweep time. Several sweeps were superimposed, then ECO was added. Fasciculations appeared about 3 min later and persisted for about 30 min. In Figure 5 it can be seen that, although the discharge was irregular, after stimulation of the nerve there tended to be fewer spontaneous twitches. It is concluded that stimulation of the nerve decreased the overall amount of fasciculation. The reduction was not quantified so it is not possible to equate this reduction in fasciculatory synaptic activity with that extra activity due to stimulation.

The effect of elevated $[Mg^{2+}]$ on the accumulation of Ca after ecothiopate

The ECO-induced changes in the contractile response to nerve stimulation i.e. enhancement of the twitch due to repetitive firing of the muscle cells, a prolonged contraction at the endplate and fascicu-

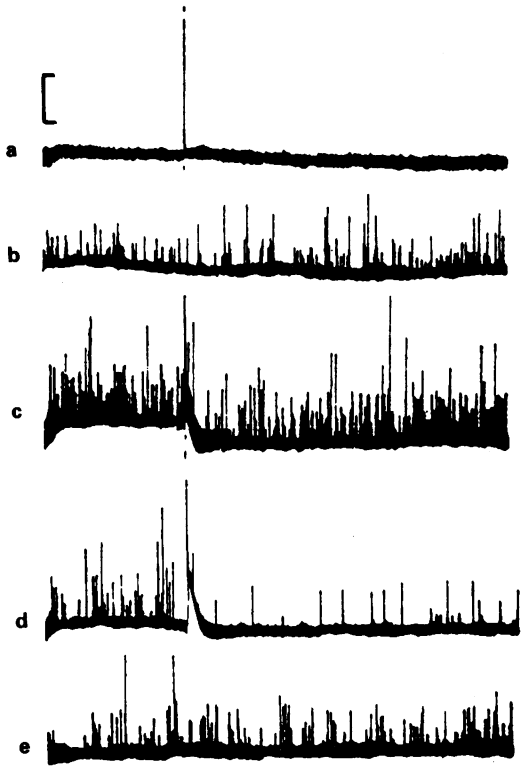


Figure 5 Mouse phrenic nerve-diaphragm preparation set up to display isometric tension on a storage oscilloscope, a.c.-coupled to minimize baseline drift, sweep time 95 s. Each trace is of several superimposed sweeps. Calibration 1 g. (a) Trace of 3 superimposed twitches elicited at 0.01 Hz. No ecothiopate (ECO). (b) Trace of 3 superimposed sweeps from 200 s to 500 s after ECO, no stimulation. Note spontaneous fasciculations. (c) Trace of 5 superimposed sweeps from 500 s to 1000 s after ECO with fasciculations and contractions evoked at 0.01 Hz. Note the prolonged localized contraction recorded differentiated as an elevated then depressed baseline, at which time there was a small reduction of the fasciculations. (d) Trace of 4 superimposed sweeps from 1000 s to 1400 s after ECO. Note the clear reduction of fasciculations after stimulation. (e) Trace of 9 superimposed sweeps from 1400 s to 2300 s after ECO, no stimulation. Note fairly even distribution of a generally reduced fasciculatory activity.

lation were not seen in experiments in which the saline contained 3.5 mM Mg^{2+} (Burd & Ferry, 1987), but m.e.p.ps were increased in amplitude and duration.

The effect of this concentration of Mg^{2+} on the accumulation of Ca after ECO was investigated. Five hemidiaphragms were incubated in 3.5 mM Mg^{2+} with ECO for 30 min, then they were prepared for assay of ^{45}Ca. It was found that the endplate accumulation of Ca (EP_{Ca}) was -0.003 ± 0.132 nmol mg^{-1} which was no different from that found in control preparations incubated in ^{45}Ca-saline for 30 min and was significantly less than that of preparations exposed to ECO in the physiological saline containing 1 mM Mg^{2+} (Table 1).

It is concluded that elevation of $[Mg^{2+}]_o$ prevented the accumulation of Ca at the endplate expected in the presence of ECO which exerted its anticholinesterase effect.

Discussion

There can be little doubt that there was accumulation of Ca derived from the bathing medium at the endplate after ECO, but our experiments do not provide direct evidence of the location of this accumulated Ca, which does not wash out and could have been sequestered by both presynaptic and postsynaptic structures. It is considered that the Ca may be sequestered intracellularly in the subsynaptic region of the muscle cells for the following reasons. If the Ca accumulated at the endplate were to have been sequestered by extracellularly-accessible structures, then it would be expected that such accumulation would have occurred in the absence of ECO, which is not the case. The role of ECO seems to be to make the site of Ca sequestration accessible from the extracellular space. Muscles incubated in ECO or in carbachol have a swollen endplate region. On microscopical examination, such regions show a considerable hypercontraction of the subsynaptic sarcomeres with swollen sarcoplasmic reticulum and mitochondria, an appearance attributable to Ca overload (Cullen & Ferry, 1982) with apparently normal nerve terminals.

Other workers have shown that Ca^{2+} enters the endplate of vertebrate skeletal muscle as a consequence of agonist action on the endplate channels (Jenkinson & Nicholls, 1961; Takeuchi, 1963; Evans, 1974; Miledi *et al.*, 1980). In our experiments with ECO the agonist is probably ACh released from the nerve terminals. The lack of effect of ECO on the washout of ^{45}Ca suggests it did not open channels permeable to Ca^{2+}. In the presence of an anticholinesterase the ACh acting on the endplate channels may have been released in several ways. The spontaneous release of ACh includes that released quantally which causes m.e.p.ps and that released non-quantally to cause a small depolarization of the endplate region equivalent to that produced by about 10^{-8} M ACh in the frog (Katz & Miledi, 1977), 10^{-7} M in mice (Vyskočil *et al.*, 1983) and 6×10^{-9} M in rats (Smith, 1984). In unstimulated tissues, the amount released non-quantally from the nerve is about ten times greater than that released quantally (Miledi *et al.*, 1983). The evoked release is quantal

and gives rise to the endplate potentials; included in this category is the ACh released by excitation of the nerve terminal during the fasciculations which arise after ECO. The non-quantal release of ACh is not dependent on Ca^{2+} and is not affected by TTX (Vizi & Vyskočil, 1979; Doležal & Tuček, 1983; Stanley & Drachman, 1983) and is from the cytoplasm of the nerve (Molenaar *et al.*, 1987).

The experiments with TTX showed that about half of the EP_{Ca} persisted and was associated with a local hypercontraction of the cell. This suggests that the m.e.p.ps and the non-quantal release together could account for about half of the accumulation of Ca at the endplate, the other half presumably being due to transmitter action following excitation of the nerve.

The experiments with stimulation of the nerve indicate that the accumulation at the endplate (EP_{Ca}) in preparations which were fasciculating and were stimulated was not different from those which only fasciculated. Taken with the experiments which showed a reduction of fasciculation after stimulation of the nerve it may be concluded that the responses evoked at 0.02 Hz which usually were associated with prolonged localized contractions at the endplate were responsible for some of the accumulation of Ca although this cannot be quantified. This conclusion is supported by experiments with more vigorous stimulation of the nerve by trains of 5 pulses at 50 Hz every 5 s in which the Ca content of the endplate assayed by atomic absorption spectrophotometry was elevated 5 min after ECO (Ferry, unpublished).

The result of the experiments with 3.5 mM $[Mg^{2+}]_o$ was that the accumulation of Ca at the endplate was reduced to zero. Half of the reduction is attributable to the prevention of fasciculation, as in the experiments with TTX. The remainder of the reduction of Ca accumulation may be due to prevention of the effects of ACh released non-quantally and quantally, by competition between Mg^{2+} and Ca^{2+} in the synaptic cleft reducing Ca^{2+} entry through the opened endplate channels (Adams *et al.*, 1986). In this regard, Jenkinson & Nicholls (1961) and Takeuchi (1963) reported abolition by Mg^{2+} of the local contracture normally found after ACh. Evans (1974) found that an increased $[Mg^{2+}]_o$ reduced the accumulation of Ca in the junctional region. Because ACh is released nonquantally in larger amounts than that causing m.e.p.ps, despite the increased frequency of the latter (Laskowski & Dettbarn, 1975), it may be that the nonquantal release of ACh is the most important spontaneous process in the accumulation of Ca at the endplate.

The accumulation at the endplate EP_{Ca} in 10^{-4} M carbachol is similar to that found after ECO, which suggests that the carbachol was about twice as effective as the non-quantally released ACh in loading the endplate region with Ca, even though the concentration of carbachol was 10^3–10^4 times that estimated for non-quantally released ACh. This inconsistency may be explained if the effect of carbachol were to have been limited by desensitization of the endplate cholinoceptors. In our experiments with ECO, m.e.p.ps were present, but the m.e.p.ps of rat diaphragm 'promptly disappeared' in the presence of 10^{-4} M carbachol (Creese & Mitchell, 1981), and the endplate current due to carbachol applied to guinea-pig diaphragm declined rapidly over the first 5 min (Creese *et al.*, 1987). Furthermore, Bray *et al.* (1982) found that m.e.p.ps were unaffected by 10^{-7} M carbachol but reduced by 10^{-6} M. Thus it is considered that the accumulation of Ca after carbachol was limited by desensitization and the accumulation due to non-quantal release of ACh after ECO was not limited to the same extent.

To conclude, although stimulation of the nerve after ECO caused an additional accumulation of Ca at the endplate which may or may not have been involved in the prolonged contraction at the end plate in our experiments with a very low frequency (0.02 Hz) of stimulation, the action of non-quantally released ACh is responsible for about half of the total accumulation at the endplate.

It may be that this accumulation of Ca is associated with the structural changes at the endplate in anticholinesterase-induced myopathy, in particular that after ECO reported briefly by Cullen & Ferry (1982).

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Kallikrein-kinin system in the plasma of the snake *Bothrops jararaca*

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1 *Bothrops jararaca* venom (BJV) caused a fall in the carotid artery blood pressure of the anaesthetized snake. This effect was tachyphylactic and was potentiated by captopril, a kininase II inhibitor; it was partially antagonized by promethazine plus cimetidine and was not affected by atropine.

2 Similar hypotensive effects were obtained by administration of trypsin or a partially purified BJV kininogenase to the snake.

3 Incubation of *Bothrops jararaca* plasma (BJP) with trypsin released a substance (or substances) that produced hypotension in the snake but not in the rat; this hypotensive effect was also potentiated by captopril.

4 The trypsinised plasma contracted *Bothrops jararaca* isolated uterus, a pharmacological preparation weakly sensitive to bradykinin. Trypsinised plasma was inactive on pigeon oviduct and rat uterus and displayed a weak action on the guinea-pig ileum. Similar effects were observed with incubates of a fraction of BJP, containing globulins, with a partially purified BJV kininogenase.

5 Like mammalian kinins, the substance(s) was(were) dialysable, thermostable in acid but not in alkaline pH, and inactivated by chymotrypsin but not by trypsin. Its(their) inactivation by BJP or BJP kininase II was inhibited by captopril.

6 These findings strongly suggest that, besides releasing histamine, BJV or trypsin release a kinin-like substance (or substances) from the snake plasma.

7 Since BJV and other kininogenases active on mammalian plasmas were shown to be unable to release kinins from BJP, in experiments conducted on pharmacological preparations suitable for the assay of mammalian kinins, these data also suggest that the snake *Bothrops jararaca*, like birds, may have developed its own kallikrein-kinin system.

Introduction

It is well known that the venom of *Bothrops jararaca* (Serpentes, Crotalinae) or trypsin decreases blood pressure of various mammalian species as a consequence of the release of histamine and bradykinin (Rocha e Silva *et al.*, 1949; Suzuki & Iwanaga, 1970). However, experiments conducted on pharmacological preparations suitable for the assay of mammalian kinins have shown that *Bothrops jararaca* venom, trypsin, and some other kininogenases, active on mammalian plasmas, were unable to release kinins from *Bothrops jararaca* plasma. After treating this plasma, in order to inhibit or destroy kininases, no evidence could be found that it contained either active factor XII (Hageman factor), prekallikrein or kininogen. Only two components of the kallikrein-kinin system were detected in *Bothrops jararaca* plasma: kininases and kininogenase inhibitors (Lavras *et al.*, 1979).

Similarly, avian plasma does not possess factor XII (Ratnoff, 1979), a factor that is involved in the activation of the blood clotting, the fibrinolytic, the C-1 complement and the kallikrein-kinin systems. However, avian plasma contains all the other components of the latter system, since a kinin is released when avian plasma is shaked with glass beads containing adsorbed mammalian or alligator factor XII (Erdös *et al.*, 1967; Seki *et al.*, 1973). In addition, Werle & Hürter (1936), Erdös *et al.* (1967), Werle & Leysath (1967) and Seki *et al.* (1973) reported that a kinin liberated from chicken plasma by pancreatic kallikrein of birds (ornitho-kallikrein) was different from mammalian kinins, in terms of its amino acid

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composition and pharmacological activity. This was an indication that birds possessed their own kallikrein-kinin system. Recently, Kimura *et al.* (1987) have purified ornitho-kininogen from chicken plasma and have determined the primary structure of the kinin liberated from this substrate by bovine plasma kallikrein as being Arg-Pro-Pro-Gly-Phe-Thr-Pro-Leu-Arg. This peptide, a little different from bradykinin, induced a contraction of chicken smooth muscle and had a strong hypotensive effect in the chicken, but did not contract the rat isolated uterus.

Therefore, it was thought worthwhile to investigate the possibility of the occurrence of a kallikrein-kinin system specific for snakes not revealed by pharmacological preparations responsive to mammalian kinins. To verify such a possibility, this work started with the study of the effects of *Bothrops jararaca* venom or trypsin on the snake's own blood pressure. Further, the action of the product(s) released by incubating *Bothrops jararaca* venom or trypsin with *Bothrops jararaca* plasma was then investigated on the snake's own blood pressure and on isolated uterus.

Methods

The snakes (*Bothrops jararaca*, Serpentes, Viperidae, Crotalinae) (BJ) used in this work were collected from nature. After a quarantine of at least 15 days, they were maintained under controlled environmental conditions with a photoperiod of 12 h of light and 12 h of darkness, at a temperature of 26°C and 65% relative humidity.

Bothrops jararaca *venom* (*BJV*)

This was a pool of samples of vacuum dried venom from the Butantan Institute and contained 870 μg protein mg^{-1} of dry powder, presenting a LD_{50} equal to 2.1–2.5 μg protein g^{-1} of mouse, injected intraperitoneally. A fraction (VF) was obtained from this pool of venom, by 0.60–0.90 ammonium sulphate saturation and contained a two–three times purified kininogenase, active on rat carotid artery blood pressure.

Bothrops jararaca *plasma* (*BJP*)

Blood was collected from decapitated snakes, into polyethylene tubes containing heparin (10 iu ml^{-1} of blood) or sodium citrate (3.8 mg ml^{-1} of blood) as anticoagulant and immediately centrifuged at 8.000 r.p.m., for 30 min at 5–10°C. The resulting plasma was used fresh or after storage at −20°C.

A fraction GF, containing globulins, was obtained from this plasma by 0.0–0.5 ammonium sulphate saturation.

A purified kininase II (ACE), hydrolysing 83.3 μg bradykinin min^{-1} ml^{-1} was obtained from BJP by 0.60–0.90 ammonium sulphate saturation (Lavras *et al.*, 1980).

Pigeon plasma (*PP*)

Blood was collected from decapitated pigeons, into polyethylene tubes containing sodium citrate (3.8 mg ml^{-1} of blood). After centrifugation at 2500 r.p.m., for 15 min at 5–10°C, the plasma obtained was used fresh or after storage at −20°C.

Blood pressure recording

The carotid artery blood pressure of adult male rats (280–300 g) and of adult male or female BJ (150–600 g), anaesthetized with sodium pentobarbitone (90 mg kg^{-1}, for rats and 30 mg kg^{-1}, for snakes), i.p., was recorded on a smoked drum, with the aid of a mercury manometer. Administration of drugs was made through a polyethylene catheter introduced into the iliac vein of the rats or into the renal vein of the snakes. As the snake preparations were set up for well over 60 min, standard doses of angiotensin II (1.5 μg kg^{-1}), bradykinin (300 μg kg^{-1}) or histamine (50 μg kg^{-1}) were administered from time to time and at the end of the experiments, in order to monitor the sensitivity of the assay system.

Isolated smooth muscle preparations

Smooth muscles isolated from mammals, birds and snakes were used in the experiments *in vitro*. The rat uterus was prepared according to Gaddum & Hameed (1954) and the guinea-pig ileum as described by Henriques *et al.* (1962).

The non-atrophied oviduct of adult pigeons was removed and immediately suspended in a 10 ml chamber containing Ringer-Locke solution aerated and maintained at 42°C (Crossley *et al.*, 1980). Contractions were recorded on a smoked drum with the aid of an isotonic lever having a 6 fold magnification under a load of 0.5–1.0 g.

Pieces of the uterus (3–4 cm long) of *Bothrops jararaca* were removed from decapitated and exsanguinated snakes (150–300 g) and suspended in a 10 ml bath containing Ringer solution for snakes (Beyenback, 1984) at 30°C. Contractions were recorded under a load of 1.0–1.5 g, as described for the pigeon oviduct.

Release of active substance(s) by incubation

This was achieved by incubating either the GF fraction of BJP with VF, in the conditions described by Hamberg & Rocha e Silva (1957) or BJP with trypsin, according to Diniz & Carvalho (1963). The

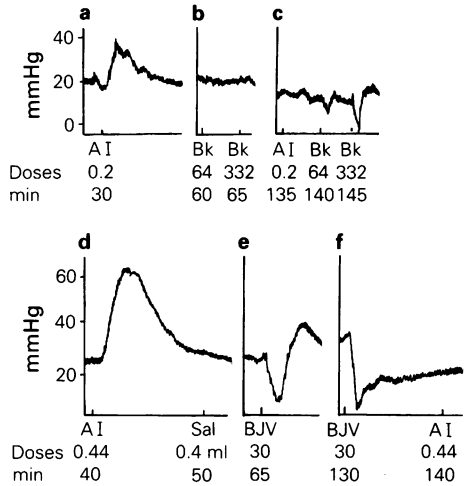


Figure 1 Action of *Bothrops jararaca* venom (BJV), bradykinin (BK) and angiotensin I (AI) on the snake's own carotid artery blood pressure. Female snakes (170 g, above and 140 g, below) were anaesthetized with pentobarbitone (30 mg kg^{-1}) i.p. and heparinized (400 iu kg^{-1}). Captopril (0.1 mg kg^{-1}) was administered at 70 min, between (b) and (c) or (e) and (f). Angiotensin II (1.5 μg kg^{-1}) produced constant effects throughout the experiment. Bk (doses in μg kg^{-1}) caused hypotension only after the administration of captopril. This drug inhibited plasma kininase II or AI-converting enzyme, since the response to AI (doses in μg kg^{-1}) disappeared following its administration. In such conditions, the hypotension caused by BJV (in mg kg^{-1}) is prolonged. Sal, 0.9% NaCl solution.

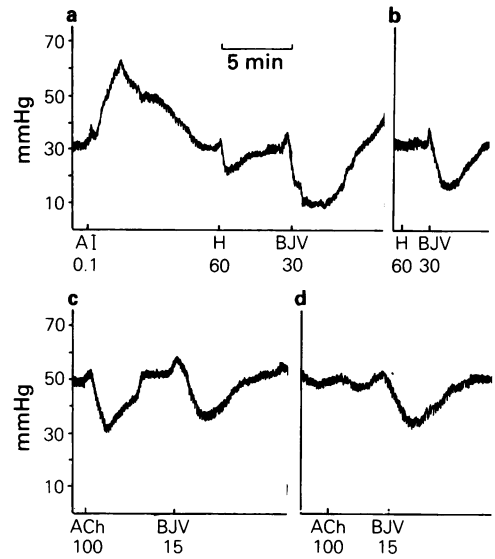


Figure 2 Effect of histamine antagonists and atropine upon the hypotension caused by *Bothrops jararaca* venom (BJV) on the same species of snake: (a) and (b) female snake (130 g); (c) and (d) male snake (120 g). Same conditions as in Figure 1. Promethazine (4 mg kg^{-1}) and cimetidine (15 mg kg^{-1}) were simultaneously administered between (a) and (b); atropine (1 mg kg^{-1} s.c. and 1 mg kg^{-1} i.v.) was injected between (c) and (d); AI – angiotensin I (doses in μg kg^{-1}). Responses to angiotensin II (1.5 μg kg^{-1}), were constant throughout the experiment.

The hypotension caused by BJV (in mg kg^{-1}) was partially blocked by the simultaneous administration of the histamine (H, in μg kg^{-1}) antagonists. Atropine did not affect the hypotension caused by the venom, in a dose that completely blocked acetylcholine (ACh, in μg kg^{-1}).

released substance(s) was(were) dried, diluted in 2 ml of 0.9% NaCl and assayed on BJ carotid artery blood pressure and rat or BJ uterus. When trypsin was incubated with BJP, the substance(s) released was(were) also tested on pigeon oviduct and guinea-pig ileum.

Pigeon plasma was incubated with trypsin according to Diniz & Carvalho (1963) and the released substance was assayed on pigeon oviduct.

Properties of the released substance(s)

The released substance(s) was(were) submitted to the following five procedures: (a) dialysis against 0.9% NaCl, at 4°C, for 24 h; (b) heating in a boiling water bath either at pH 10–11, for 5 and 10 min or at pH 1.5–2.0, for 30 and 60 min (Prado *et al.*, 1956); (c) incubation with trypsin (110 μg ml^{-1}) or chymotrypsin (37 μg ml^{-1}) (Prado *et al.*, 1956); (d) incubation at 37°C, for 15 min, with either 0.3 ml of 25 fold diluted BJP or 0.15 ml ACE and 0.2 or 0.05 ml of 0.05 M Tris-maleate buffer pH 7.4, the reaction being interrupted with boiling Ringer for snakes (1.5 or 0.8 ml respectively); (e) experiments similar to those described in (d) but BJP or ACE previously treated with captopril (170 μg ml^{-1} for BJP or 13.5 μg ml^{-1} for ACE) was used.

Drugs and reagents

The following drugs and reagents were used: sodium pentobarbitone, a gift from Abbott Laboratories of Brazil Ltd.; sodium heparin (Liquemine, Roche Laboratories, Brazil); promethazine hydrochloride (Phenergan, Rhodia Laboratories, Brazil); cimetidine (Tagamet, SKF Enila Ltd., Brazil); captopril (Capoten, SQ 14225), kindly supplied by Squibb Laboratories, Brazil; angiotensin I and bradykinin triacetate, synthesized at the Department of Biophysics, Escola Paulista de Medicina, Brazil; angiotensin II (Ciba Laboratories, Switzerland); hista-

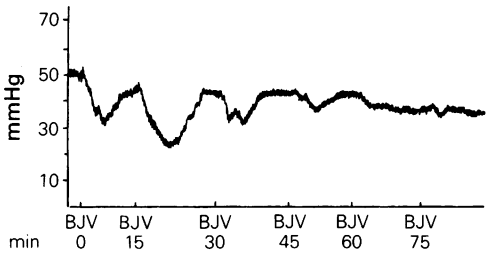


Figure 3 Tachyphylaxis to the effect of *Bothrops jararaca* venom (BJV) on the snake's blood pressure. Female snake (170 g); same conditions as in Figure 1. Responses to angiotensin II (1.5 $\mu g\,kg^{-1}$), histamine (50 $\mu g\,kg^{-1}$) and bradykinin (300 $\mu g\,kg^{-1}$), at the beginning and at the end of the experiment, were similar.

The hypotension caused by BJV diminished or even disappeared when the same dose of venom (30 $mg\,kg^{-1}$) was given repeatedly within a short interval of time.

mine free-base, acetylcholine hydrochloride, bovine pancreas trypsin (2 × crystallized, type III) and chymotrypsin (3 × crystallized, type II), (Sigma Chem. Co., U.S.A.); atropine sulphate, calcium chloride and dipotassium phosphate (E. Merck, Germany); monopotassium phosphate (J.T. Baker, U.S.A.); sodium citrate (Ecibra, Brazil).

Results

Effects of BJV, BJV kininogenase or trypsin on the snake blood pressure

As may be seen in Figure 1, BJV caused a fall in the carotid artery blood pressure of the snake, in doses as high as 10–40 $mg\,kg^{-1}$. A dose of 15 $mg\,kg^{-1}$ was equivalent to 60 $\mu g\,kg^{-1}$ of histamine or 100 $\mu g\,kg^{-1}$ of acetylcholine in producing a 20 mmHg drop in blood pressure. BJV produced a similar effect in the rat, with doses of 3–5 $mg\,kg^{-1}$. In the rat, doses of 3–6 $\mu g\,kg^{-1}$ of bradykinin produced a hypotensive effect, whereas this polypeptide showed weak effects or was even inactive on BJ blood pressure.

The hypotension caused by BJV lasted longer if the snake was treated beforehand with captopril (0.05–0.10 $mg\,kg^{-1}$), an inhibitor of angiotensin I-converting enzyme or kininase II, which in such

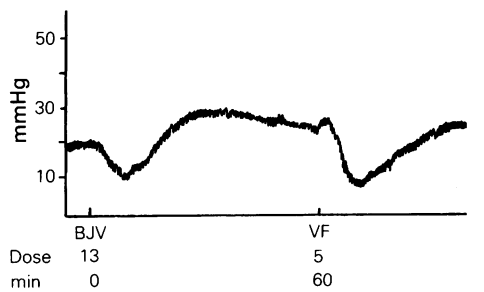


Figure 4 Effect of *Bothrops jararaca* venom (BJV) and of a partially purified venom kininogenase (VF) on the snake carotid artery blood pressure. Female snake (180 g), was anaesthetized and heparinized and treated with captopril (0.1 $mg\,kg^{-1}$), promethazine (4 $mg\,kg^{-1}$) and cimetidine (15 $mg\,kg^{-1}$). VF is a two–three times purified kininogenase obtained from BJV by 0.60–0.90 ammonium sulphate saturation. Angiotensin II (1.5 $\mu g\,kg^{-1}$) was used to check the sensitivity of the preparation. Equipotent effects were produced by BJV (in mg protein kg^{-1}) and a dose of the kininogenase almost three times smaller.

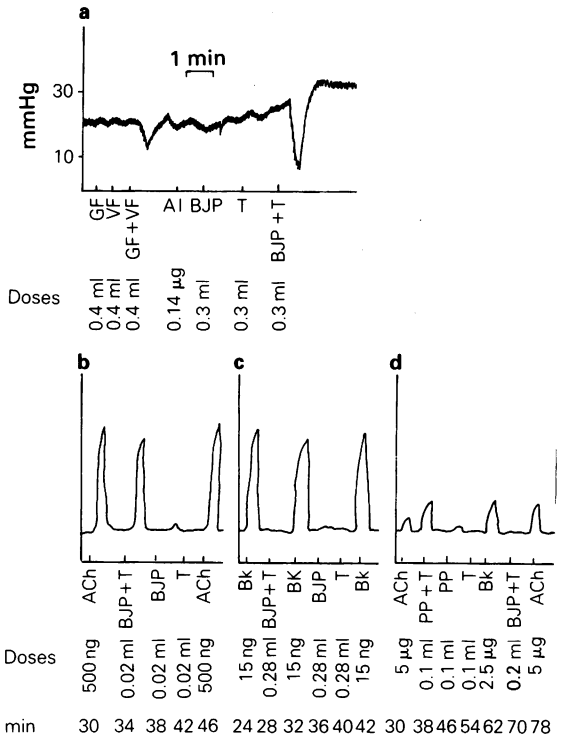


Figure 5 Effect of the substance(s) released by incubating either *Bothrops jararaca* plasma (BJP) with trypsin (T) or a fraction of BJP containing globulins (GF) with VF, a two–three times purified *Bothrops jararaca* venom kininogenase, upon the own snake's blood pressure (a) and isolated uterus (b), the rat uterus (c) and the pigeon oviduct (d). AI – angiotensin I; ACh – acetylcholine. The female snake (150 g), anaesthetized and heparinized, used for recording blood pressure, was treated beforehand with captopril (0.05 $mg\,kg^{-1}$) and gave normal responses to angiotensin II (1.5 $\mu g\,kg^{-1}$). The released substance(s), dried and diluted in 2 ml of 0.9% NaCl, was hypotensive in the snake, contracted the snake uterus but was inactive on rat uterus and pigeon oviduct. This last preparations contracted after addition of the incubate of pigeon plasma (PP) with T.

doses blocked the angiotensin I pressor response and allowed bradykinin to produce hypotension in doses that had previously been inactive (Figure 1).

After the simultaneous administration of promethazine ($4\,mg\,kg^{-1}$) and cimetidine ($15\,mg\,kg^{-1}$) to BJ, the effect of BJV was reduced. It was not modified by pretreating the snake with atropine ($2\,mg\,kg^{-1}$) (Figure 2).

As in mammals, the hypotension caused by BJV diminished or even disappeared (Figure 3) when the same dose of venom was given repeatedly to the snake within a short period of time.

Figure 4 shows that VF, a fraction of BJV containing a two–three times purified kininogenase, produced a hypotensive effect in the snake similar to that obtained with a dose of BJV two–three times greater. This effect was also potentiated by captopril ($0.05–0.10\,mg\,kg^{-1}$); it was diminished after the administration of promethazine ($4\,mg\,kg^{-1}$) plus cimetidine ($15\,mg\,kg^{-1}$) and was not modified by atropine ($2\,mg\,kg^{-1}$).

Administration of trypsin ($4\,mg\,kg^{-1}$) to BJ also caused hypotension, partially antagonized by promethazine plus cimetidine ($4\,mg\,kg^{-1} + 15\,mg\,kg^{-1}$) and not modified by atropine ($2\,mg\,kg^{-1}$).

Release of active substance(s) by incubation of GF with VF or BJP with trypsin

Incubation of GF, a fraction of BJP containing globulins, with VF, under the conditions used by Hamberg & Rocha e Silva (1957) for the liberation of kinins, resulted in the release of a substance (or substances) that caused hypotension in BJ (Figure 5). This figure also shows that a substance (or substances) with similar effect was(were) obtained by incubating BJP with trypsin, under the conditions used by Diniz & Carvalho (1963) for the determination of kininogen in various animal species. The hypotension caused by this(these) substance(s) in BJ was potentiated by a previous treatment of the snake with captopril ($0.05–0.10\,mg\,kg^{-1}$) (Figure 6). The substance(s) relased was(were) inactive on rat blood pressure.

This(these) substance(s) contracted BJ uterus (Figure 5), a preparation that does not respond to bradykinin in concentrations up to $200–300\,ng\,ml^{-1}$. The released substance(s) was(were) inactive on pigeon oviduct and rat uterus (Figure 5), but in high doses caused a weak contraction of the guinea-pig ileum. While rat uterus and guinea-pig ileum are known to react to very low doses of bradykinin, pigeon oviduct is less sensitive but contracts under the influence of the substance released by incubating pigeon plasma with trypsin.

The substance(s) released in the incubation of BJP with trypsin was(were) dialysable, showed thermostability in acid pH but not in alkaline pH. Furthermore, it(they) was(were) inactivated by chymotrypsin, BJP or ACE but not by trypsin. The inactivation by BJP or ACE was inhibited by previous incubation of BJP or ACE with captopril (Table 1).

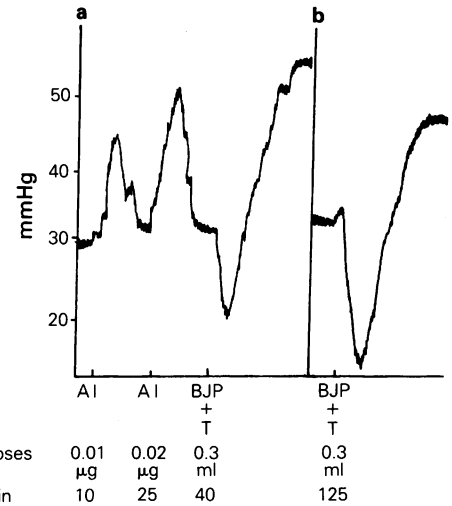


Figure 6 Effect of captopril upon the hypotension caused by the substance(s) released in the incubation of *Bothrops jararaca* plasma (BJP) with trypsin (T). Male snake (170 g) anaesthetized and heparinized; between (a) and (b), at 60 min, captopril ($0.1\,mg\,kg^{-1}$) was administered. Responses to angiotensin II ($1.5\,\mu g\,kg^{-1}$) were constant throughout the experiment. AI – angiotensin I. The hypotension produced by the released substance(s) was greater after inhibition of AI-converting enzyme by captopril.

Discussion

In mammals, small doses of venoms extracted from *Bothrops jararaca* and other snakes from the same genus mainly produce haemorrhage and hypotension (Suzuki & Iwanaga, 1970), this last effect being attributed to the release of histamine and bradykinin (Feldberg & Kellaway, 1937a,b; Rocha e Silva *et al.*, 1949) by the venoms.

In the *Bothrops jararaca* itself, BJV also caused hypotension. The decrease or even disappearance of this hypotensive effect after repeated administration of venom to the snake does not seem to be due to tachyphylaxis at receptor level since, after the disappearance of the effect, the preparation reacts to standard doses of acetylcholine, histamine or bradykinin in the same degree as before the first dose of venom. This fact suggests that, as in mammals, the venom is acting indirectly, releasing hypotensive substances

Table 1 Properties of the substance(s) released by incubating *Bothrops jararaca* plasma (BJP) with trypsin and those of bradykinin

Procedure	*Temperature* (°C)	*pH*	*Time*	*Released substance(s)*	*Bradykinin*
Dialysis against 0.9% NaCl	4	6.5	24 h	dial.	dial.
Heating	98	1.5–2.0	30–60 min	stable	stable
		10.0–11.0	5–10 min	unst.	unst.
Incubation with:					
Chymotrypsin (37 $\mu g\,ml^{-1}$)	37	6.5	5 min	inact.	inact.
Trypsin (110 $\mu g\,ml^{-1}$)	37	6.5	60 min	resist.	resist.
BJP (1:25)	37	7.4	15 min	inact.	inact.
ACE*	37	7.4	15 min	inact.	inact.
BJP (1:25) + Capt† (170 $\mu g\,ml^{-1}$)	37	7.4	15 min	resist.	resist.
ACE + Capt (13.5 $\mu g\,ml^{-1}$)	37	7.4	15 min	resist.	resist.

dial. – dialysable; unst. – unstable; inact. – inactivated; resist. – resistant.
*ACE – a purified kininase II, hydrolysing 83.3 μg bradykinin $min^{-1}\,ml^{-1}$, obtained from BJP.
†Capt – captopril (*Capoten.* SQ 14225).

and exhausing plasma substrates. Acetylcholine is not involved in this effect since it is not affected by atropine. As the duration of the hypotension decreased after the simultaneous administration of promethazine and cimetidine, H_1 and H_2 receptor blockers respectively, the release of histamine has to be considered. However, as these blockers antagonized the effect of the venom only partially, the simultaneous release of some other hypotensive substance(s) besides histamine is suggested. Taking into account the high kininase and antikininogenase activities detected in BJP (Lavras *et al.*, 1979) and the high doses of BJV necessary to produce hypotension in BJ, a kinin-like substance may be supposed to be released. It is noteworthy that high doses of bradykinin were also necessary to produce hypotension in BJ, an effect that was only achieved if the snake had previously been treated with captopril, a powerful kininase II inhibitor. The prolongation of the effect of the venom on the snake by captopril also indicates the release of a kinin-like substance.

This hypothesis is reinforced by the data showing that doses of VF two–three times smaller than those of BJV, produced similar hypotensive effects in BJ. VF contained a two–three times purified kininogenase, assayed on the rat blood pressure. In addition trypsin, a known kininogenase (Prado, 1970), caused effects in BJ, similar to those produced by BJV.

In order to confirm such a hypothesis, some *in vitro* experiments were conducted which showed that incubates of BJP or one of its fractions containing globulins, with trypsin or VF, respectively, contained a substance (or substances) that was(were) practically inactive on rat blood pressure and uterus, on pigeon oviduct and on guinea-pig ileum but that was(were) hypotensive in BJ and caused contraction of BJ uterus. Like kinins (Prado, 1970), this(these) substance(s) was(were) dialysable, thermostable at acid but not at alkaline pH, inactivated by chymotrypsin and BJ kininases but not by trypsin. Like bradykinin, the inactivation of this(these) released substance(s) by BJ kininases was inhibited by the kininase II inhibitor captopril.

All these findings strongly suggest that, as in birds (Seki *et al.*, 1973), a kinin-like substance (or substances) is(are) released in BJ by its own venom kininogenase or trypsin, a fact indicative of the presence of kininogen in the snake plasma. Furthermore, the data here presented seem to indicate that BJ has actually developed its own kallikrein-kinin system, inactive in mammals and birds and therefore, not revealed in pharmacological preparations suitable for the assay of mammalian kinins, as those used by Lavras *et al.* (1979).

The purification and characterization of the released substance(s), attempts to activate the intrinsic kallikrein-kinin system in *Bothrops jararaca* plasma and the extension of these studies to other snake species must be carried out, in order to conclude if snakes, in general, possess a specific kallikrein-kinin system.

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The pharmacological evaluation of LY170680, a novel leukotriene D_4 and E_4 antagonist in the guinea-pig

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1 This paper describes the evaluation of LY170680 (5-<3-[2(**R**) (carboxyethylthio)-1(S)-hydroxypentadeca 3(E)5(Z)-dienyl]phenyl > 1H tetrazole, as an antagonist of the cysteinyl leukotrienes C_4, D_4, E_4, in a variety of *in vitro* and *in vivo* models.

2 *In vitro*, LY170680 was a potent, selective, and competitive antagonist of LTD_4, and LTE_4. It produced a concentration-dependent rightward displacement of the concentration-response curves elicited by either LTD_4 or LTE_4 on both the guinea-pig ileal and tracheal preparation. The pA_2 values for LY170680 were estimated to be 8.1 ± 0.2 ($n = 8$) and 8.1 ± 0.1 ($n = 6$) on trachea, and 8.7 ± 0.1 ($n = 12$) and 9.0 ± 0.3 ($n = 6$) on ileum for both LTD_4 and LTE_4 respectively. The slopes of the Schild plots in these studies were all close to unity.

3 LY170680 was shown to be a modest antagonist of the LTC_4-induced responses on guinea-pig ileum (pA_2 7.0 ± 0.2 $n = 5$), but had no discernable effects against contractions induced by histamine, prostaglandin E_2 (PGE_2), $PGF_{2\alpha}$ or acetylcholine. The compound also reduced the LTC_4-induced responses on trachea, but in a non competitive manner.

4 Intravenous LY170680 reduced in a dose-dependent manner (ED_{50} $3.8\,mg\,kg^{-1}$, 60 min pretreatment) the fall in compliance in the anaesthetized guinea-pig, and the rise in total pulmonary resistance (TPR) (ED_{50} $2.0\,mg\,kg^{-1}$, 30 min pretreatment) in the artificially ventilated guinea-pig, produced by intraveous LTD_4.

5 LY170680 ($5\,mg\,kg^{-1}$, i.v.) was also effective in reducing the increase in TPR to intravenous antigen in sensitized animals pretreated with mepyramine, indomethacin and propranolol.

6 The compound was only moderately effective (ED_{50} $40\,mg\,kg^{-1}$ p.o.) in preventing the rise in TPR induced by intravenous LTD_4, when given orally.

7 Inhaled LY170680 was particularly effective in preventing the increase in TPR produced by an exposure to aerosolised LTD_4. An estimated 1–2 μg of LY170680 delivered to the airways by nebuliser 1 hour beforehand, produced a 6 fold lateral displacement to the right of the dose-response curve to LTD_4.

8 Studies in conscious animals indicated that inhaled LY170680 produced a dose-dependent reduction in the increased volume of gas, trapped in the lung following exposure to aerosolised LTD_4. Duration studies also indicated that an estimated inhaled dose of 10 μg LY170680 significantly reduced the LTD_4-induced' increase in trapped lung gas volume for at least 4 h. Inhaled LY170680 also reduced LTC_4-induced increase in gas trapping in a manner similar to LTD_4. However, histamine-induced gas trapping was unaffected.

Introduction

The cysteinyl leukotrienes are a group of related compounds (leukotriene C_4 (LTC_4), LTD_4, LTE_4) believed to be important mediators in the pathogenesis of asthma (Lewis *et al.*, 1982). They are released from human lung by immunological and non-immunological stimuli, and their pharmacology resembles many of the pathophysiological features of the disease (Samuelsson, 1983). Leukotrienes are potent bronchoconstrictor substances in man, being significantly more potent than histamine. In addition they interfere with mucus clearance and cause oedema (Holroyde *et al.*, 1981; Weiss *et al.*, 1982; Marom *et al.*, 1982; Woodward *et al.*, 1983). Consequently the pharmacological intervention of the actions of leukotrienes in the airways may provide therapeutic benefits in the treatment of asthma.

This study describes the pharmacology of (5-<3-[2(**R**) (carboxyethylthio)-1(S)-hydroxypentadeca

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3(E)5(Z)-dienyl]phenyl > 1H tetrazole (LY170680), a potent inhaled leukotriene antagonist, which was found to be effective in preventing both leukotriene- and antigen-induced bronchospasm in guinea-pigs.

Methods

Guinea-pig ileum

Male Dunkin-Hartley guinea-pigs (350–450 g) were killed by cervical dislocation and a portion of terminal ileum removed. Short segments of ileum (2 cm) were suspended in a 5 ml organ bath containing Tyrode solution at 37°C aerated with 95% O_2 and 5% CO_2. The ileum was equilibriated for approximately 1 h under a maintained resting tension of 0.5 g.

Guinea-pig trachea

The trachea was removed from a freshly killed male Dunkin-Hartley guinea-pig (350–450 g) and cut longitudinally to expose the strip of muscle. The preparation was cut into four segments across the muscle. Each segment was suspended in a 25 ml organ bath by attaching, with thread, to a stationary steel rod and to a Harvard isometric force displacement transducer. The tissue bath contained Krebs solution maintained at 37°C and aerated with 95% O_2 and 5% CO_2. The preparations were equilibrated for approximately 2 h under a maintained resting tension of 2 g before drugs were tested. Trachea were pretreated with indomethacin (3×10^{-6} M) to reduce the formation of prostaglandins and thromboxane (Orehek *et al.*, 1973; Weichman *et al.*, 1982).

Concentration-response curves to LTC_4 on ileum were performed in the presence of FPL55712 (5×10^{-6} M). This level of FPL55712 was used to block the LTD_4/E_4 receptor, thus eliminating the biological consequence of any conversion of LTC_4 to LTD_4 during the studies. Experiments with LTC_4 on trachea were conducted in the presence of acivicin (3×10^{-5} M) a gamma glutamyl transpeptidase inhibitor (Ibe & Campbell, 1988). FPL55712 was used on ileum as gamma glutamyl transpeptidase inhibitors such as serine-borate, and acivicin tended to affect the contractivity of the preparation. Experiments with LTD_4 on trachea were performed in the presence of L-cysteine (3×10^{-3} M) to prevent conversion of LTD_4 to LTE_4.

Cumulative concentration-response curves to agonists were generated for both tissues and the effects of different concentrations of LY170680 investigated. Only one concentration-response curve was generated per preparation. The responses were expressed in terms of percentage of maximal response, in an attempt to reduce inter-tissue variation.

The dissociation constant (K_B) of the receptor-inhibitor complex was calculated by the method of Furchgott (1972) using the following equation:

$$K_B = \frac{[\text{antagonist}]}{\text{Dose-ratio} - 1}$$

Dose-ratio refers to the concentration of agonist required to elicit 50 percent of the maximum response (ED_{50}) in the presence of the antagonist, divided by the ED_{50} in the absence of the antagonist. This formula can only be used when agonist and antagonist are competitive with one another. Such a relationship exists when the K_B values are independent of the antagonist concentration and the slope of the Schild plot (Arunlakshana & Schild (1959), log (dose ratio – 1) vs-log [antagonist], is close to the theoretical value of 1. The intercept of this straight line with the abscissa scale represents the pA_2 value.

In vivo

Pulmonary mechanics in anaesthetised guinea-pigs Pulmonary function in free breathing anaesthetized guinea-pigs was measured by the method described by Amdur & Mead (1958).

Male Dunkin Hartley guinea-pigs (350–450 g) were anaesthetized with sodium pentobarbitone (38 mg kg^{-1}) given intraperitoneally. Cannulae were surgically introduced into the right jugular vein for drug administration and into the carotid artery to record blood pressure. The trachea was cannulated and connected to a Grass PT5A volumetric transducer via a Fleisch pneumotachograph to record pulmonary airflow. Intrapleural pressure signals from a saline filled catheter in the pleural space were fed into a respiratory analogue computer (Buxco Model 6) and compliance (C_{dyn}) values were calculated.

LTD_4 administered intravenously to guinea-pigs pretreated with indomethacin (3 mg kg^{-1}) produced a dose-related fall in dynamic lung compliance (Leith *et al.*, 1983). LY170680 was administered intravenously prior to LTD_4. In these studies the level of LTD_4 used (1 μg kg^{-1} i.v.) was chosen to produce a fall in compliance of approximately 50%.

Total pulmonary resistance

Male Dunkin-Hartley guinea-pigs (350–450 g) were anaesthetized and cannulated as described above. The tracheal cannula was connected to a small animal ventilator and the animal artificially respired at a tidal volume of 10 ml kg^{-1} body weight and at a rate of 50 breaths min^{-1}. Total pulmonary resist-

ance (TPR) corresponding to intratracheal pressure, an index of bronchomotor tone, was measured with a Statham pressure transducer connected to the side arm on the tracheal cannula. The increase in TPR was expressed as a percentage of the pressure recorded with the tracheal cannula totally occluded.

Initial experiments investigated the effects of LY170680 given intravenously on the increase in TPR produced by intravenous LTD_4 in the presence of indomethacin ($3\,mg\,kg^{-1}$, i.v.). In these experiments the dose of LTD_4 ($1\,\mu g\,kg^{-1}$, i.v.) used was chosen to produce a reproducible increase in TPR of approximately 50%.

LY170680 was given orally to fasted animals which were later anaesthetized and surgically prepared such that 1 h after dosing, the increase in TPR to intravenous LTD_4 ($1\,\mu g\,kg^{-1}$, i.v.) could be measured.

In a further modification of this experiment, animals were sensitized to ovalbumin by an intraperitoneal injection of hyperimmune serum (0.5 ml) (Fleisch *et al.*, 1985) given 48 h before, and the TPR in response to a bolus injection of intravenous antigen was measured. To ensure that a significant portion of the increase in TPR was mediated by leukotrienes, the guinea-pigs were pretreated with mepyramine ($5\,mg\,kg^{-1}$, i.v.) a histamine H_1 receptor antagonist, propranolol ($1\,mg\,kg^{-1}$, i.v.) a β-adrenoceptor antagonist, and indomethacin ($3\,mg\,kg^{-1}$, i.v.) a cyclo-oxygenase inhibitor. The dose-response to antigen (ovalbumin) was always very steep and the sensitivity varied with each batch of guinea-pigs. Consequently each dose of LY170680 was run with its own controls. The dose of ovalbumin used in this study was in the range of 5–$10\,\mu g\,kg^{-1}$.

In a further series of studies, a DeVilbiss Pulmosonic ultrasonic nebuliser was connected in series with the respiratory pump after the method of Payne & de Nucci (1987) such that agents could be aerosolized into the airways of the ventilated animal. A dose-response to LTD_4 aerosolized to the animal for 10 s was established, and compared with a dose-response to LTD_4 in animals that had been exposed to aerosolized LY170680 (nebuliser concentration $1\,mg\,kg^{-1}$) for 20 s, 1 h previously.

Excised lung gas volume

Male Dunkin-Hartley guinea-pigs (550 ± 50 g body weight) were exposed to aerosolized LTD_4 or LTC_4 for 10 min. The aerosol, generated by a Wright nebuliser using compressed air at a pressure of 15 lbs per sq in, was passed into a 10 litre steel and perspex exposure chamber which contained groups of 3 guinea-pigs. The airflow through the nebuliser was $7\,l\,min^{-1}$ and the flow through the chamber was adjusted to $10\,l\,min^{-1}$. The particle size (mass median diameter $5.0 \pm 0.05\,\mu m$) generated by the nebuliser was measured with a Malvern 2600 HSD particle sizer. During the 10 min exposure to leukotriene, (nebuliser concentration LTD_4 $1.5\,\mu g\,ml^{-1}$, LTC_4 $2.5\,\mu g\,ml^{-1}$) the guinea-pigs exhibited symptoms of respiratory distress, characterized by an increased rate and depth of respiration followed by a convulsive cough. The animals were removed from the chamber and immediately killed by cervical dislocation. The lungs and trachea were removed and weighed on a Sartorius electronic balance. The lungs were then immersed in saline and the volume estimated by displacement. The volume of gas trapped within the lung was estimated to be, the difference between the weight of the displaced saline and the weight of the lungs, and was expressed as $ml\,kg^{-1}$ body weight. In all of these experiments, the volume of residual gas trapped in the lungs of control animals exposed to an aerosol of saline was similarly estimated and subtracted from animals exposed to leukotriene. Thus, the expressed results are a direct estimation of the increase in volume of trapped gas caused by leukotriene treatment. In these experiments, guinea-pigs were given propranolol ($5\,mg\,kg^{-1}$, i.p.) 30 min before the leukotriene challenge. Propranolol was included in these experiments as it had been previously found to improve the reproducibility of the LTD_4 response (Stengel *et al.*, 1987).

Exposure chamber concentrations of LY170680 were estimated by the addition of a known percentage (w/v) of fluorescein to the nebuliser. The nebulised material was passed into the exposure chamber, collected on a paper filter for a known period of time, the fluorescein was eluted from the filter with a known volume of sodium bicarbonate (0.1 M) solution and quantitated by u.v. absorption (493 nm).

Drugs and solutions

The composition of the Tyrode solution used in these studies was ($g\,litre^{-1}$): NaCl 8.0, KCl 0.2, $MgCl_2$ 0.1, $CaCl_2$ 0.8, NaH_2PO_4 0.05, $NaHCO_3$ 1.0 and glucose 1.0. The composition of the Krebs (Hukovic) solution was ($g\,litre^{-1}$): NaCl 6.6, KCl 0.35, $MgSO_4 \cdot 7H_2O$ 0.29, KH_2PO_4 0.16, $NaHCO_3$ 2.1, $CaCl_2$ 0.28 and glucose 2.0.

LY170680 was prepared as described by Baker *et al.* (1988). FPL55712 (7-[3-(4-acetyl-3-hydroxy-2-propylphenoxyl-2-hydroxypropoxy]-4-oxo-8-propyl-4H-1-benzopyran-2 carboxylic acid), LTC_4, LTD_4 and LTE_4 were prepared by Lilly Research Laboratories. Pentobarbitone sodium was purchased from May and Baker; acetylcholine, L-cysteine, histamine, indomethacin, mepyramine, ovalbumin, PGE_2, $PGF_{2\alpha}$ and propranolol were all purchased from Sigma Chemical Company.

Unless stated otherwise drugs were dissolved in Tyrode solution for experiments with guinea-pig ileum, Krebs Hukovic solution for experiments with trachea, and saline for all *in vivo* studies.

LY170680 was administered *in vitro* and *in vivo* as an aqueous solution. For the *in vitro* studies the compound was initially dissolved to give a concentration of 10^{-4} M in a sodium bicarbonate solution (0.5 M). Further dilutions were made in physiological solutions. LY170680 was prepared in a solution of sodium bicarbonate up to concentrations of 10 mg ml^{-1} for *in vivo* studies. The solutions were degassed to stabilise the pH and any subsequent dilutions were prepared in saline.

Statistical analysis

Results are expressed as arithmetic means ± standard error (s.e.). Statistical analysis was assessed by using a two tailed unpaired Student's *t* test. *P* values of less than 0.05 were considered statistically significant. The activity of LY170680 *in vivo* was expressed as an ED_{50} value together with 95% confidence limits, calculated by linear regression analysis of data from treated animals compared to vehicle-treated controls.

Results

In vitro *studies*

LY170680 caused a concentration-related shift of the LTD_4 and LTE_4 concentration-response curves on both ileum and trachea to the right. The calculated pK_B values for LY170680 against both LTD_4 and LTE_4 on both preparations were independent of the concentration of LY170680 used. Consequently the slope of the Schild plot and pA_2 value for each experiment was calculated (Table 1). The mean group value for the Schild plot slope for antagonism of both LTD_4 and LTE_4 on both smooth muscle preparations (regression coefficient > 0.92 in all experiments) was close to unity indicating LY170680 was a competitive antagonist of both LTD_4 and LTE_4. Increasing concentrations of LY170680 also caused a parallel shift in the LTC_4 concentration-response curves on guinea-pig ileum. However, LY170680 was approximately 50 times less potent in this respect than against LTD_4-induced responses. The effect of the compound against LTC_4 on trachea appeared to be non-competitive as the slopes of the Schild plots were less than one and the pKb values obtained were dependent on the antagonist concentration.

A series of experiments was conducted to determine whether LY170680 was a selective leukotriene receptor antagonist. These studies indicated that at concentrations 100 times greater than those required to antagonize LTD_4, LY170680 (3×10^{-6}–10^{-5} M) did not affect histamine, PGE_2, $PGF_{2\alpha}$, or acetylcholine-induced contractions of guinea-pig ileum.

During these studies it was noticed that the antagonism of the leukotrienes seemed very persistent and this was investigated in a series of washout experiments. Pieces of ileum treated with LY170680 (3×10^{-8} M) for 15 min required frequent and lengthy washing (1–2 h) before sensitivity to LTD_4 returned to normal.

Table 1 The *in vitro* effect of LY170680 on leukotriene-induced contractions of guinea-pig ileum and trachea

Agonist *Ileum*	pA_2	*Slope Schild plot*
LTC_4	7.0 ± 0.2 (5)	0.8 ± 0.2
LTD_4	8.7 ± 0.1 (12)	0.85 ± 0.1
LTE_4	9.0 ± 0.3 (6)	1.2 ± 0.2
Trachea		
LTD_4	8.1 ± 0.2 (8)	1.0 ± 0.1
LTE_4	8.1 ± 0.1 (6)	1.1 ± 0.1
LTC_4	pK_B	
3×10^{-7} M[a]	7.1 (2)	
10^{-6} M	6.8 ± 0.1 (6)	
3×10^{-6} M	6.6 ± 0.1 (6)	
10^{-5} M	6.2 ± 0.1 (4)	

pA_2, pK_B and slope values are the mean ± s.e. for each experiment. The figures in parentheses are the number of experiments, each of which was performed with at least three concentrations of LY170680.

[a] Molar concentrations of LY170680. Experiments with trachea were performed in the presence of indomethacin (3×10^{-6} M). Experiments with LTC_4 on ileum were performed in the presence of FPL 55712 (5×10^{-6} M) and on trachea in the presence of acivicin (3×10^{-5} M)

During these studies it was noticed that the antagonism of the leukotrienes seemed very persistent and this was investigated in a series of washout experiments. Pieces of ileum treated with LY170680 (3×10^{-8} M) for 15 min required frequent and lengthy washing (1–2 h) before sensitivity to LTD_4 returned to normal.

In vivo *studies*

LTD_4-induced changes in pulmonary mechanics in anaesthetized guinea-pigs LY170680, administered intravenously as a bolus before LTD_4, produced a pretreatment- and dose-dependent reduction of the LTD_4-induced fall in compliance (Figure 1). When administered 15 min prior to LTD_4, LY170680 at doses of 2.5–10 mg kg^{-1} almost completely abolished the LTD_4 response. LY170680, given 60 min prior to LTD_4, produced a dose-dependent reduction of the fall in compliance induced by LTD_4. The ED_{50} for LY170680 at a 60 min pretreatment time was estimated to be 3.8 mg kg^{-1}.

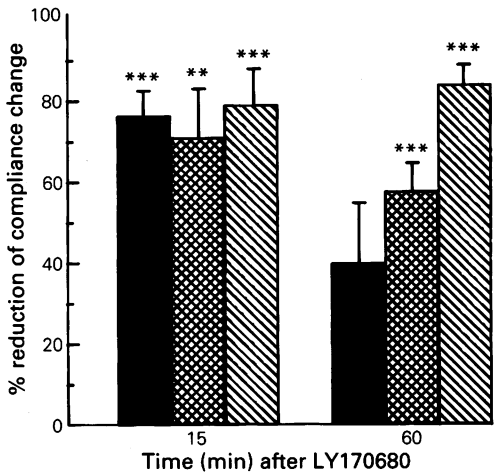


Figure 1 The effect of increasing intravenous concentrations of LY170680, on the fall in compliance induced by leukotriene D_4 (LTD_4, 1 $\mu g\,kg^{-1}$, i.v.). Anaesthetized spontaneously breathing guinea-pigs, were pretreated, intravenously with increasing concentration of LY170680: solid columns, 2.5 $mg\,kg^{-1}$; cross-hatched columns, 5.0 $mg\,kg^{-1}$; hatched columns, 10 $mg\,kg^{-1}$ at 15 min or 60 min before giving LTD_4. All animals were pretreated with indomethacin (3 $mg\,kg^{-1}$ i.v.). Each value represents mean of the data from at least 6 animals, and the vertical lines indicate the standard error of the mean. Statistical significance is calculated using unpaired t test (**$P < 0.01$; ***$P < 0.001$).

Intravenous LTD_4-induced increase in total pulmonary resistance LY170680 given as a bolus intravenously, produced a dose-related reduction of the LTD_4-induced increase in TPR (Table 2). The calculated ED_{50} of LY170680 administered 30 min before LTD_4 was estimated to be 2.0 $mg\,kg^{-1}$. The efficacy of LY170680 given 60 min before LTD_4 was less pronounced but significant inhibition of the leukotriene response was observed at 5 $mg\,kg^{-1}$ (43% reduction, $P = <0.01$).

LY170680 produced a dose-related reduction in the increase in TPR when given orally 60 min before intravenous LTD_4. LY170680 (50 $mg\,kg^{-1}$, p.o.) reduced the control TPR value from 50.6 ± 7.8% to 18.3 ± 4.7% (a reduction of 64%; $P = <0.02$), and at 25 $mg\,kg^{-1}$ p.o. from 24.4 ± 6% to 16.4 ± 5.4% (a reduction of 32%, P = not significant). The estimated ED_{50} value for LY170680 obtained from this limited study corresponded to approximately 40 $mg\,kg^{-1}$.

Antigen-induced increase in total pulmonary resistance LY170680 given intravenously 5 $mg\,kg^{-1}$ prior to antigen produced a time-dependent inhibition of the increase in TPR caused by antigen (Table 3). The degree of inhibition of the response to antigen appeared comparable to that against the LTD_4 response described in the previous section.

Aerosol LTD_4-induced increase in total pulmonary resistance Aerosolised LTD_4 from a nebuliser incorporated in the ventilator circuit produced a dose-related increase in TPR. In a study to examine the duration of LY170680 the dose of LTD_4 was adjusted to produce a reproducible increase in TPR of approximately 50%. This increase was reduced by prior inhalation of LY170680 in a time-dependent manner. The nebuliser concentration of LY170680 used in these studies was 1 $mg\,ml^{-1}$ inhaled for 30 s, a level which delivered an estimated dose of

Table 2 The effect of LY170680 given intravenously on the increase in total pulmonary resistance (TPR) produced by intravenous leukotriene D_4 (LTD_4)

Group	n	*% increase in TPR*	*% reduction*	P
LTD_4 control	11	46 ± 7	—	—
LY170680				
5.0 $mg\,kg^{-1}$	5	15 ± 10	68	<0.05
1.0 $mg\,kg^{-1}$	7	26 ± 5	44	<0.01
0.5 $mg\,kg^{-1}$	5	41 ± 10	11	NS

LY170680 was administered intravenously as a bolus, 30 min before intravenous LTD_4. Increases in TPR are expressed as a % of pressure recorded with the trachea cannula closed. ED_{50} value with 95% confidence limits calculated as 2.0 $mg\,kg^{-1}$ (0.02-152) by linear regression analysis, regression coefficient = 0.95.
All animals pretreated with indomethacin (3 $mg\,kg^{-1}$, i.v.)
Results are expressed as mean ± s.e. mean.
Statistical comparison between LY170680 groups and control by unpaired Student's t test (NS = not significant, $P = >0.05$); n = number of animals.

Table 3 Effect of LY170680 (5 mg kg^{-1}) given intravenously against intravenous antigen-induced increases in total pulmonary resistance (TPR)

Predose time (min)	% increase *TPR for OA control*	% increase *TPR for LY170680*	% *redn.*	P
30	32 ± 4	5 ± 1	83	<0.001
60	19 ± 3	9 ± 3	51	<0.100
90	28 ± 6	21 ± 7	24	N.S.

All animals passively sensitized to ovalbumin by an intraperitoneal injection of anti-ovalbumin hyperimmune serum. All animals pretreated with indomethacin (3 mg kg^{-1}), mepyramine (5 mg kg^{-1}) and propranolol (1 mg kg^{-1}).
OA = ovalbumin, (5 μg kg^{-1}, i.v.) $n = 6$.
For other details see legends in Table 2.

3 μg kg^{-1} body weight. This dose of LY170680 was sufficient to reduce the pronounced LTD_4 bronchospasm by 80% ($P < 0.001$) after 30 min. The inhibition of LTD_4-induced increase in TPR by inhaled LY170680 decreased with time, such that pretreating with LY170680 60 min beforehand produced a 50% ($P < 0.01$) reduction of the response to LTD_4 and at 90 min a 33% ($P > 0.05$) reduction.

This study was extended to investigate the effect of a single aerosol concentration of LY170680 on a range of LTD_4 aerosol doses. In this experiment LY170680 at a nebuliser concentration of 1 mg ml^{-1} was inhaled for 20 s by the guinea-pigs 60 min before giving LTD_4 aerosol. The results showed a parallel shift of the LTD_4 dose-response curve to the right by a factor of approximately 6 (Figure 2). The amount of LY170680 introduced into the lungs was approximately 2 μg kg^{-1} body weight. The amount of compound delivered to the lung in these studies was estimated by adding a known concentration of fluorescein to the nebuliser and quantitating by u.v. absorption the amount leaving the end of the tracheal cannula.

Leukotriene-induced increase in excised lung gas volume (ELGV) LY170680 given orally at doses up to 100 mg kg^{-1} failed to modify the LTD_4-induced increase in ELGV. However, LY170680 delivered by aerosol protected guinea-pigs against the increase in ELGV in a dose- and time-dependent manner (Figures 3 and 4). Guinea-pigs exposed 6 h previously to an aerosolised atmosphere of LY170680 generated from a nebuliser concentration of 10 mg ml^{-1} (estimated aerosol concentration 40 μg l^{-1}; initial lung dose of 2–3 μg g^{-1} wet weight), were significantly protected (40%, $P < 0.05$) against the bronchoconstrictor effect of LTD_4. The dose of

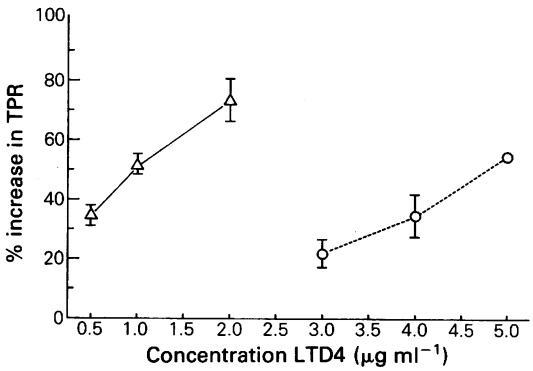


Figure 2 The displacement by a prior inhalation of LY170680 of the dose-response curve for the rise in total pulmonary resistance induced by inhaled leukotriene D_4 (LTD_4). Artificially ventilated guinea-pigs were exposed to different levels of inhaled LTD_4 for 10 s and the increase in TPR measured. A further dose-response curve was constructed after the animals had been exposed for 20 s 60 min earlier, to an aerosolised atmosphere of LY170680 generated from a nebuliser concentration of 1 mg ml^{-1}. The vertical lines show s.e.means. Each point is the mean of the data from at least 5 animals.

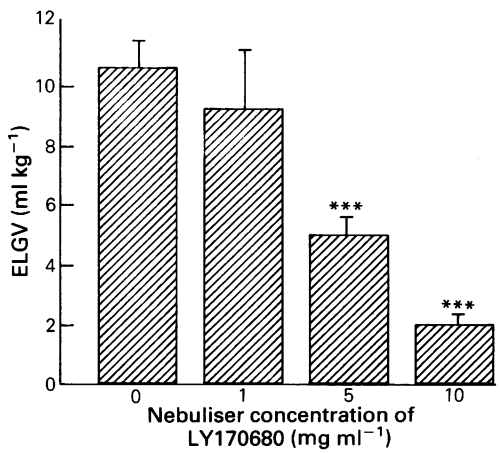


Figure 3 The dose-related effect of inhaled LY170680 on the increase in excised lung gas volume (ELGV) in conscious guinea-pigs induced by a 5 min exposure to inhaled leukotriene D_4 (LTD_4). LY170680 was administered for 10 min by aerosol, 4 h before the LTD_4. The arosolised atmosphere of LTD_4 in these studies originated from a nebuliser concentration of 1.5 μg ml^{-1}. All animals pretreated with propranolol (5 mg kg^{-1}, i.p.). The vertical lines show s.e.mean. Each experiment is the mean of data from at least 6 animals. Statistical significance was calculated using unpaired t test (***$P < 0.001$).

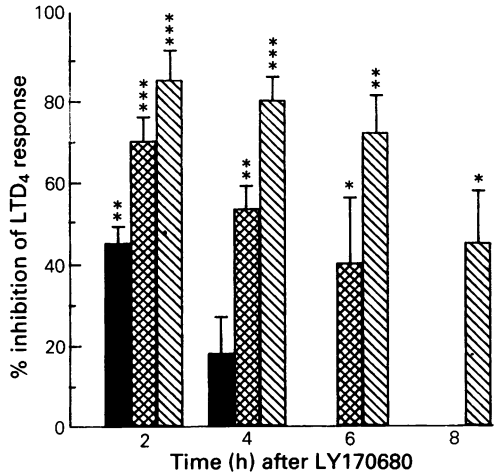


Figure 4 The dose- and time-related effect of inhaled LY170680 on the increased gas trapping (ELGV) induced by inhaled leukotriene D_4 (LTD_4). An aerosolised atmosphere of LY170680 generated from different nebuliser concentrations: solid columns, $1\,mg\,ml^{-1}$; cross-hatched columns, $5\,mg\,ml^{-1}$; hatched columns, $10\,mg\,ml^{-1}$ was administered for 10 min to conscious guinea-pigs at different times before inhaled LTD_4 (5 min, $1.5\,\mu g\,ml^{-1}$). All animals pretreated with propranol ($5\,mg\,kg^{-1}$, i.p.). Results represent means of data from at least 3 experiments each of 6 animals. Vertical lines show s.e.mean. Statistical significance is calculated using unpaired t test. ($^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

LTD_4 used in these studies was chosen to produce a pronounced and reproducible bronchospasm which was equivalent to $70 \pm 10\%$ maximum of ELGV. The lung levels of LY170680 in these studies were estimated by use of radiolabelled LY170680. The aerosol concentration was estimated with fluorescein and corresponded to $4.5\,\mu g\,l^{-1}$ from a $1\,mg\,ml^{-1}$, $20\,\mu g\,l^{-1}$ from $5\,mg\,ml^{-1}$, and $38\,\mu g\,l^{-1}$ from $10\,mg\,ml^{-1}$ nebuliser concentration.

A series of experiments was conducted to determine whether LY170680 could also prevent LTC_4-induced increase in ELGV. The results (Table 4) demonstrated that LY170680 (nebuliser concentration $5\,mg\,ml^{-1}$) was equally effective in preventing LTC_4-induced bronchospasm (73% protection, $P = < 0.001$ inhaled 4 h previously).

The relative selectivity of LY170680 for leukotrienes was demonstrated using this system as this antagonist failed to protect guinea-pigs against an increase in ELGV produced by histamine at a dose that almost completely abolished the leukotriene response. (Table 4).

Table 4 The effect of LY170680 by aerosol on excised lung gas volume (ELGV) induced by leukotriene C_4 (LTC_4) and histamine

	Predose time (h)	*ELGV* ($ml\,kg^{-1}$)	*% redn.*	P
LTC_4 alone	—	11.7 ± 2.0	—	—
+LY170680	2	1.2 ± 0.4	90	<0.001
+LY170680	4	3.2 ± 0.9	73	<0.001
Histamine alone	—	12.9 ± 1.0	—	—
+LY170680	2	10.2 ± 1.3	20	N.S.

LY170680 (nebuliser concentration $5\,mg\,ml^{-1}$, aerosol concentration $20\,\mu g\,l^{-1}$) delivered by nebuliser for 10 min prior to inhaled LTC_4 ($2.5\,\mu g\,ml^{-1}$) or histamine ($80\,\mu g\,ml^{-1}$) for 5 min. All animals pretreated with propranolol ($5\,mg\,kg^{-1}$ i.p.). The excised lung gas volume is expressed in $ml\,kg^{-1}$ and measured by displacement of saline.
$n = 5$. For other details see Table 2.

Discussion

It has been proposed that leukotrienes are important mediators in the pathophysiology of disorders such as asthma. Consequently the discovery and development of specific leukotriene antagonists should offer a useful therapeutic approach.

The present study indicates that LY170680 is a potent antagonist of the contractile actions of LTD_4 and LTE_4. The nature of the antagonism appears to be selective and competitive for both leukotrienes. The selectivity of action of LY170680 for LTD_4/LTE_4 was indicated by the lack of effect against other smooth muscle agonists, although there was some inhibition of the contractile action of LTC_4.

The similar pA_2 values for LTD_4 (in the presence of L-cysteine) and LTE_4 on trachea indicates similar functional receptor requirements, although the difference in pA_2 values on gut and airway smooth muscle, would seem to indicate differences in receptor affinities between the two organs. The *in vitro* antagonism of LTD_4/LTE_4 by LY170680 appears very persistent as repeated washing of the isolated preparation was required for 1–2 h before sensitivity to leukotrienes was restored. During the *in vitro* studies, no discernible relaxation of the preparations occurred in the presence of high levels of LY170680. This was considered to be indirect evidence that LY170680 was not a phosphodiesterase inhibitor, unlike acetophenone antagonists typified by LY171883 which are phosphodiesterase inhibitors and which caused relaxation of respiratory smooth muscle.

The relatively low activity observed with LY170680 following oral dosing correlated with the low blood levels of radioactivity measured after oral dosing with [^{14}C]-LY170680. This study indicated peak blood levels occurred 30 min to 1 h after oral dosing, before falling quickly to very low levels (Colvin unpublished observations 1989). Consequently the lack of oral efficacy observed with LY170680 in the ELGV model 2 h after dosing was not surprising.

LY170680 was much more active by the intravenous route in preventing leukotriene- and antigen-induced bronchospasm and particularly active by the inhaled route in preventing leukotriene-induced bronchospasm.

The time course of the intravenous studies with LY170680 indicated a reduction in efficacy with time, in line with the intravenous half-life of the compound (Colvin unpublished observations 1989). The reduction of antigen-induced bronchospasm occurred in animals pretreated with mepyramine, propranolol and indomethacin. This pretreatment combination has been previously shown to result in an antigen-induced leukotriene-mediated bronchospasm (Anderson *et al.*, 1983).

LY170680 was particularly effective by the inhaled route in reducing the increase in TPR produced by inhaled LTD_4. A 6 fold parallel shift to the right of the LTD_4 dose-response curves was achieved after an estimated exposure of 1–2 μg per animal given 1 h previously. Similarly, studies with the ELGV model indicated a dose- and time-dependent reduction of the LTD_4-induced increase in trapped gas. Guinea-pigs exposed to an aerosolised atmosphere of LY170680 for 10 min were protected against leukotriene induced airway obstruction for 4–6 hours depending on the nebuliser concentration used. Studies with radiolabelled LY170680 indicated a direct relationship between efficacy and lung levels of radiolabel. In these studies a nebuliser concentration of 10 mM resulted in a ^{14}C equivalent lung dose of 2–3 $\mu g\,g^{-1}$ wet weight.

The compound was also equally effective in antagonizing the LTC_4-induced increase in gas trapping. This finding contrasts with the *in vitro* data which demonstrates LY170680 was a preferential LTD_4/LTE_4 antagonist. This protection against LTC_4 is presumably due to the rapid conversion *in vivo* of LTC_4 to LTD_4 by gamma glutamyl transpeptidase. A similar finding was reported by Fleisch *et al.* (1985). The selectivity of the compound was demonstrated by the lack of efficacy against histamine-induced increases in gas trapping at a level of LY170680 which had previously been shown to be active against LTD_4.

In conclusion LY170680 is a potent, selective and competitive antagonist of leukotriene-induced responses in guinea-pig airways. It is particularly active by the inhaled route with a long duration of action. It may be therapeutically useful in pathological conditions of the airways such as asthma where involvement of leukotrienes have been implicated.

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A dual model for cardiac arrhythmias: coexistence of re-entry and abnormal automaticity and effects of antiarrhythmic agents

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1 We have developed a dual model for arrhythmia in anaesthetized dogs. The model consists of an inducible re-entrant atrial tachycardia and spontaneous ventricular ectopies in the same heart.

2 The model for re-entrant atrial tachycardia was created by crushing the right atrium longitudinally in the intercaval region and transversely in the front free wall parallel to the atrioventricular groove. Ventricular abnormal automaticity was produced by prior (20 ~ 24 h) left anterior descending coronary artery occlusion. The ventricular arrhythmia was partially suppressed during rapid pacing-induced atrial tachycardia and resumed after atrial re-entry was terminated.

3 Mapping experiments indicate that the atrial tachycardia was due to circus movement occurring in the tissue around the tricuspid ring. This re-entrant circuit was identical to that induced in the model created by the incision method.

4 Clofilium (0.75 mg kg^{-1}, $n = 5$) increased the cycle length of atrial re-entry by $14 \pm 4\%$ from 139 ± 12 to 159 ± 18 ms ($P < 0.05$). Flecainide (1.8 ± 0.9 mg kg^{-1}, $n = 5$) prolonged the cycle length of the tachycardia by $114 \pm 57\%$ from 158 ± 11 to 332 ± 66 ms ($P < 0.05$).

5 Both drugs terminated the atrial arrhythmia, but re-entry could be reinduced only in flecainide-treated dogs. Flecainide reduced ventricular ectopies by $89 \pm 19\%$, whereas clofilium did not change ventricular abnormal automaticity or maximum pacing cycle length that is necessary to overdrive the ventricle fully.

6 These data indicate that the dual model provides coexisting arrhythmias of different mechanisms in the same heart and that Class I and Class III anti-arrhythmic drugs may be differentiated from each other by the distinct patterns of pharmacological activities produced in this test system.

Introduction

Re-entry and abnormal automaticity are two primary mechanisms responsible for the genesis of cardiac arrhythmias (Hoffman & Rosen, 1981). Ventricular arrhythmias in dogs after occlusion of a coronary artery for 20–24 h are believed to be due to abnormal automaticity, which occurs in a *de novo* or triggered fashion, in the endocardial Purkinje fibres of the infarcted zone (Friedman *et al.*, 1973a,b; Lazzara *et al.*, 1973; 1978; El-Sherif *et al.*, 1983). This type of arrhythmia is frequently used to assess the antiarrhythmic potential of new chemical entities. Class I antiarrhythmic agents such as lignocaine, procainamide, quinidine and flecainide have been shown to suppress multifocal ectopies in the 1-day myocardial infarcted dog model. Recently, a conscious model for re-entry arrhythmia in the dog has been developed (Frame *et al.*, 1986; Wu & Hoffman, 1987). The arrhythmia is a result of re-entrant excitation propagated in a partially repolarized atrial tissue around the tricuspid ring (Frame *et al.*, 1986; 1987; Wu *et al.*, 1989a). The circus movement is easy to induce, stable in cycle length, and persistent. Various antiarrhythmic agents have been studied in this model of arrhythmia (Wu & Hoffman, 1986; Frame & Colatsky, 1987; Spinelli & Hoffman, 1987; Wu & Hoffman, 1987).

It was our objective to combine the model for ventricular abnormal automaticity due to prior (20 ~ 24 h) coronary artery occlusion and the model for re-entrant atrial tachycardia in the same canine heart. In addition, we used the sodium channel blocker, flecainide, and the potassium channel blocker, clofilium, to examine the antiarrhythmic patterns of these agents in this dual model for arrhythmia.

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Methods

Dogs of either sex weighing 10–16 kg were fasted overnight, anaesthetized with pentobarbitone (35 mg kg^{-1}, i.v.), and ventilated artificially. The left side of the chest was opened at the fifth intercostal space and a segment of 0.5 ~ 1.0 cm of left anterior descending (LAD) coronary artery proximal to the first diagonal branch was dissected free and occluded by an Ameroid plastic constrictor, according to the method described by Smith *et al.* (1973). Twenty to 24 h after each dog had recovered from the surgery, the animal was reanaesthetized by slow infusion of pentobarbitone (30 mg kg^{-1}, i.v.) and artificially ventilated with its temperature maintained at 37 ± 0.5°C by a heating pad. The chest was opened at the right side through the fourth intercostal space. The intercaval tissue, extending longitudinally for 5 to 7 cm, was crushed by hemostat clamps. Continuous crushes of 5 to 7 cm long were also performed at the front right atrium branched from the intercaval crushes toward the atrial appendage and in parallel to the atrioventricular groove. Two or 3 pairs of bipolar electrodes were sewn on the posterior wall of the right atrial appendage near the aortic root and Bachmann's bundle for stimulation and recording of local atrial electrograms. For mapping of the activation sequence during stable and regular re-entrant tachycardia, a bipolar roving probe (4 mm in diameter and 10 cm in length) was used for the recording of the epicardial and endocardial electrograms. The roving probe was inserted into the right atrium through a purse string aperture at the tip of appendage to perform endocardial mapping. The location of the recording site was guided and verified by probing with an index finger on the epicardium.

Lead II electrocardiogram (ECG) and local atrial electrograms were displayed and recorded on a Electronics for Medicine (Model DR-8) recorder and Grass polygraph (Model 7). Electrical stimulation of the atrium was provided by a digital stimulator and constant current stimulation isolation unit (Model DTU-201, Bloom Associates). The effective refractory period of the right atrium (AERP) was measured at a paced cycle length of 300 ms. A series of 8 impulses (S1), 1 ms in duration and 2 times the diastolic threshold, followed by a premature stimulus (S2) of the same strength was delivered through a bipolar electrode. The S1–S2 intervals were decreased progressively by 5 or 10 ms. The AERP was defined as the shortest S1–S2 interval that produced a propagated response. Re-entrant atrial tachycardia was induced by rapid pacing of the right atrium with a roving bipolar electrode connected to a Grass stimulator and a stimulation isolation unit (Model S48, 2 ms pulse width, 4–8 volts, 100 to 120 ms cycle length). Conduction velocity (m s^{-1}) of the induced atrial tachycardia was estimated by dividing post mortem tricuspid ring circumference by the cycle length of the atrial re-entry.

Ventricular arrhythmias in postinfarcted dogs were quantitated as % ectopies of total resting rhythm over a period of 10–20 s. Ventricular ectopic beats were identified by the abnormal R waves of the ECG in which the preceding local atrial electrograms do not occur at normal P–R intervals before the QRS complexes. The maximum pacing cycle length (MPCL) that produced 100% suppression of the ventricular ectopies was obtained by pacing at the right atrium beginning at 250 ms and increased at intervals of 50 ms. The presence of the overdrive suppression of ventricular abnormal automaticity was determined by atrial pacing at 250 ms cycle length and expressed as intervals from the last paced QRS complex to the first spontaneous QRS complex derived from an ectopic focus. In 3 experiments involving flecainide, the right vagal nerve was dissected free in the cervical region and electrically stimulated with a Grass stimulator to inhibit atrial overdrive, thereby revealing residual ventricular abnormal automaticities during resting rhythm before and after drug administration.

Heart rate, MPCL, AERP, and % ventricular ectopies were measured before and after intravenous infusion of clofilium tosylate or flecainide acetate with a Harvard infusion pump. The infusion rate of clofilium and flecainide was 0.25 and 0.2 mg kg^{-1} min^{-1}, respectively. The infusion of clofilium was continued for 3 min in all trials (0.75 mg kg^{-1}), whereas flecainide was given until atrial re-entry was terminated or a total of 1.6 mg kg^{-1} was delivered. AERP was determined at the end of infusion followed by pacing and other measurements. All post-drug measurements were completed at 5 ~ 15 min after drug administration. Changes in the cycle length of the atrial tachycardia were also recorded during drug administration. Data are expressed as the mean ± standard deviation (s.d.) and differences between treatment groups were analyzed by Student's t test with significance level chosen at $P < 0.05$. A paired t test was also used for comparisons between predrug controls and postdrug effects within the same treatment group. Clofilium tosylate and flecainide acetate were kindly supplied by Eli Lilly and Riker Laboratories, respectively. Doses are expressed as the salt form of the compounds.

Results

One day following LAD coronary artery occlusion, the incidence of ventricular ectopic activity was 77 ± 25% at an average heart rate of 174 ± 24 beats

per min ($n = 10$) under open-chest and anaesthetized conditions. The MPCL required to overdrive fully ventricular arrhythmia was 280 ± 35 ms. Overdrive suppression was 387 ± 77 ms following atrial pacing at a cycle length of 250 ms. The first returning spontaneous beat following 250 ms atrial pacing was always ectopic. Atrial re-entry induced by rapid pacing showed stable and regular cycle lengths of 151 ± 12 ms ($n = 10$). During atrial tachycardia, ventricular ectopic activities were partially suppressed (see Figures 2b and 3b) but resumed after atrial circus movement was terminated by rapid pacing. Because we employed crushes instead of incisions as the method for preparing the re-entry model, we confirmed that the current model had the same pathway for circus movement as that created by the incision method. For this purpose, we performed Mines' tests (Mines 1913; 1914) for re-entry by applying tension around the tricuspid ring on either the left or right side of the AV groove with a ligature. The atrial tachycardia was first slowed down and then terminated in all trials. We also conducted mapping experiments in 3 dogs by using a roving probe to record local activation sequence on the epicardium and endocardium of the right atrium. Figure 1 depicts a right atrial isochrone map characterizing a single episode of a counterclockwise re-entrant tachycardia. The right atrial endocardium was displayed two-dimensionally, in a similar way to that described by Frame *et al.* (1987), by cutting open the intercaval region on the left side and from the tip of the appendage towards the superior vena cava and aortic root. The activation times at each epicardial and endocardial site were sequenced in relation to a fixed electrode. Note that bridging of the activation sequence in the whole tachycardia cycle occurred around the tissue of the tricuspid ring. Also, the circulating impulses spread away from the tricuspid ring tissue toward the surrounding portion of the atrium. This circuit path for re-entry is identical to that induced in the model created by the incision method (Frame *et al.*, 1986; 1987).

Clofilium, at the infusion dose of 0.75 mg kg^{-1} 3 min^{-1}, significantly prolonged cycle length (Table 1) and terminated the atrial arrhythmia in all dogs. In all cases, neither rapid pacing nor premature stimulation could reinitiate the re-entrant tachycardia. AERP showed significant increases that were greater than the degree of cycle length prolongation (Table 1). Conduction velocity, estimated from tachycardia cycle length and the tricuspid ring circumference, showed decreases that are comparable to cycle length changes (Table 1). Clofilium increased ventricular ectopies in 2 of 5 dogs. In the remaining 3 dogs, the ectopic frequency in the presence of clofilium did not deviate by more than 10%. Following clofilium, heart rate during resting rhythm was

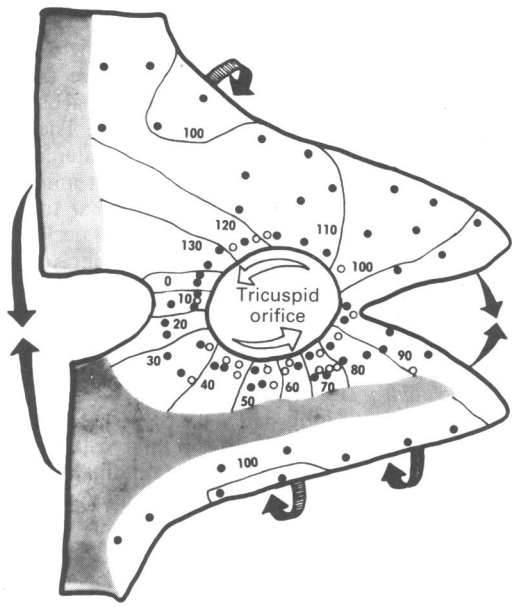


Figure 1 Isochrone map of the two-dimensional right atrium during a counterclockwise re-entrant atrial tachycardia (indicated by the open arrows). Thin lines are isochrones labelled with activation times (in ms) at 10 ms intervals; (●) and (○) represent recording sites on the epicardium and endocardium, respectively. Shading indicates crushed area. Solid arrows on the left and right margins link the continuous portion of the tissue, i.e., intercaval tissue and appendage, that was cut apart for display. Shaded arrows on the top and bottom margins indicate continuous wavefronts propagated from the opposite wall of the atrial appendage.

increased in 1 dog and decreased in 4 dogs. MPCL, which was needed to overdrive the ventricle, was not significantly changed by clofilium (Table 1). Figure 2 is representative of the effect of clofilium on this dual model for arrhythmia. In Figure 2a, over two-thirds of the rhythm showed dissociation of ventricular and atrial activities with the first, second, and ninth as sinus beats. Panel (b) displays the rhythm during re-entrant atrial tachycardia at a cycle length of 145 ms with a 3:2 A-V ratio. Panel (c) shows that clofilium prolonged atrial re-entry to a cycle length of 158 ms. Ten seconds later the tachycardia was terminated. Panel (d) is the resting rhythm indicating that ventricular arrhythmia was not corrected after administration of clofilium.

At average doses of 1.8 ± 0.9 mg kg^{-1}, flecainide prolonged atrial tachycardia cycle length (Table 1) and terminated the re-entry in all cases. The doses of flecainide that were required for the termination of re-entry were more variable than clofilium (ranged from 0.64 to 1.6 mg kg^{-1}). In those dogs in which the termination of arrhythmia occurred before the 8th

Table 1 Effects of clofilium and flecainide in a dual model for arrhythmia in the dog

Parameter	*Control* ($n = 5$)	*Clofilium* ($n = 5$)	*Control* ($n = 5$)	*Flecainide* ($n = 5$)
Heart rate (bpm)	187 ± 26	148 ± 22	161 ± 15	153 ± 16
% ectopy	78 ± 28	89 ± 15	76 ± 25	9 ± 13*†
% change (ectopy)		14 ± 24		−89 ± 19
MPCL (ms)	280 ± 27	290 ± 22		
ARCL (ms)	139 ± 12	159 ± 18*	158 ± 11	332 ± 66*†
% change ARCL		14 ± 4		114 ± 57†
CV ($m\,s^{-1}$)	0.64 ± 0.06	0.56 ± 0.06*	0.58 ± 0.06	0.29 ± 0.08*
% change (CV)		−12 ± 3		−50 ± 12*†
AERP (ms)	121 ± 13	158 ± 22*	118 ± 13	141 ± 15†
% change (AERP)		31 ± 12[a]		20 ± 4
Reinitiated ARCL after termination		Not reinducible		193 ± 12

Values are mean ± s.d.
* Significant difference ($P < 0.05$) between treatment and control.
† Significant difference ($P < 0.05$) between clofilium and flecainide.
[a] Significant difference ($P < 0.05$) as compared with % change ARCL of clofilium group.
ARCL = cycle length of atrial re-entry; CV = conduction velocity;
MPCL = maximum pacing length cycle; AERP = effective refractory period of the right atrium.

minute, flecainide was infused until $1.6\,mg\,kg^{-1}$ had been given. Note that the magnitude of cycle length prolongation by flecainide was significantly greater than that produced by clofilium (Table 1). The final cycle, in most instances, was usually drastically lengthened before termination of re-entry. Atrial re-entry could always be reinitiated after its termination by flecainide. However the reinitiated rhythm had a cycle length that was shorter than the maximum cycle length achieved before termination by flecainide. The slowing of conduction velocity was greater than the extent of AERP prolongation (Table 1). Following flecainide, ventricular abnormal beats were fully ablated in 3 dogs and reduced by more than 70% in other 2 dogs. Flecainide did not significantly alter resting heart rate in these dogs. Vagal stimulation in 3 dogs revealed ventricular ectopies with an average cycle length of 431 ± 64 ms. This cycle length was significantly ($P < 0.05$) longer than that obtained during the control period (309 ± 89 ms). Figure 3 is representative of the effects of flecainide on this dual model for arrhythmia. During the control period, ectopic activities in resting rhythm (Figure 3a) were partially overdrive suppressed during atrial re-entry at a cycle length of 145 ms (panel b). During flecainide administration, the atrial re-entry slowed down to a cycle length of 274 ms without any ventricular ectopies (panel c). The tachycardia was terminated about 1 min later and the ectopic rhythm was fully corrected to a sinus rhythm (panel d).

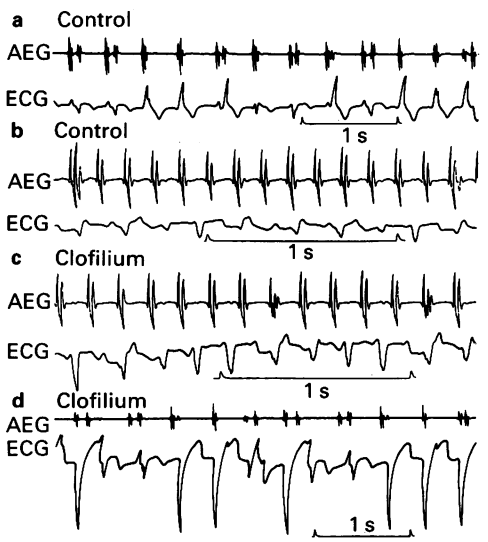


Figure 2 Effects of clofilium on the dual model for arrhythmia: (a) and (b) are predrug control; (c) and (d) are postdrug effects. Panels (b) and (c) show rhythms of re-entrant atrial tachycardia. AEG: atrial electrograms (see text for discussion).

Discussion

We have demonstrated that a re-entrant atrial tachycardia can be induced in 1-day myocardial infarcted dogs. Although tissue crushing instead of the incision method was used to accomplish the injury, the circuit for re-entrant excitation appears identical to the ones reported for the incision method (Frame *et*

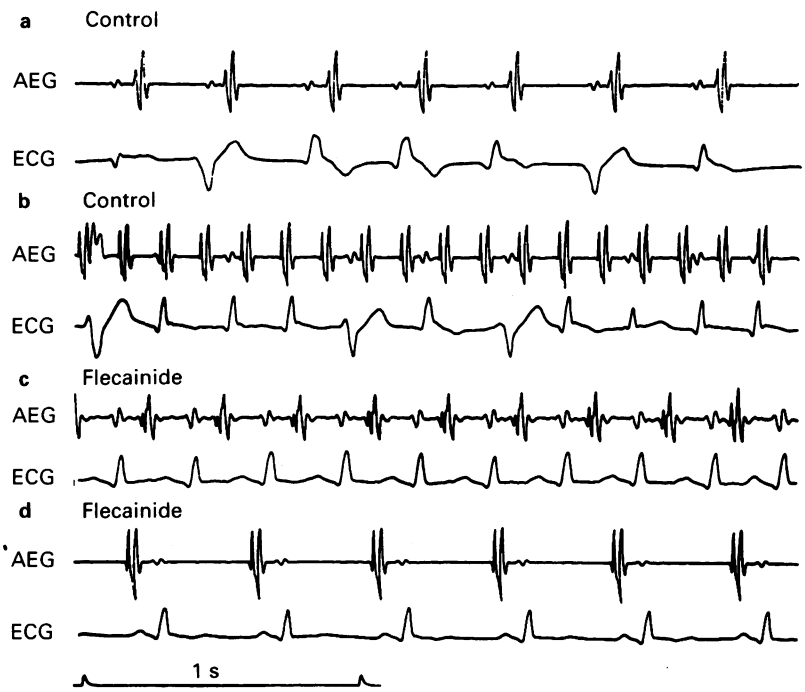


Figure 3 Effects of flecainide on the dual model for arrhythmia: (a) and (b) are control, (c) and (d) are effects of flecainide. Re-entrant atrial tachycardia is shown in (b) and (c). AEG: atrial electrograms (see text for discussion).

al., 1986; 1987). Mines' tests for re-entry are satisfied in that (1) block of the circulating impulse by tension upon the pathway terminated the re-entry and (2) continuous activation during a cycle was identified and confirmed in the tricuspid ring by roving probe mapping. Thus, for acute studies, the tissue crushing procedure is simpler and less traumatic than the incision method because it eliminates the tedious suturing required to achieve complete atrial closure or to minimize bleeding and reduces the large traumatized area produced by the clamping of tissue before the cut.

Despite the fact that the models for re-entrant atrial tachycardia and the 24 h post infarction ventricular arrhythmias have been characterized under separate conditions, experimental benefits can be derived by the use of this dual model which provides two contrasting arrhythmias in the same heart. This model offers an obvious economic advantage for the evaluation of the antiarrhythmic efficacy and mechanism of action of new agents since the pharmacological profile can be achieved with half the number of animals. The dual model also provides the opportunity to evaluate not only the specificity of a drug against either type of arrhythmia, but also can be useful in determining the sensitivity of a drug for each type of arrhythmia. Although separate arrhythmia models could be used for such experiments there is always the problem of interanimal variability. The scientific advantage of this dual model is that the antiarrhythmic action of a drug against either arrhythmia can be analyzed under the same pharmacodynamic and pharmacokinetic conditions. From a clinical standpoint, patients with acute ventricular myocardial infarction may concurrently exhibit re-entrant atrial flutter (Jewitt *et al.*, 1967; Pasternak *et al.*, 1988). The latter often intensifies haemodynamic deterioration. The dual model provides an experimental facsimile of the clinical conditions and could prove useful in determining optimal therapeutic remedies for this cardiovascular problem.

In the present study, clofilium did not produce any significant decrease in ventricular ectopic frequency. Clofilium also failed to reduce the incidence of premature ventricular complex in patients with a history of ventricular arrhythmias (Platia & Reid, 1984). Further, it is known that phase 4 depolarization of normal automatic canine Purkinje fibres is not altered by clofilium (Steinberg *et al.*, 1981). In contrast to its lack of effect on automatic rhythm, clofilium is effective against re-entry. The latter action is presumably due to its effect on delaying repolarization and thus prolonging refractoriness

(Steinberg & Molloy, 1979). Since clofilium has no effect on upstroke velocity of the propagating impulse, it should produce little changes in conduction velocity (Steinberg *et al.*, 1981; 1984). However, during circus movement while the action potential was gradually prolonged by clofilium, takeoff potential of the next impulse shifted to a more depolarized level of membrane potential. This may indirectly cause a reduction in upstroke velocity and a slowing in the conduction of the circulating impulse, as shown by the fact that the cycle length of re-entry was prolonged by clofilium.

Termination of the circus movement should occur when repolarization has been delayed and refractoriness of the tissue increased to an extent that the wavefront of the next impulse is blocked. In the present study, we have observed significant increases in refractory periods that exceeded maximum cycle length achieved after clofilium. These data apparently account for the termination of tachycardia and support the above explanation for the action of clofilium.

In contrast to clofilium, flecainide significantly reduced ventricular ectopies in our model for arrhythmia. This is in agreement with previous studies that have shown flecainide to be very effective in ablating canine arrhythmias induced by ouabain and coronary artery ligation (Kvam *et al.*, 1984). In the re-entry portion of the dual model, flecainide was also effective in terminating the atrial tachycardia. Flecainide depresses phase 0 upstroke and produces a marked slowing in conduction (Kvam *et al.*, 1984) as reflected by the drastic increases in re-entrant cycle length observed in the present studies. Recently, we have also shown that flecainide produces a longer cycle length than clofilium in the same atrial re-entry model in conscious dog (Wu *et al.*, 1989b). The termination of re-entry by flecainide should occur when the excitability of the tissues is insufficiently recovered for impulse propagation. The fact that flecainide increases the refractory period has been reported in human (Viko-Mo & Ohm, 1982) and canine atrium (Hodess *et al.*, 1979). However, in the present study, the ERP was not prolonged to the extent of the cycle length that was achieved after flecainide. Our results, thus, do not appear to support the above reasoning for the action of flecainide on re-entry termination. The exact mechanisms responsible for the above phenomena warrant further investigation.

To summarize, the pharmacological profiles showed that clofilium was active against re-entry but not abnormal automaticity, while flecainide was active in both types of arrhythmias and produced a longer cycle length than clofilium on re-entry termination.

In conclusion, we have shown that re-entrant atrial tachycardia can be induced in 1-day infarcted dogs that have spontaneous ventricular ectopic activities. While it is evident that other antiarrhythmic agents need to be evaluated in this dual model to realize fully its predictive utility, the sodium channel blocker, flecainide, and the potassium channel blocker, clofilium, could be differentiated from each other based upon their distinctive patterns of activities. Since 2 primary types of arrhythmias can be induced in the same heart, the dual model may provide an excellent tool to evaluate Class I and III antiarrhythmic agents.

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Inotropic response to DPI 201-106 in the failing human heart

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1 The present study was designed to characterize the positive inotropic response to DPI 201-106 in isolated papillary muscle strips obtained from heart failure patients undergoing surgery.

2 The positive inotropic responses to isoprenaline and milrinone and cardiac β-adrenoceptor density were also determined.

3 DPI 201-106 increased the force of contraction in papillary muscle strips from patients with moderate (NYHA II-III) and severe (NYHA IV) heart failure, in a concentration-dependent manner. This positive inotropic effect was more pronounced in tissues from NYHA IV patients. Furthermore, these responses were greater than those produced by milrinone or isoprenaline. The positive inotropic effects of isoprenaline and milrinone were reduced in NYHA IV compared to NYHA II–III. Consistently, there was also a smaller density of β-adrenoceptors in myocardium from NYHA IV than in NYHA II–III. The positive inotropic effect of Ca^{2+} was similar in tissues from both groups of patients.

4 The positive inotropic effect of DPI 201-106 was not antagonized by adenosine or carbachol, whereas both compounds reduced the positive inotropic effect of isoprenaline.

5 DPI 201-106 did not increase the Ca^{2+}-sensitivity of chemically skinned ventricular fibres, whereas a significant increase of the Ca^{2+}-sensitivity was obtained with trifluoperazine.

6 It is concluded that DPI 201-106 produces significant positive inotropic effects in tissue excised from the failing human heart. The lack of inhibition by adenosine and carbachol might contribute to its greater effectiveness in NYHA IV than NYHA II–III and indicates that its mechanism of action is cyclic AMP-independent. A sensitization of the contractile proteins to Ca^{2+} does not appear to be important for the positive inotropic action of DPI 201–106 in the failing human heart.

Introduction

DPI 201-106 is a novel cardiotonic agent, which produces positive inotropism by acting on myocardial sodium channels (Buggisch *et al.*, 1985; Scholtysik *et al.*, 1986). The compound increases coronary blood flow and does not release catecholamines from cardiac stores (Scholtysik *et al.*, 1985). It has been shown to sensitize porcine contractile proteins to Ca^{2+} (Herzig & Quast 1984). Recent clinical studies provide evidence that DPI 201-106 produces haemodynamic improvement, when given intravenously (Thornmann *et al.*, 1986) or orally (Kostis *et al.*, 1987) in patients with severe heart failure. However, demonstration of a direct positive inotropic effect in the human heart is lacking.

The present study was performed to compare the positive inotropic effect of DPI 201–106 in isolated myocardial tissue from patients with moderate (NYHA II–III) or severe (NYHA IV) heart failure. Isoprenaline and milrinone were also studied for comparison. Furthermore, the effects of adenosine and carbachol, both of which reduce the positive inotropic effect of adenylate cyclase stimulators and phosphodiesterase inhibitors (Schrader *et al.*, 1977; Schmitz *et al.*, 1985), on the positive inotropic effect of DPI 201-106 and isoprenaline were studied. The effect of DPI 201–106 on the Ca^{2+}-sensitivity of

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Table 1 Age, diagnoses and haemodynamic measurements of the patients studied

No.	*Sex*	*NYHA*	*Diagnosis*	*Age*	*LVEDP*	*LVEDV*	*EF*	*CI*
1	m	II–III	MI > MS	54	14	165	41	1.7
2	m	II–III	MI > MS	81	26	276	34	2.3
3	m	II–III	MS = MI	65	12	146	53	2.6
4	f	II–III	MS > MI	68	12	121	67	2.5
5	f	II–III	MS > MI	67	10	182	50	2.4
6	f	II–III	MS > MI	62	16	165	55	1.8
7	f	II–III	MS > MI	65	10	133	63	2.6
8	f	II–III	MI > MS	33	16	196	77	3.3
9	f	II–III	MS > MI	42	12	144	50	2.5
10	f	II–III	MI > MS	67	16	82	50	2.3
11	f	II–III	MI = MS	57	6	131	51	2.9
12	f	II–III	MS > MI	59	10	146	60	2.6
Mean ± s.e.mean				60 ± 3.6	13.3 ± 1.4	157.3 ± 13.8	54.3 ± 3.3	2.5 ± 0.1
13	f	IV	iCM	47	19	178	30	1.7
14	f	IV	dCM	49	12	200	26	2.2
15	m	IV	dCM	37	34	243	26	2.3
16	m	IV	dCM	51	16	268	21	2.5
17	m	IV	iCM	48	20	238	19	2.7
18	m	IV	dCM	47	22	395	22	2.4
19	m	IV	dCM	23	26	244	22	2.1
20	m	IV	dCM	48	34	220	20	1.1
21	m	IV	dCM	27	28	278	19	2.2
22	m	IV	dCM	56	22	292	18	2.6
23	m	IV	iCM	48	15	178	28	1.9
24	m	IV	dCM	36	22	596	15	2.6
Mean ± s.e.mean				43 ± 2.9*	22.5 ± 2.0*	277.5 ± 33.5*	22.2 ± 1.3*	2.3 ± 0.1*

* $P < 0.05$ vs NYHA II–III.
NYHA II–III: moderate heart failure; NYHA IV: severe heart failure; m: male; f: female; MI: mitral incompetence; MS: mitral stenosis; iCM: ischaemic cardiomyopathy; dCM: dilated cardiomyopathy; age in years; LVEDP: left ventricular end diastolic pressure (mmHg); LVEDV: left ventricular end diastolic volume (ml); EF: ejection fraction (%); CI: cardiac index ($l\,min^{-1}\,m^{-2}$).

human skinned myocardial fibres was also investigated.

Methods

The experiments were performed on human isolated, electrically stimulated, papillary muscle strips. Tissue was obtained during either mitral valve replacement or heart transplantation. For this study, patients have been grouped as having moderate ('NYHA II–III') or severe ('NYHA IV') heart failure. Patients with heart failure were classified as NYHA II–III on the basis of clinical symptoms and signs as judged by the attending cardiologist shortly before operation. This group of patients with moderate heart failure was not further divided into NYHA II and NYHA III because of the subjective nature of this division, especially in mitral valve disease. Patients suffering from heart failure class NYHA IV were studied for comparison. The underlying disease was dilated cardiomyopathy. All patients gave written informed consent before surgery. All patients receiving β-adrenoceptor antagonists or catecholamines were withdrawn from the study. Medical therapy consisted of diuretics, nitrates and cardiac glycosides in patients with NYHA II–III and NYHA IV. All patients with NYHA IV also received enalapril. All experiments were performed on fresh tissue. The clinical diagnosis and the age of all patients are summarized in Table 1.

Radioligand binding experiments

Papillary muscles (0.8–1.0 g wet weight) were chilled in 30 ml ice-cold homogenization buffer (10 mmol l^{-1} Tris-HCl, 1 mmol l^{-1} EDTA, pH 7.4). Connective tissue was removed and myocardial tissue was minced with scissors and homogenized with a motordriven glass-teflon homogenizer for 1 min at 400 r.p.m. The membrane preparation was further homogenized by hand for 1 min with a glass-glass

homogenizer. This homogenate was diluted with an equal volume of ice-cold 1 mol l^{-1} KCl and stored on ice for 10 min. The solution was centrifuged for 45 min at 100,000 *g*. The pellet was resuspended in 50 volumes of incubation buffer (50 mmol l^{-1} Tris-HCl, 10 mmol l^{-1} $MgCl_2$, pH 7.4) and homogenized for 1 min with a glass-glass homogenizer. This suspension was recentrifuged at 100,000 *g* for 45 min and the pellet was used as a source of membranes after resuspension in incubation buffer (50 volumes). β-Adrenoceptors in cardiac tissue were investigated using [^{3}H]-CGP-12177 ((−)-4-(3-*t*-butylamino-2-hydroxy-propoxy)-[5,7-^{3}H]benzimidazol-2-one)) as radiolabelled ligand (specific activity 51 Ci mmol $^{-1}$). Homogenate (150 μg per tube) was incubated with different concentrations of [^{3}H]-CGP-12177 (0.05–10.0 nmol l^{-1}) at 37°C for 60 min. The total assay volume was 250 μl. These conditions allowed complete equilibration of the receptor with the radioligand. The reaction was terminated by rapid vacuum filtration through Whatman GF/C filters. The filters were washed immediately three times with 6 ml of ice-cold incubation buffer. Filters were dried at 90°C and placed in 10 ml scintillation fluid (Qickszint 501, Zinsser analytics, Frankfurt, F.R.G.) and radioactivity determined in a liquid scintillation counter. All determinations were performed in triplicate. Specific binding was defined as the difference in binding in the absence and the presence of 3 μmol l^{-1} (−)-propranolol. The maximal density (B_{max}) and apparent affinity (K_D) of binding sites were obtained in individual experiments from Scatchard plots determined by linear regression analysis. Protein concentration was determined according to Lowry *et al.* (1951) using bovine serum albumin as standard.

Contraction experiments

Left ventricular papillary muscle strips were attached to a bipolar platinum electrode and suspended in a modified Tyrode solution (pH 7.4) containing (mmol l^{-1}): NaCl 136.9, KCl 5.4, $MgCl_2$ 1.05, $CaCl_2$ 1.8, $NaHCO_3$ 22.6, NaH_2PO_4 0.42, glucose 5.5, ascorbic acid 0.28, Na_2-EDTA 0.05. The temperature was maintained at 35°C and the Tyrode solution was bubbled with 95% O_2 and 5% CO_2. Isometric contractions were measured with an inductive force transducer (W. Fleck, Mainz, F.R.G.) attached to a Hellige Helco Scriptor or Gould recorder. Each muscle was stretched to the length at which force of contraction was maximal. The resting force (approximately 5 mN) was kept constant throughout the experiment. The preparations were stimulated using field stimulation (frequency 1 Hz, duration 5 ms, intensity 10–20% above threshold) and were allowed to equilibrate to allow complete mechanical stabilization before drug addition. From every specimen 6–10 strips were prepared. Different agents were studied in strips from one heart.

All compounds were applied in a cumulative fashion. Isoprenaline and Ca^{2+} were present for 5 min, milrinone for 10 min and DPI 201–106 for 60 min at each concentration. During this time the positive inotropic effect of the compounds had reached equilibrium at each concentration. In experiments involving adenosine or carbachol, the drugs were added after peak effects had been obtained with DPI 201-106 or isoprenaline. The incubation time was 5 min at each concentration. Only one concentration-contractile response curve was obtained in each preparation.

Skinned fibres

Left ventricular papillary muscles from patients with severe heart failure (NYHA IV) were chemically skinned in solution A containing: 50% glycerol, 20 mmol l^{-1} imidazole, 10 mmol l^{-1} NaN_3, 5 mmol l^{-1} ATP, 5 mmol l^{-1} $MgCl_2$, 4 mmol l^{-1} EGTA, 2 mmol l^{-1} dithioerythrit, pH 7.0 at 4°C for 1 h. The preparations were then incubated for 24 h in solution A containing 1% Triton-X 100 at 4°C before storage at −20°C in solution A without detergent. Tissues were mounted and isometric contractions recorded using a force transducer (AME 801, Senso Nor, Horten, Norway). Fibres were slightly stretched (sarcomere length ~2 μm) in relaxation solution containing (mmol l^{-1}): ATP 5, $MgCl_2$ 6, EGTA 5, HEPES 50, NaN_3 5, phosphocreatine 10 and 380 u ml^{-1} creatine kinase (Boehringer, Mannheim, F.R.G.) at pH 7.0. The contraction solution had the same composition as the relaxation solution except that EGTA was substituted by 5 mmol l^{-1} Ca-EGTA. The desired free Ca^{2+} concentration was obtained by mixing appropriate proportions of relaxation and contraction solutions. The free Ca^{2+} concentration was calculated according to Fabiato & Fabiato (1979).

Materials

Drugs used were: (±)-isoprenaline-HCl (Boehringer Ingelheim, F.R.G.), adenosine (Boehringer Mannheim, F.R.G.) and carbachol (Sigma Chemical Co München, F.R.G.). DPI 201-106 (4-(3-(4-diphenylmethylpiperazine-1-yl)-2-hydroxypropoxy)-1H-indole-2-carbonitrile) was kindly provided by Professor Scholtysik from Sandoz Coop (Basel, Switzerland). Milrinone was supplied by Sterling-Winthrop (Hamburg, F.R.G.). DPI 201-106 was dissolved in an equimolar solution of tartaric acid and 10% N-methyl-pyrrolidine. Milrinone was dissolved in dimethylformamide and further diluted in bathing solution.

All other compounds were freshly dissolved in Tyrode or contraction solution and did not change the pH of the medium. All other chemicals were of analytical or best commercial grade available.

Statistics

The data are shown as means ± s.e.m. and statistical significance was estimated with Student's t test for paired or unpaired observations. A P value of less than 0.05 was considered significant. The drug concentrations producing 25% inhibition (IC_{25}) or 50% of the maximal effect (EC_{50}) were determined graphically in each individual experiment. The IC_{25} and EC_{50} values are given with 95% confidence limits. In experiments on skinned fibres, concentration-response curves to Ca^{2+} were fitted by nonlinear regression to the Hill equation. In these experiments, the EC_{50} values for Ca^{2+} are given as pCa.

Results

Cardiac β-adrenoceptors

In myocardial membranes from 5 patients with NYHA II–III and 5 patients with NYHA IV the density of cardiac β-adrenoceptors was compared. There was a significant reduction ($P < 0.05$) in the number of cardiac β-adrenoceptors in NYHA IV (Figure 1). No difference in the antagonist affinity of [^{3}H]-CGP 12177 was observed.

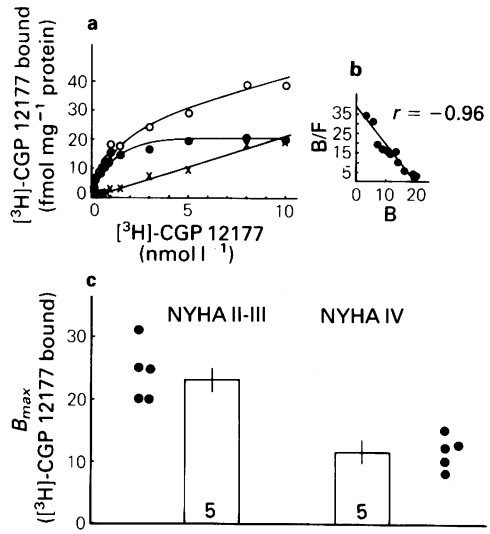


Figure 1 (a) Binding of [^{3}H]-CGP 12177 to cardiac membranes of a patient with mitral valve disease (NYHA II–III). (b) Linear transformation of the saturation experiment. [^{3}H]-CGP 12177 bound mg^{-1} protein is plotted as a function of the ratio of bound to free (B/F) of [^{3}H]-CGP 12177 (nmol l^{-1}). The intercept with the abscissa scale is the density of binding sites (B_{max}), the slope is the apparent affinity (K_D). Each point represents the mean of triplicate observations. (c) Density of β-adrenoceptors in membranes from patients with moderate (NYHA II–III) and severe (NYHA IV) heart failure. Ordinate scale: maximal number of binding sites in fmol [^{3}H]-CGP 12177 bound mg^{-1} protein. In (a), (○) total binding, (●) specific binding, (×) non-specific binding. Binding experiments were performed in patients numbers 2, 4–7, (NYHA II–III) and 14–18 (NYHA IV) of Table 1.

Effects of DPI 201-106 and Ca^{2+}

DPI 201-106 increased force of contraction in a concentration-dependent manner in human isolated papillary muscle strips (Figure 2). The effect was more pronounced in preparations from patients with severe heart failure (NYHA IV) than in preparations from patients with moderate heart failure. The positive inotropic effect of Ca^{2+} was measured in the same preparations in order to investigate the maximal increase in each individual preparation. The Ca^{2+} concentration-response curve was determined before DPI 201-106 because the positive inotropic effect of DPI 201-106 was not reversed after washout with drug-free bathing solution. The positive inotropic effect of Ca^{2+} was not different in NYHA II–III and NYHA IV. DPI 201-106 maximally increased force of contraction to 24 ± 3% and 41 ± 5% of the maximal increase induced by 15 mmol l^{-1} Ca^{2+} in NYHA II–III and NYHA IV, respectively ($P < 0.05$). The EC_{50} value was significantly ($P < 0.05$) higher in NYHA II–III (1.2 (0.96–1.4) μmol l^{-1}, $n = 7$) than in NYHA IV (0.7 (0.62–0.81) μmol l^{-1}, $n = 6$). The positive inotropic effect reached maximum after about 60 min at each concentration. The solvent (tartaric acid and N-methylpyrrolidine) reduced force of contraction by about 20–25% within 60 min (not shown). In this respect, it is noteworthy that in 4 preparations from NYHA II–III (out of 14 preparations) no effect of DPI 201-106 was observed. In NYHA IV, DPI 201-106 increased force of contraction in all preparations. The positive inotropic effect of isoprenaline was reduced in NYHA IV compared with NYHA II–III by about 50%. Similar results were obtained with the phosphodiesterase inhibitor milrinone (Figure 2).

Effects of adenosine and carbachol

The effects of adenosine and carbachol on the positive inotropic effects of DPI 201-106 or isoprenaline

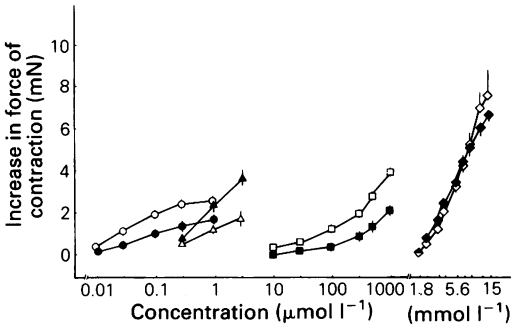


Figure 2 Cumulative concentration-response curves for the effects of, isoprenaline (○, ●), milrinone (□, ■), DPI 201–106 (△, ▲) and Ca^{2+} (◇, ◆) in isolated, electrically driven (1 Hz) papillary muscle strips from patients with moderate (open symbols, NYHA II–III) and severe (solid symbols, NYHA IV) heart failure. In the experiments with DPI 201-106, the solvent (tartaric acid and 10% N-methylpyrolidine) decreased force of contraction by 18.3 ± 8.5% ($n = 7$) in NYHA II–III and 18.4 ± 7.6% ($n = 6$) in NYHA IV ($n = 5$). Basal values for force of contraction were: NYHA II–III: 1.9 ± 0.2 mN ($n = 39$; from 12 hearts) NYHA IV: 1.7 ± 0.2 mN ($n = 45$, from 10 hearts).

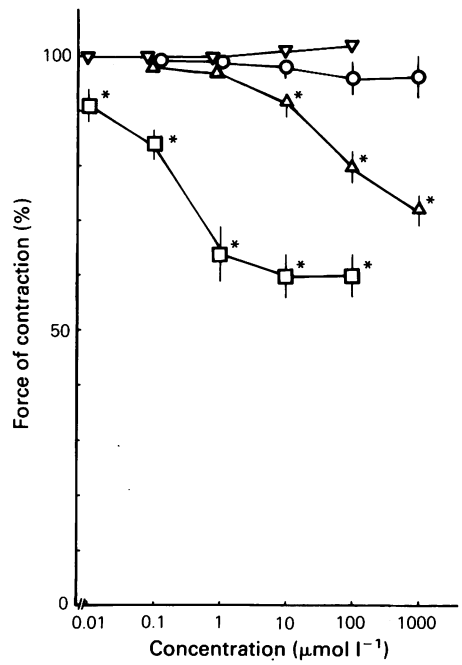


Figure 3 Cumulative concentration-response curves for the effect of adenosine (0.1–1000 μmol l^{-1}) and carbachol (0.01–100 μmol l^{-1}) in the presence of DPI 201–106 (3 μmol l^{-1}) or isoprenaline (0.01 μmol l^{-1}) in isolated, electrically driven (1 Hz) papillary muscle strips from patients with moderate heart failure (NYHA II–III). (○) Adenosine + DPI 201-106 ($n = 7$), (▽) carbachol + DPI 201-106 ($n = 7$), (△) adenosine + isoprenaline, (□) carbachol + isoprenaline. Ordinate scale: force of contraction in % of the value before addition of adenosine or carbachol. Abscissa scale: concentration of adenosine or carbachol in μmol l^{-1}. Basal force of contraction was 3.1 ± 0.5 mN ($n = 34$). Isoprenaline and DPI 201-106 increased force of contraction to 216 ± 13% predrug value ($n = 20$) and to 186 ± 24% predrug value ($n = 14$), respectively. Asterisks denote significant differences ($P < 0.05$) from the values before addition of adenosine or carbachol.

in papillary muscle strips from NYHA II–III patients are illustrated in Figure 3. Both adenosine and carbachol failed to reduce the positive inotropic effect of DPI 201-106. In contrast, concentration-dependent, negative inotropic responses were obtained with adenosine and carbachol after stimulation with isoprenaline. The IC_{25} values were 152 (102–335) μmol l^{-1} ($n = 10$) for adenosine and 0.3 (0.2–0.6) μmol l^{-1} ($n = 10$) for carbachol, respectively. Adenosine and carbachol alone did not reduce force of contraction (not shown).

Effect of DPI 201-106 on Ca^{2+} sensitivity of skinned fibres

Figure 4a shows that 1 μmol l^{-1} DPI 201-106 did not shift the Ca^{2+} concentration-response curve obtained in skinned fibre preparations. The EC_{50} values obtained in the presence (5.82 ± 0.02 pCa) or absence (5.81 ± 0.02 pCa) of DPI 201-106 (cf. Figure 4b) were not significantly different. Furthermore, the effects of various concentrations of DPI 201-106 (0.0001–10 μmol l^{-1}) were studied in the presence of 1.44 μmol l^{-1} Ca^{2+} (approximate EC_{50} value). The original recording (Figure 5a) and the concentration-response curves (Figure 5b) for all concentrations of DPI 201–106 showed that DPI 201-106 failed to increase tension development of the fibres. Trifluoperazine (100 μmol l^{-1}), which is known to increase the sensitivity of skinned fibres to Ca^{2+} (Tkachuk *et al.*, 1987), increased tension development by about 50%.

Discussion

DPI 201-106 increased force of contraction concentration-dependently in human myocardial tissues. In preparations from patients with severe

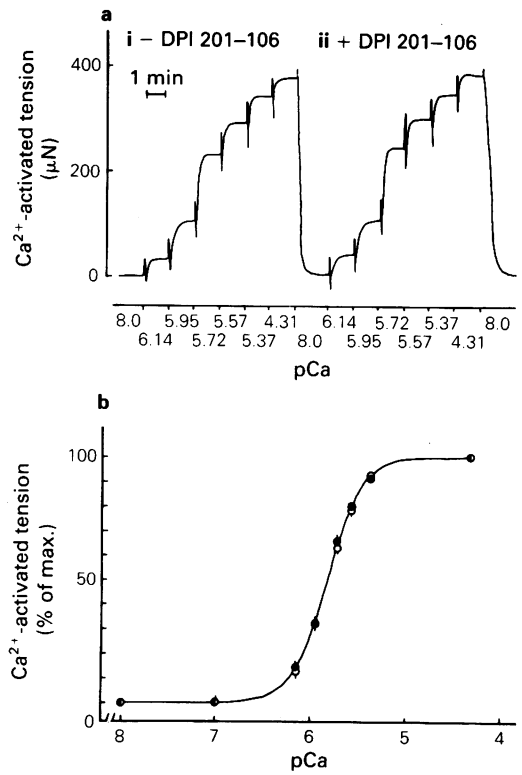


Figure 4 (a) Original records illustrating developed force of a chemically skinned fibre after exposure to increasing concentrations of Ca^{2+} with (ii) and without (i) $1\,\mu mol\,l^{-1}$ DPI 201-106 and (b) concentration-response curve for Ca^{2+} alone and in the presence of $1\,\mu mol\,l^{-1}$ DPI 201-106. In control experiments, the same concentration of solvent (tartaric acid and N-methylpyrrolidin) was present. Fibres were prepared from hearts of patients with severe heart failure (NYHA IV). (○) –DPI 201-106, (●) $+1\,\mu mol\,l^{-1}$ DPI 201-106. Experiments were performed on 7 fibres from 4 hearts. Ordinate scales: Ca^{2+}-activated tension in mN or as % of maximally developed tension. Abscissae: concentration of Ca^{2+}.

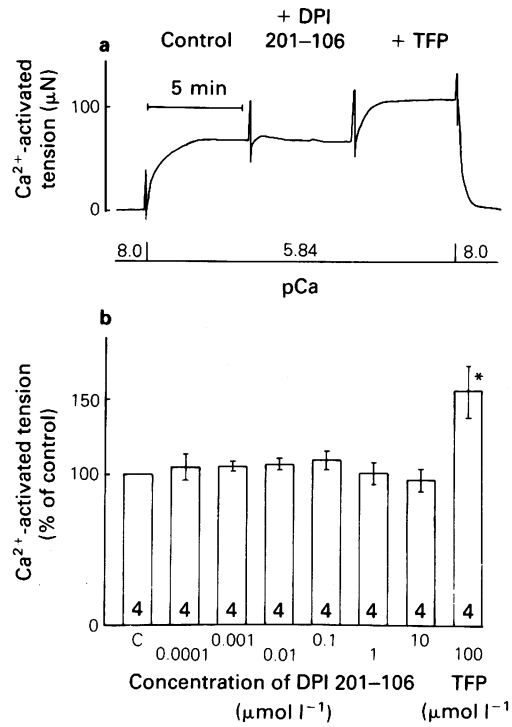


Figure 5 (a) Original record illustrating the developed force of a chemically skinned fibre after exposure to Ca^{2+} (pCa 5.84, DPI 201-106 ($1\,\mu mol\,l^{-1}$) and trifluoperazine (TFP, $100\,\mu mol\,l^{-1}$). (b) Effect of DPI 201-106 ($0.0001–10\,\mu mol\,l^{-1}$) and trifluoperazine ($100\,\mu mol\,l^{-1}$) at $1.44\,\mu mol\,l^{-1}$ Ca^{2+} on developed force of chemically skinned fibres. Values are given as % of the value before addition of DPI 201-106 or trifluoperazine. Experiments were performed in 4 fibres from 4 hearts with severe heart failure. Ordinates: Ca^{2+}-activated tension in $\mu mol\,l^{-1}$ or % of control. Abscissae: concentration of Ca^{2+}, DPI 201-106 or trifluoperazine. Asterisk denotes significant difference ($P < 0.05$) from control.

heart failure of functional class NYHA IV, this effect was more pronounced than in tissue from patients with moderate heart failure. In similar preparations from patients with heart failure functional class NYHA IV, the positive inotropic effects of isoprenaline and milrinone were reduced. Consistently, the number of cardiac β-adrenoceptors was also reduced. Adenosine and carbachol did not reduce the positive inotropic effect of DPI 201-106 but did reduce that of isoprenaline. DPI 201-106 had no effect on Ca^{2+}-sensitivity of skinned fibres.

In the failing heart, there is a reduced number of cardiac β-adrenoceptors (Bristow *et al.*, 1982). This and other alterations, such as an increase of the inhibitory guanine-nucleotide binding protein g_i (Feldman *et al.*, 1987), impair the effectiveness of positive inotropic agents such as β-adrenoceptor agonists (Bristow *et al.*, 1982; Böhm *et al.*, 1988a) and phosphodiesterase inhibitors (Feldman *et al.*, 1987; Böhm *et al.*, 1988c) in the human failing heart. The number of β-adrenoceptors and the positive inotropic effects of isoprenaline and milrinone were reduced in NYHA IV compared to NYHA II–III. The age of the patients is considerably different in

NYHA II–III and NYHA IV (Table 1). Therefore, one might argue that aging has an influence on the density of β-adrenoceptors or the inotropic responses. However, it has been shown previously in a larger number of patients that there is no correlation between the number of β-adrenoceptors or the effectiveness of inotropic stimulation to the age of patients with heart failure (Böhm *et al.*, 1988c). Moreover, patients with moderate heart failure (NYHA II–III) suffered from different valve lesions (Table 1). However, from another series of experiments it became evident that the reduction of β-adrenoceptors is related to the severity but not the underlying cause of heart failure (Erdmann & Böhm, 1988). Therefore, these findings indicate that our experimental model is sensitive to detect differences in inotropic responsiveness between the degrees of heart failure of functional class NYHA II–III and NYHA IV.

In the present study, DPI 201-106 increased force of contraction in both NYHA II–III and NYHA IV. The positive inotropic effect was slightly more pronounced in NYHA IV than in NYHA II–III. Similar results were observed in animal models of cardiomyopathy. In Syrian hamsters with cardiac hypertrophy (Salzmann *et al.*, 1986) or in pressure overloaded ferret hearts (Hajjar *et al.*, 1987), DPI 201-106 causes a greater increase in force of contraction than in hearts from the respective control animals. However, in spontaneously hypertensive rats (SHR) with cardiac hypertrophy, the positive inotropic effect of DPI 201-106 was similar to control rats but greater than the effect of isoprenaline (Böhm *et al.*, 1988b). The present study indicates that a significant responsiveness to DPI 201-106 occurs in severe human heart failure as in the above mentioned animal models for cardiomyopathy. This is different from the effects of positive inotropic agents such as isobutylmethylxanthine (Böhm *et al.*, 1988a) or milrinone (Feldman *et al.*, 1987; Böhm *et al.*, 1988c) which are less active in tissues from severe heart failure patients. The positive inotropic effects of DPI 201-106 in severe heart failure (NYHA IV) has also been shown to be more pronounced than that of dobutamine and isobutylmethylxanthine (Böhm *et al.*, 1988a).

In patients with dilated cardiomyopathy (NYHA III–IV), there was a marked improvement of haemodynamic parameters after injection of DPI 201-106 intravenously (Thormann *et al.*, 1986). Moreover, there was a greater number of responders to DPI 201-106 than to dobutamine. A recent double blind, randomized study on orally administered DPI 201-106 showed a significant, plasma level-related, improvement of stroke work index in patients with ischaemic or idiopathic cardiomyopathies (Kostis *et al.*, 1987). The findings presented in this study provide evidence that the improvement of haemodynamic measurements could be due to direct positive inotropic effects on the heart rather than pre- or afterload reduction. This is in line with experiments on whole animals in which only minor effects of DPI 201-106 on peripheral resistance were observed (Salzmann *et al.*, 1986).

Beside a reduction of β-adrenoceptors there are endogenous inhibitory mechanisms which might further limit the effectiveness of positive inotropic stimulation in the human heart. Adenosine is released from the heart during increased cardiac workload (Foley *et al.*, 1978), ischaemia (Fox *et al.*, 1974), catecholamine exposure (Schrader *et al.*, 1977) and heart failure (Newman *et al.*, 1984). It reduces positive inotropic effects of adenylate cyclase stimulating agents (Schrader *et al.*, 1977) and phosphodiesterase inhibitors (Schmitz *et al.*, 1985) in the mammalian heart including man (Böhm *et al.*, 1985). The effects of adenosine are shared by M-cholinoceptor agonists (Watanabe & Besch, 1975) such as carbachol. Moreover, in the failing heart, there is an increase of the 40 kDa pertussis toxin substrate G_i (Feldman *et al.*, 1988). Increased levels of these regulatory components are likely to produce augmented effects after stimulation of inhibitory receptors such as A_1-adenosine receptors or M-cholinoceptors on force of contraction in heart failure. Both adenosine and carbachol reduced the positive inotropic effect of isoprenaline in papillary muscle strips from heart failure patients. No effect of these agents could be observed in the presence of DPI 201-106. Hence, a reduction of the positive inotropic effectiveness of DPI 201-106 by inhibitory mechanisms is most unlikely to occur in the human heart. The lack of inhibition could contribute to the greater effectiveness in NYHA IV than NYHA II–III. Moreover, the lack of inhibition by A_1-adenosine receptor or M-cholinoceptor stimulation indicates that the mechanism of action of DPI 201-106 is cyclic AMP-independent.

DPI 201-106 has been shown to increase the Ca^{2+} sensitivity of skinned fibres from porcine (Herzig & Quast, 1984) and ferret (Hajjar *et al.*, 1987) hearts. Moreover, an increased sensitization of the contractile proteins for Ca^{2+} by DPI 201-106 was observed in the hypertrophied ferret heart (Hajjar *et al.*, 1987). However, no effect of DPI 201-106 was observed in skinned fibres from failing human hearts. Since the skinning procedures used in this study and in the study of Herzig & Quast (1984) are similar, this discrepancy is most likely explained by species differences. These results indicate that DPI-201 106 does not apparently act through this mechanism in human heart.

In conclusion, DPI 201-106 increases force of contraction in myocardium from patients with NYHA

IV. The positive inotropic effect of DPI 201-106 was more pronounced than the increase in force produced by isoprenaline or milrinone. The inotropic response was unaffected by carbachol or adenosine. It is conceivable that the lack of involvement of cyclic AMP-dependent mechanisms may contribute to the positive inotropic effectiveness of DPI 201-106 in heart failure, during hypoxia or increased workload. A sensitization of the contractile proteins for Ca^{2+} by DPI 201-106 was not observed in the human heart. This indicates that in the human heart the compound primarily acts by another mechanism e.g. prolongation of the open state of the sodium channel.

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The modulation by chlormethiazole of the $GABA_A$-receptor complex in rat brain

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1 The interactions of chlormethiazole with γ-aminobutyric acid (GABA) synthesis and release, and with ligand binding to sites associated with the $GABA_A$-receptor complex and the $GABA_B$-receptor have been studied in the rat. The $GABA_A$-receptor was studied using [^{3}H]-muscimol, [^{3}H]-flunitrazepam was used to label the benzodiazepine modulatory site, and [^{35}S]-butylbicyclophosphorothionate ([^{35}S]-TBPS) to label the chloride channel.

2 Chlormethiazole had no effect on GABA synthesis in the cortex, hippocampus and striatum or on GABA release from cortical slices *in vitro*. Chlormethiazole did not displace [^{3}H]-baclofen binding to the $GABA_B$-receptor.

3 Chlormethiazole (IC_{50} = 140 μM) and pentobarbitone (IC_{50} = 95 μM) both inhibited [^{35}S]-TBPS binding by increasing the rate of [^{35}S]-TBPS dissociation. In addition, chlormethiazole caused an apparent decrease in the affinity of [^{35}S]-TBPS binding.

4 Chlormethiazole enhanced the binding of [^{3}H]-muscimol but had no effect on [^{3}H]-flunitrazepam binding. In contrast, the sedative barbiturate pentobarbitone enhanced both [^{3}H]-muscimol and [^{3}H]-flunitrazepam binding.

5 It is concluded that the sedative and anticonvulsant effects of chlormethiazole are probably mediated through an action at the $GABA_A$-receptor. However, chlormethiazole does not interact with the $GABA_A$-receptor complex in an identical manner to the sedative barbiturate pentobarbitone.

Introduction

Chlormethiazole is used clinically as a sedative, hypnotic and anticonvulsant agent (Evans *et al.*, 1986). Many compounds with this profile of action have been shown to interact with the γ-aminobutyric $acid_A$ ($GABA_A$)-receptor complex, and potentiate responses to GABA (see Simmonds & Turner, 1987 for review). The $GABA_A$-receptor is an oligomeric protein containing an ion channel permeable to Cl^- ions. The activity of the ion channel is modulated not only by the binding of GABA to a specific binding site but also by the binding of several chemically distinct compounds (Schwartz, 1988 for review). Benzodiazepines bind to a specific site on the $GABA_A$-receptor and increase the frequency of the opening of the Cl^- channel (Study & Barker, 1981), whereas barbiturates bind to a distinct and separate site and prolong the length of opening of the channel (Barker & McBurney, 1979). Convulsant compounds such as picrotoxin and the cage convulsants such as *t*-butylbicyclophosphorothionate (TBPS) antagonise the effects of GABA by blocking channel opening. Barbiturates and some related compounds have been shown to interact with the TBPS/picrotoxinin binding site labelled by [^{35}S]-TBPS in brain membranes (Squires *et al.*, 1983). In addition, sedative barbiturates will potentiate the binding of [^{3}H]-muscimol and [^{3}H]-flunitrazepam to the $GABA_A$-receptor (Leeb-Lundberg *et al.*, 1980; Whittle & Turner, 1982; Thyagarajan *et al.*, 1983; Lohse *et al.*, 1987).

Electrophysiological studies have shown that chlormethiazole can interact with $GABA_A$-receptors in a manner apparently analogous to that of barbiturates (Harrison & Simmonds, 1983; Hedlund *et al.*, 1987). In the present study we have examined the effects of chlormethiazole on ligand binding to sites

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associated with the $GABA_A$-receptor in rat brain cortex membranes. In addition, the effect of chlormethiazole on the synthesis of GABA in rat brain and on GABA release from rat cortical tissue prisms has also been examined.

A preliminary account of some of these findings has been presented to the British Pharmacological Society (Cross *et al.*, 1988).

Methods

Animals

Male Lister hooded rats (about 200 g) were used. They were housed in groups of 6 under a 12 h light/dark cycle with food and water available *ad libitum*.

[^{35}S]-TBPS binding

The cerebral cortex was homogenised by an Ultra-Turrax in 50 volumes of 50 mM Tris-HCl pH 7.4 and centrifuged at 20,000 *g* for 20 min at 4°C. The resulting pellet was resuspended in 50 mM Tris-citrate buffer pH 7.4 containing NaCl (100 mM) and centrifuged at 20,000 *g* for 20 min at 4°C. The next resulting pellet was washed a further 4 times by resuspension in 50 mM Tris-citrate, NaCl (100 mM) buffer and centrifuged as previously. The final crude membrane pellet was resuspended in 40 volumes of the buffer and used immediately.

Binding assays contained 5 nM [^{35}S]-TBPS, 160 μl of membrane preparation and displacing agents in a total volume of 200 μl of 50 mM Tris-citrate buffer. Incubations were carried out in plastic microtitre plates, for 90 min at room temperature. Bound ligand was separated by rapid filtration and washing over glass fibre filters by use of a Skatron cell harvester (Hall & Thor, 1979). Non-specific binding was defined as that not displaced by 10 μM picrotoxin.

Saturation studies employed a range of [^{35}S]-TBPS concentrations from 2.5 to 200 nM, achieved by diluting [^{35}S]-TBPS with unlabelled TBPS. For studies of the dissociation of bound [^{35}S]-TBPS, membranes were incubated with 5 nM [^{35}S]-TBPS for 90 min at room temperature. Dissociation was initiated by the addition of unlabelled TBPS (final concentration 5 μM) with or without drugs under study and incubations were filtered at various times thereafter.

[^3H]-flunitrazepam binding

Cerebral cortex was homogenised in 20 volumes of 0.32 M sucrose and centrifuged at 1000 *g* for 10 min at 4°C. The resulting supernatant was centrifuged at 20,000 *g* for 20 min at 4°C. This pellet was washed four times by resuspension in 10 mM sodium phosphate buffer containing 200 mM NaCl pH 7.4.

Binding assays contained membrane preparation, [^{3}H]-flunitrazepam (1 nM final concentration) and drugs in 200 μl 10 mM sodium phosphate buffer, which contained 200 mM sodium chloride pH 7.4. Incubations were performed in plastic microtitre plates, for 30 min on ice and terminated by rapid filtration. Non-specific binding was defined as that not displaced by 10 μM diazepam.

[^3H]-muscimol and [^3H]-(−)-baclofen binding

A frozen osmotically-shocked membrane preparation from rat cortex was used for both [^{3}H]-(−)-baclofen and [^{3}H]-muscimol binding assays.

Frozen cortex was homogenized in 6 volumes ice-cold 10 μM Tris-HCl, pH 7.4, containing 0.32 M sucrose, by use of a Potter Elvehjem homogeniser. The homogenate was centrifuged at 500 *g* for 10 min. The resulting supernatant was stored on ice and the pellet re-homogenised and re-centrifuged twice so that the three pooled supernatants gave a total suspension of 18 volumes per g tissue. The supernatant was centrifuged at 30,000 *g* for 30 min at 4°C and the resulting pellet resuspended in 60 volumes of ice-cold distilled water by use of an Ultra Turrax homogeniser, this suspension was centrifuged and resuspended in distilled water as before. Membrane pellets were harvested by centrifugation at 30,000 *g* for 12 min at 4°C and stored at −20°C for [^{3}H]-(−)-baclofen binding. For [^{3}H]-muscimol binding, after freezing at −20°C overnight, membrane pellets were thawed at room temperature, resuspended in 90 volumes of distilled water and centrifuged at 30,000 *g* for 12 min at 4°C. All membrane pellets were stored at −20°C for up to 2 months before their use in binding assays.

For [^{3}H]-muscimol binding (Williams & Risley, 1979), frozen membrane pellets were thawed, resuspended in ice-cold 50 mM Tris-HCl, pH 7.4 by use of an Ultra-Turrax homogeniser and centrifuged at 30,000 *g* for 10 min at 4°C. The resulting pellet was resuspended in buffer (1–1.5 mg protein ml^{-1}) and used immediately in the assay. The assay, which contained 10 nM [^{3}H]-muscimol and approximately 0.2 mg membrane protein in 200 μl 50 mM Tris HCl (containing 50 mM KCl pH 7.4), was incubated on ice for 10 min and terminated by filtration.

[^{3}H]-baclofen binding was studied by using a modification of the method described by Bowery *et al.* (1983), in particular the binding assay was terminated by filtration rather than centrifugation. Frozen membrane pellets were thawed, resuspended in assay buffer (50 mM Tris-HCl, pH 7.4 containing $CaCl_2$, 2.5 mM) and incubated at room temperature for 45 min. The membranes were centrifuged at 30,000 *g*

for 12 min at 20°C and then washed in assay buffer a further three times, with 15 min incubations between each centrifugation. Final equlibrated membrane pellets were resuspended in assay buffer (1–2 mg protein ml^{-1}) and assayed immediately. The binding assay was performed in 96 well microtitre plates in a total incubation volume of 200 μl, and routinely utilized 30 nM [^{3}H]-baclofen, and tissue homogenate (0.25 mg protein). After 10 min incubation at 25°C the binding was terminated by filtration through glass fibre filters by use of a Skatron semi-automatic cell harvester. Filters were rapidly washed with approximately 1 ml ice-cold assay buffer in a 2 s wash time. After drying, the radioactivity on the filters was measured by liquid scintillation counting. The non-specific binding of both [^{3}Hl-baclofen and [^{3}H]-muscimol was defined with an excess of GABA.

GABA synthesis

The synthesis of GABA in the cortex, hippocampus and striatum was assessed by measuring the accumulation of GABA following an injection of aminooxyacetic acid (AOAA), a method recently described and validated by Green *et al.* (1987). Briefly, animals were injected with chlormethiazole (35 mg kg^{-1}, i.p.). Animals were killed 60 min after administration of AOAA, by exposure of the head to a focussed high intensity microwave beam of power density 0.7 kW for 2.2 s. Cortex, hippocampus and striatum were removed from the brain and the GABA extracted in 2 mM HCl at 120°C for 15 min. Extracts were centrifuged at 5000 *g* for 5 min and the GABA concentration in the resultant supernatants was assayed by a slight modification of the enzymatic-fluorimetric assay of Baxter (1972), as described by Bowdler & Green (1982).

GABA release from rat cortical tissue prisms

Tissue prisms from whole cortex were prepared by cutting at 0.3 mm intervals in 2 planes (45° angle) with a McIlwain tissue chopper. The pooled slices were suspended in 30 ml Krebs bicarbonate buffer, pH 7.4 and bubbled with 95% O_2/5% CO_2 at 37°C. The medium composition was as follows (mM): NaCl 118.5, KH_2PO_4 1.18, $MgSO_4$ 1.18, $NaHCO_3$ 25, glucose 10 and $CaCl_2$ 2.54. 'Basal' release was measured in the presence of 1 mM KCl and 'K^+-evoked' release in 35 mM KCl. Tissue prisms were preincubated at 37°C for 1 h, the buffer being changed every 15 min. The buffer was discarded leaving a packed slice suspension. Aliquots of the slice suspension (60 μl, 1 mg protein) were distributed evenly into baskets made from plastic test tubes with nylon mesh bases. The baskets of slices were preincubated in 1 ml of Krebs buffer for 10 min at 37°C. This, and all subsequent incubations contained 100 μM nipecotic acid. The baskets were blotted and immersed in a second set of vials which contained 1 ml fresh, pregassed buffer (containing drug where appropriate) and incubated for 5 min (basal release). The baskets were then blotted and transferred into another set of vials which contained 35 mM K^+ buffer (containing drug where appropriate) for a further 5 min (K^+-evoked release). After this incubation, the baskets were blotted and put into vials containing 1 ml of 0.1 M NaOH solution, SDS (0.7% w/v) and $NaCO_3$ (2% w/v), to solubilize the slices before analysis of the protein content by a phenol reagent technique (Dawson & Heatlie, 1984). The supernatants (800 μl) were removed and centrifuged at 4000 *g* for 5 min. The concentration of GABA in the supernatants was measured by h.p.l.c. as described by Procter *et al.* (1988).

Chemicals

[^{35}S]-TBPS (70–80 Ci $mmol^{-1}$), [^{3}H]-flunitrazepam (80–85 Ci $mmol^{-1}$), [^{3}H]-muscimol (15–20 Ci $mmol^{-1}$) and unlabelled TBPS were obtained from New England Nuclear, U.K. Phenobarbitone, pentobarbitone, diazepam, picrotoxin and GABA were supplied by Sigma Chemical Co., Poole. Chlormethiazole was obtained from Dr Ulf Lindberg, Astra Alab, Sweden.

Results

Ligand binding to the GABA receptor

Chlormethiazole (100 μM) did not inhibit [^{3}H]-muscimol binding to the $GABA_A$-receptor or [^{3}H]-baclofen binding to the $GABA_B$-receptor. In addition, chlormethiazole (100 μM) did not affect [^{3}H]-flunitrazepam binding (data not shown, but see next section).

[^{35}S]-TBPS binding was fully displaced by GABA, picrotoxin and chlormethiazole (Figure 1). Pentobarbitone was also an effective inhibitor of [^{35}S]-TBPS binding, whereas phenobarbitone was less active (Figure 1).

[^{35}S]-TBPS binding to rat cortical membranes was saturable, the K_D being 70 ± 11 nM. In the presence of GABA, binding affinity was unchanged whereas the number of binding sites was significantly reduced (Table 1), consistent with non-competitive inhibition. Both chlormethiazole and picrotoxin decreased the apparent affinity of binding without significantly affecting the number of binding sites, consistent with an apparent competitive inhibition (Table 1).

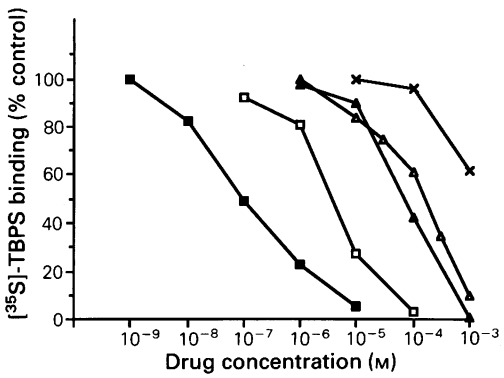


Figure 1 Inhibition of [^{35}S]-butylbicyclophosphorothionate ([^{35}S]-TBPS) binding by γ-aminobutyric acid (GABA) and drugs active at the GABA receptor. (■) picrotoxin, (□) GABA, (▲) pentobarbitone, (△) chlormethiazole, (×) phenobarbitone. Each curve is the mean of 3–4 experiments, s.e.mean for all points was less than 8%. IC_{50} values calculated from Hill plots were: GABA, 4.1 ± 0.4 μM; picrotoxin, 105 ± 20 nM; chlormethiazole, 140 ± 30 μM and pentobarbitone, 95 ± 24 μM.

Under control conditions the dissociation of [^{35}S]-TBPS was biphasic (Figure 2). The addition of chlormethiazole (100 μM and 1 mM) increased the rate of dissociation, in the presence of 1 mM chlormethiazole only a single rapid dissociation was apparent. A similar change in the dissociation kinetics was observed in the presence of 300 μM pentobarbitone (Figure 2).

Stimulation of [^{3}H]-muscimol and [^{3}H]-flunitrazepam binding

Chlormethiazole, like pentobarbitone, stimulated [^{3}H]-muscimol binding in a dose-dependent manner (Figure 3a). Both compounds were approximately

Table 1 Interaction of chlormethiazole with the [^{35}S]-butylbicyclophosphorothionate ([^{35}S]-TBPS) binding site

	[^{35}S]-TBPS binding constants	
Compound	K_D (nM)	B_{max} (fmol)
Control	70 ± 11	126 ± 17
GABA	78 ± 17	78 ± 9*
Picrotoxin	203 ± 32*	165 ± 30
Chlormethiazole	233 ± 72*	142 ± 30

Values are the mean ± s.e.mean of 3 experiments. Binding constants were derived from Scatchard plots using linear regression. *$P < 0.01$ vs control (Student's t test, 2-tailed).

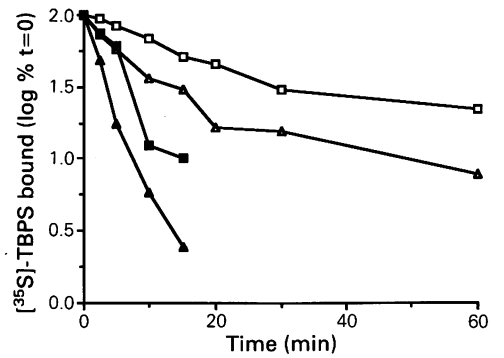


Figure 2 Dissociation of bound [^{35}S]-butylbicyclophosphorothionate ([^{35}S]-TBPS) in the presence of chlormethiazole and pentobarbitone. Membranes were incubated with [^{35}S]-TBPS to equilibrium. Dissociation was initiated by the addition of an excess (5 μM) of unlabelled TBPS, either alone (□) or in the presence of 100 μM chlormethiazole (△), 1 mM chlormethiazole (▲) or 1 mM pentobarbitone (■). The data are from a representative experiment, which was repeated at least 3 times.

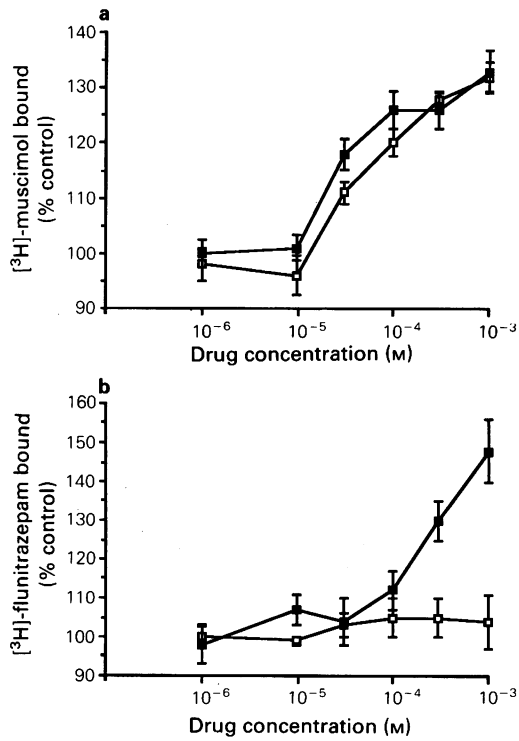


Figure 3 (a) Stimulation of [^{3}H]-muscimol binding by pentobarbitone (■) and chlormethiazole (□). (b) Effects of pentobarbitone (■) and chlormethiazole (□) on [^{3}H]-flunitrazepam binding. Each point is the mean of 3 experiments, vertical lines represent s.e.mean.

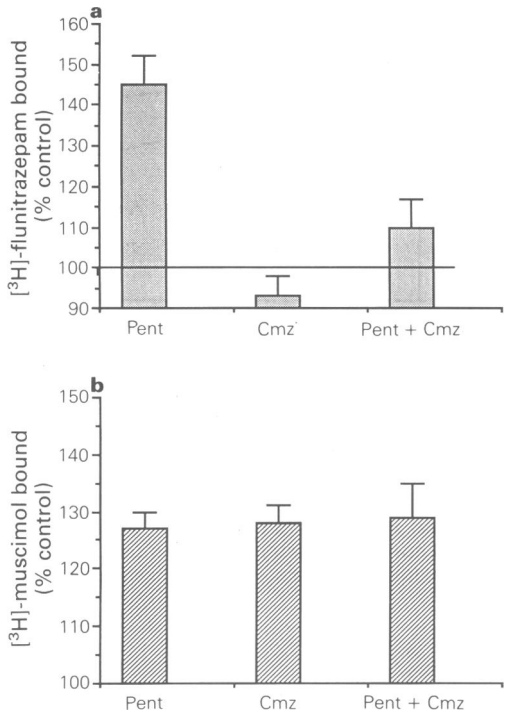


Figure 4 Effects of chlormethiazole (Cmz) in combination with pentobarbitone (Pent) on [^{3}H]-flunitrazepam binding (a) and [^{3}H]-muscimol binding (b). Both drugs were added at a final concentration of 1 mM. Results are the mean of 3 experiments, bars represent s.e.mean.

equipotent. The stimulation of [^{3}H]-muscimol binding by pentobarbitone (1 mM) was not significantly altered by 1 mM chlormethiazole (Figure 4). Pentobarbitone also enhanced [^{3}H]-flunitrazepam binding (Figure 3b). In contrast, chlormethiazole was without effect on [^{3}H]-flunitrazepam binding.

Table 3 The effect of chlormethiazole (Cmz) on γ-aminobutyric acid (GABA) release from rat cortical slices

	Release of endogenous GABA	
	Basal (1 mM K^+)	*Stimulated* (35 mM K^+)
Control	2.08 ± 0.27	5.26 ± 1.2
Cmz (100 μM)	2.17 ± 0.40	6.15 ± 0.78

Values are expressed as pmol GABA mg^{-1} protein $5\,min^{-1}$; mean ± s.d. of 4 experiments. GABA release in the presence of Cmz was not significantly different from controls (Student's *t* test, 2-tailed).

However, chlormethiazole (1 mM) inhibited the stimulation of [^{3}H]-flunitrazepam binding produced by 1 mM pentobarbitone (Figure 4).

The effect of chlormethiazole on GABA synthesis

Acute administration of chlormethiazole ($35\,mg\,kg^{-1}$, i.p.) did not alter the concentration of GABA in the cortex, striatum and hippocampus 40 min later (Table 2). The rate of synthesis of GABA, as indicated by the accumulation of GABA following an injection of aminooxyacetic acid, was also unaffected by chlormethiazole (Table 2).

The effect of chlormethiazole on GABA release

Chlormethiazole (100 μM) had no effect on the release of GABA from cortical slices under basal or depolarising (35 mM K^+) conditions (Table 3).

Discussion

In agreement with a previous study (Ogren, 1986), a single injection of chlormethiazole ($35\,mg\,kg^{-1}$, i.p.) did not alter the concentration of GABA in the

Table 2 The effects of chlormethiazole (Cmz) on basal concentrations and aminooxyacetic acid (AOAA)-induced accumulation of γ-aminobutyric acid (GABA) in rat brain

	Hippocampus		*Striatum*		*Cortex*	
	Control	*Cmz*	*Control*	*Cmz*	*Control*	*Cmz*
Basal	2.13 ± 0.20 (10)	2.03 ± 0.03 (4)	2.74 ± 0.03 (10)	2.76 ± 0.21 (4)	1.46 ± 0.16 (10)	1.37 ± 0.11 (4)
AOAA	4.15 ± 0.35 (8)	3.83 ± 0.46 (8)	4.27 ± 0.70 (8)	4.14 ± 0.61 (9)	3.37 ± 0.56 (8)	3.32 ± 0.60 (8)
Synthesis rate	2.02	1.80	1.53	1.38	1.91	1.95

GABA concentrations are nmol g^{-1} tissue, mean ± s.d. The number of samples is given in parentheses. Synthesis rate is nmol g^{-1} tissue h^{-1}. There were no significant differences between controls and chlormethiazole-treated animals (one way analysis of variance).

brain. Moreover the rate of synthesis of GABA, as estimated by the aminooxyacetic acid method, was also unaltered. Treatment of cortical slices with chlormethiazole (100 μM) was without effect on either the basal or potassium-evoked release of GABA. It would seem therefore that if the effects of chlormethiazole are mediated via the GABA system, this does not involve presynaptic mechanisms.

Chlormethiazole (100 μM) did not inhibit the binding of [^{3}H]-muscimol to the $GABA_A$-receptor or [^{3}H]-baclofen binding to the $GABA_B$-receptor. It is unlikely therefore that chlormethiazole is a direct agonist at either the $GABA_A$- or the $GABA_B$-receptor. However, the results of the present study strengthen the previous suggestions that chlormethiazole interacts with the Cl^- channel of the $GABA_A$-receptor as it was an effective inhibitor of [^{35}S]-TBPS binding. Although the potency of chlormethiazole at the [^{3}H]-TBPS binding site appears weak in comparison with many other drug-receptor interactions, serum concentrations of chlormethiazole can reach 100–200 μM at hypnotic doses (Kim & Khanna, 1983). The IC_{50} of chlormethiazole in [^{35}S]-TBPS binding (140 μM) was very similar to the potency of chlormethiazole in potentiating the electrophysiological response to GABA in rat cuneate nucleus (Harrison & Simmonds, 1983) and cultured rat spinal cord neurones (Hedlund *et al.*, 1987). The affinity of chlormethiazole in [^{32}S]-TBPS binding was similar to that of pentobarbitone (see legend to Figure 1).

Activity at [^{35}S]-TBPS binding clearly does not relate to anticonvulsant activity, as phenobarbitone is an effective anticonvulsant but is virtually inactive in [^{35}S]-TBPS binding, while pentobarbitone had a potency similar to chlormethiazole. It has been noted by others that the sedative actions of barbiturates may correlate with their activity at the site on the $GABA_A$-receptor labelled with [^{35}S]-TBPS (Whittle & Turner, 1982). By analogy with these barbiturates it would seem likely that the interaction of chlormethiazole with the [^{35}S]-TBPS binding site may relate to its sedative effect. This conclusion is strengthened by observations that the sedative effects of chlormethiazole are potentiated by muscimol and aminooxyacetic acid (Ogren, 1986).

Saturation analysis suggested that both picrotoxin and chlormethiazole were competitive inhibitors of [^{35}S]-TBPS binding. This effect of chlormethiazole differs from that obtained for sedative barbiturates which lower the B_{max} of [^{35}S]-TBPS (Ticku & Ramanjaneyulu, 1984; Lohse *et al.*, 1987). The dissociation experiments demonstrate, however, that the interaction of chlormethiazole with the [^{35}S]-TBPS binding site is not truly competitive. Thus chlormethiazole increased the dissociation rate of bound [^{35}S]-TBPS suggesting an allosteric interaction. A similar effect has been noted previously for the sedative barbiturate, methohexitone (Lohse *et al.*, 1987), and in the present study with pentobarbitone. In this respect chlormethiazole seems to be acting in a similar manner to the sedative barbiturates. The studies on the enhancement of [^{3}H]-muscimol and [^{3}H]-flunitrazepam binding by chlormethiazole suggest that these interactions of chlormethiazole may well be distinct from that of the sedative barbiturates. Thus, whilst both chlormethiazole and pentobarbitone enhanced [^{3}H]-muscimol binding, in contrast to pentobarbitone, chlormethiazole had no effect on [^{3}H]-flunitrazepam binding (Figure 3b). It has been shown previously that chlormethiazole does not enhance [^{3}H]-diazepam binding (Leeb-Lundberg *et al.*, 1981). Moreover, when added in combination, 1 mM chlormethiazole effectively antagonised the stimulation of [^{3}H]-flunitrazepam binding induced by 1 mM pentobarbitone.

In conclusion, the present study provides *in vitro* evidence for the interaction of chlormethiazole with the $GABA_A$-receptor. This interaction may account for the sedative properties of chlormethiazole, and is similar, but not identical to the effects of barbiturates.

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Chronotropic and inotropic actions of amrinone, carbazeran and isobutylmethyl xanthine: role of phosphodiesterase inhibition

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1 The chronotropic and inotropic effects of amrinone, carbazeran and 3-isobutyl-1-methyl xanthine (IBMX) were examined in isolated preparations of papillary muscle and right atria from rabbit heart. The effects of the drugs on cardiac phosphodiesterase and cyclic nucleotide content were also examined.

2 Amrinone (2.4×10^{-4} M–2×10^{-3} M), carbazeran (9.1×10^{-6} M–1.2×10^{-3} M), and IBMX (1.8×10^{-5} M–4.5×10^{-4} M) produced concentration-dependent positive inotropic responses of papillary muscle preparations, the rank order of potency being carbazeran = IBMX > amrinone. Sub-threshold positive inotropic concentrations of all three compounds potentiated the positive inotropic effects of isoprenaline; leftward shifts in the concentration-effect curves were 5 fold (IBMX), 11 fold (amrinone) and 46 fold (carbazeran).

3 Amrinone and IBMX produced concentration-dependent positive chronotropic responses in isolated right atria and showed a similar rate selectivity to isoprenaline, but carbazeran elicited a decrease in beating frequency. None of these drugs potentiated the positive chronotropic effects of isoprenaline.

4 Concentrations of amrinone, carbazeran and IBMX that produced similar positive inotropic responses were associated with different increases in papillary muscle cyclic AMP and cyclic GMP concentrations.

5 All three compounds inhibited right atrial and ventricular phosphodiesterase, with amrinone being the least potent. There was, however, a marked difference between the IC_{50} and EC_{50} values for phosphodiesterase inhibition and positive inotropy. In contrast the positive chronotropic effects of amrinone and IBMX were observed in the same concentration ranges that produced phosphodiestrease inhibition.

6 The results indicate that amrinone possesses a similar rate/force selectivity to isoprenaline and IBMX. In contrast, carbazeran exerts both positive inotropic and negative chronotropic effects. Phosphodiesterase inhibition and elevation of intracellular cyclic AMP concentration may be involved, at least in part, in the cardiac effects of these drugs.

Introduction

A variety of cardiotonic agents that exert their pharmacological effects principally through phosphodiesterase inhibition has been described (Farah *et al.*, 1984; Sharpe, 1984; Weishaar *et al.*, 1986). The rationale underlying the use of drugs of this type in the treatment of congestive heart failure is that phosphodiesterase inhibitors have the dual effect of stimulating myocardial activity and lowering pre- and after-load through vasodilatation (Sharpe, 1984; Farah *et al.*, 1984), consequently improving the pumping efficiency of the heart. Despite the intense activity in the development of these newer inotropes, the exact role of cyclic nucleotide generation in the effects of phosphodiesterase inhibitors remains controversial (see Van Belle, 1983; Bowman *et al.*, 1985). Furthermore, some observations have indicated that the relationship between tissue adenosine 3′:5′-cyclic monophosphate (cyclic AMP) concentration and

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cardiac contractility appears to be more complex than was originally assumed (Rodger & Shahid, 1984; England & Shahid, 1987).

It has also been proposed that the chronotropic effects of adrenoceptor agonists are mediated by cyclic nucleotides (Yamasaki *et al.*, 1974; Taniguchi *et al.*, 1977). However, an analysis of the literature indicates that the information available is sparse and relatively little is known about the role of cyclic nucleotides in mediating changes in the frequency of beating.

The aims of the present experiments were to compare and study further the inotropic and chronotropic effects of the two newer compounds, amrinone (Ward *et al.*, 1983) and carbazeran (1-butyl-3-[1-(6,7-dimethoxyquinazoline-4yl)piperidin-4-yl]area) (Smith *et al.*, 1987) with those of 3-isobutyl-1-methyl xanthine (IBMX) (Korth, 1978) in rabbit papillary muscles and spontaneously beating right atria. The effects of these compounds on tissue cyclic AMP and guanosine 3′:5′-monophosphate (cyclic GMP) concentrations and on cardiac phosphodiesterase were also examined.

Methods

Male New Zealand white rabbits (Ranch Rabbits, Sussex) were stunned with a blow to the back of the neck and exsanguinated. The thorax was opened and the hearts rapidly excised and placed in a dish containing warm oxygenated Krebs-Henseleit solution. Papillary muscles were removed from the right ventricle and suspended in an organ bath containing Krebs-Henseleit solution at 32°C. The solution was of the following composition (mmol l^{-1}): NaCl 118, KCl 4.7, $MgSO_4 7H_2O$ 1.2, KH_2PO_4 1.2, $CaCl_2 6H_2O$ 2.5, $NaHCO_3$ 25 and glucose 11.7 and bubbled with a mixture containing 95% O_2 and 5% CO_2. The papillary muscles were mounted vertically between two platinum wire electrodes such that the base of each tissue was in contact with the bottom electrode whilst the tendon end, attached to a force displacement transducer (Grass FTO3C) lay just beneath the upper electrode. Initially the tissues were stretched by adjusting the diastolic tension to 1 g and allowed to stabilize for 60 min (at driving frequency of 1 Hz with pulses of 1 ms duration at a votlage 50–100% above threshold from a Grass S88 stimulator). At the conclusion of this equilibration period, any fall off in diastolic tension was corrected by resetting back to 1 g and stimulation frequency unless otherwise stated, was lowered to 0.4 Hz before the addition of a drug.

In all the experiments that involved the construction of concentration-effect curves to isoprenaline, papillary muscles were first exposed to a maximally effective concentration (2×10^{-6} M) of the agonist. This was done because it is known that the position of the first isoprenaline concentration-effect curve differs substantially from subsequent curves (Clark & Poyser, 1977; Nicholson & Broadley, 1978). Following washout of isoprenaline, a concentration-effect curve was constructed to isoprenaline which was taken as the standard curve. Potential modifiers of isoprenaline responses were added for 20 min after the tissues had fully recovered from this standard isoprenaline response. A second concentration-effect curve to isoprenaline was then constructed in the presence of the modifier. Only one concentration of each drug (modifier) was tested on each preparation.

In experiments in which $CaCl_2$ was used as the inotropic agent, the frequency of stimulation was maintained at 1 Hz but extracellular Ca^{2+} concentration ($[Ca^{2+}]$) was lowered to 2.5×10^{-4} M. The tissues were allowed to equilibrate for 20–30 min until a new steady-state contraction had been established, before the addition of $CaCl_2$ in a cumulative manner. Cumulative concentration-effect curves to $CaCl_2$ were performed before and after treatment with amrinone. Results for both isoprenaline and $CaCl_2$ were expressed as percentages of the corresponding maximum control responses.

Papillary muscle twitch shapes were recorded on an oscilloscope and photographed. The following components of the isometric contraction curve were analysed: time to peak force, relaxation time and total twitch duration.

Chronotropic activity

The chronotropic action was determined using spontaneously beating right atria from male New Zealand white rabbit hearts. The preparations were suspended (diastolic tension 1.5–2.0 g) in Krebs-Henseleit solution as described for papillary muscle experiments. After a stabilisation period of 60 min, cumulative concentration-effect curves to the different drugs were constructed. In the case of isoprenaline the procedure was identical to that described for the papillary muscle experiments. The frequency of beating was measured using a tachograph and monitored on a Grass (Model 7; Quincy, Mass) recorder.

Cyclic nucleotide measurements

These measurements were made in rabbit papillary msucles according to the procedure described in detail previously (Rodger & Shahid, 1984). The cyclic nucleotide levels were measured at the peak of the positive inotropic responses.

Preparation of cardiac phosphodiesterase and measurement of activity

A fresh enzyme preparation was made for each experiment and all procedures were performed at 4°C. Fresh ventricles (1.5–2.5 g) or right atria (0.3–0.5 g), from male New Zealand White rabbit hearts, were trimmed free of fat and connective tissue and chopped into small pieces using scissors. The tissue was homogenised (Polytron; PT20 probe: 2 × 10 s bursts at speed settings 5 and 7) in 10 volumes of buffer A (pH 6.5) containing (mmol l^{-1}): Bis-Tris 20, dithiothreitol (DTT) 1, benzamidine 2, ethylenediaminetetra acetic acid (EDTA) 2, Na-acetate 50 and phenylmethanesulphonylfluoride (PMSF) 0.1. The PMSF was added just before homogenisation. The homogenate was filtered through two layers of cheesecloth and centrifuged at 12000 *g* for 10 min (Damon IEC M60 ultracentrifuge; rotor 410). The supernatant fraction was decanted and used as the source of phosphodiesterase. The pellet fraction was usually discarded. However, for some experiments it was resuspended (to remove soluble phosphodiesterases) in 40–50 ml of buffer A (using a hand held homogeniser). The washed membranes were recollected by centrifugation (12000 *g* for 10 min) and then resuspended in a small volume (8–10 ml) of buffer A, containing 250 mM sucrose. This was used as a source of membrane bound phosphodiesterase. The enzyme preparations were used within 2 h of homogenisation.

Phosphodiesterase activity was measured according to the method described by Methven *et al.* (1980) with minor modifications. The standard assay mixture (total volume 200 μl) contained 3 mM $MgCl_2$, 1 mM 5'-AMP or 5'-GMP, 1 μM ^{3}H-labelled/unlabelled cyclic nucleotide (~200,000 d.p.m. $nmol^{-1}$), 1 mM DTT, 0.05% (w/v) bovine serum albumin in 50 mM Tris-HCl buffer, pH 7.4. The concentration of cyclic nucleotide in stock solutions was determined spectrophotometrically. The reaction was started by the addition of enzyme, and samples were incubated at 37°C for 15 min in a shaking water bath (Grant Instruments) before termination by heat (3 min in aluminium blocks set at 105°C). ^{14}C-labelled cyclic AMP or cyclic GMP (20 μl; 10^4 d.p.m.) was added to each tube immediately before boiling to act as an internal recovery marker. Cyclic nucleotide in the boiled samples was separated from other labelled products by application to alumina columns (chromatographic alumina; 1.5 g dry wt, packed into Pasteur pipettes) which had been equilibrated with 10 ml buffer (0.4 M Tris HCl, pH 7.0). Each tube was washed with 0.2 ml of the same buffer and the washings were applied to the corresponding columns. The columns were allowed to drain directly into scintillation vials and were eluted with a further 2.8 ml (for cyclic AMP) or 3.8 ml (for cyclic GMP) of buffer. The eluted material was dissolved in Picofluor-30 scintillant (Packard) and counted on a Packard RCA 2000 liquid-scintillation spectrometer. The recovery of unchanged cyclic AMP and cyclic GMP was 70–80% and 60–70% respectively. Assays were performed in duplicate and were conducted in the linear range of the reaction, where less than 25% of the initial substrate was hydrolysed. The concentrations producing 50% inhibition of phosphodiesterase activity (IC_{50} values) were determined using an iterative non-linear regression curve fitting program on a PDP 11/44 computer.

Materials

The following substances were used: (±)-isoprenaline hydrochloride (Sigma), IBMX (Aldrich), amrinone, carbazeran and dantrolene were gifts from Sterling Winthrop (UK), Pfizer (UK) and Norwich Pharmacal Company (UK), respectively. Isoprenaline solutions were prepared in acidified (pH 3.5) saline to enhance stability. Carbazeran, amrinone and dantrolene were dissolved in saline, lactic acid (0.5 N) and propylene glycol, respectively, any further dilutions were made in Krebs-Henseleit solution. [8-^{3}H]-adenosine 3':5'-cyclic monophosphate (25–30 Ci $mmol^{-1}$), [8-^{3}H]-guanosine 3':5'-cyclic monophosphate (15–20 Ci $mmol^{-1}$), [U-^{14}C]-adenosine 3':5'-cyclic monophosphate, [U-^{14}C]-guanosine 3':5'-cyclic monophosphate were obtained from Amersham International (Amersham, Bucks, UK). Chromatographic aluminium oxide was obtained from May and Baker (UK). All other chemicals were obtained either from Sigma Chemical Co or BDH Chemicals (both of Poole, Dorset, UK) and were normally of Analar or equivalent grade.

Statistical evaluation

The results are presented as mean values ± s.e.mean. In isolated tissue experiments the EC_{50} values (the concentration of drug producing 50% of its own maximum response) were calculated, from the cumulative concentration-effect curve. The significance of differences between means was determined by Student's *t* test; a *P* value of less than 0.05 was taken as being significant.

Results

Effects on ventricular force and contraction shape

In these experiments, isoprenaline was used as a reference drug to determine the papillary muscle

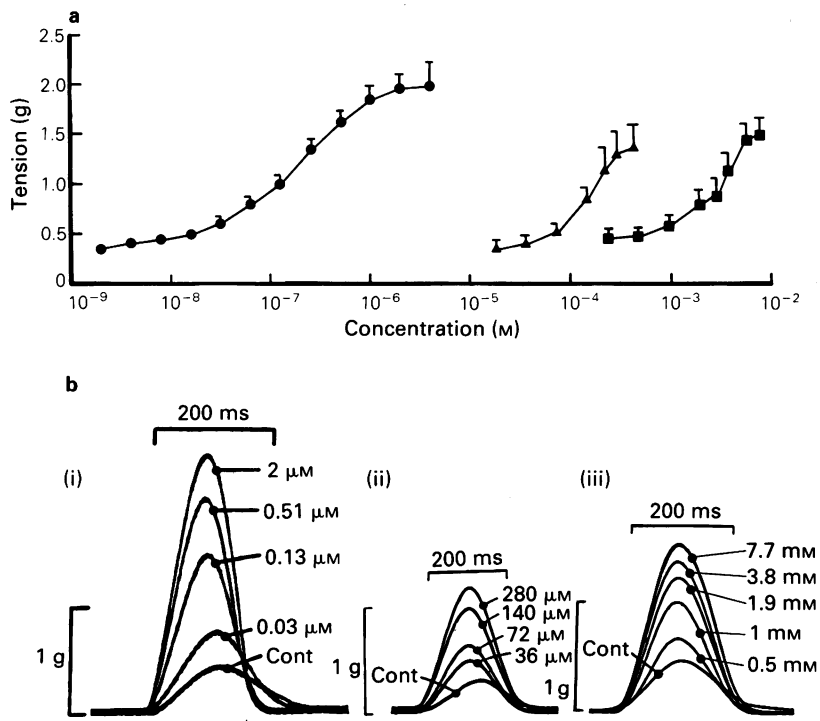


Figure 1 (a) Cumulative concentration-effect curves for the positive inotropic effects of isoprenaline (●), isobutylmethyl xanthine (IBMX, ▲) and amrinone (■) in electrically stimulated papillary muscles (0.4 Hz) of the rabbit. $n = 5$–7. Each point represents the mean and vertical lines indicate s.e.mean. (b) Concentration-related effects of (i) isoprenaline, (ii) IBMX and (iii) amrinone on papillary muscle twitch shape. Cont indicates the control contraction before addition of drug.

response to a full β-adrenoceptor agonist. Isoprenaline produced concentration-dependent increases in the force of contraction (Figure 1), which were rapid in onset (10–15 s) and reached peak effect within 60–180 s after each cumulative addition of the drug. Propranolol (10^{-7} M) competitively antagonised the effects of isoprenaline, producing a 15 fold rightward parallel shift of the concentration-effect curve, without significantly altering the maximum response. IBMX, amrinone (Figure 1) and carbazeran (Figure 2) also produced concentration-related positive inotropic responses. Compared to isoprenaline, the effects of these compounds were slow, the onset of action being 1–2 min after drug addition and 10–20 min elapsed before peak tension was achieved. Propranolol (1×10^{-7} M) did not significantly modify either the inotropic potency or the size of the maximum response produced by IBMX, amrinone or carbazeran, indicating that neither β-adrenoceptors nor noradrenaline release was involved. It is clear from the mean cumulative concentration-effect curves shown in Figures 1a and 2a that IBMX, amrinone and carbazeran were much less potent than isoprenaline at augmenting papillary muscle contractions. The EC_{50} values for the positive inotropic action are shown in Table 1 and indicate a rank order of potency of isoprenaline ≫ carbazeran = IBMX > amrinone. In addition to

Table 1 The cardiac stimulant effects of isoprenaline, isobutylmethyl xanthine (IBMX), amrinone and carbazeran.

	Chronotropic (μM)	*Inotropic* (μM)	*Chronotropic: inotropic ratio*
Isoprenaline	0.022 ± 0.004	0.35 ± 0.04	0.06
IBMX	9.5 ± 1.9	130 ± 20	0.07
Amrinone	295 ± 68	3400 ± 700	0.09
Carbazeran	NC	100 ± 20	NC

Each value is the mean EC_{50} ± s.e.mean, $n = 4$–7.
NC: negatively chronotropic.

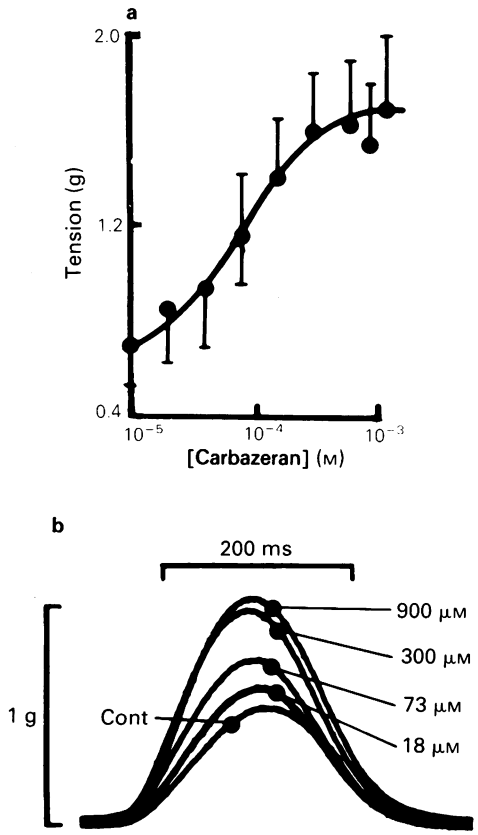


Figure 2 (a) Mean cumulative concentration-effect curve to carbazeran in papillary muscles electrically driven at 0.4 Hz. Each point represents the mean and vertical lines indicate s.e.mean. (b) Typical concentration-dependent effects of carbazeran on papillary muscle twitch shape. The stimulation frequency was 0.4 Hz and the control contraction is indicated by cont.

increasing force of contraction, both isoprenaline and IBMX stimulated the rate of tension generation (positive klinotropic action), reduced the time to peak force, accelerated the rate of relaxation (positive hisotropic action) and decreased total twitch duration in a concentration-dependent manner (Figure 1b). The effects of amrinone on papillary muscle twitch shape were similar to those of isoprenaline and IBMX (Figure 1b), although at higher concentrations ($>1 \times 10^{-3}$ M) the decrease in relaxation time was reduced. In contrast, carbazeran, whilst producing a positive klinotropic response and a reduction in total peak force, had a negative hisotropic action, which resulted in an increase in the total twitch duration (Figure 2b).

Threshold, non-inotropic, concentrations of all three drugs potentiated isoprenaline-induced positive inotropic responses. IBMX (4.5×10^{-6} M), amrinone (6×10^{-4} M) and carbazeran (3.6×10^{-5} M) produced 5, 11 and 46 fold parallel leftward shifts in the position of the isoprenaline concentration-effect curve, respectively. The positive inotropic agents ouabain and vanadate which exert their positive inotropic effects through cyclic AMP-independent mechanisms did not potentiate isoprenaline responses (data not shown).

The effect of amrinone (6×10^{-4} M) on the responsiveness of papillary muscles to [Ca^{2+}] is shown in Figure 3a. The EC_{50} values for Ca^{2+} pre- ($1.3 \pm 0.1 \times 10^{-3}$ M) and post- ($1.1 \pm 0.1 \times 10^{-3}$ M) amrinone are not significantly different, although the maximum response may have increased. Verapamil (10^{-6} M) did not significantly affect the cardiotonic action of amrinone (Figure 3b), which produced a maximum response similar to that obtained in the absence of the verapamil. The mean EC_{50} value for amrinone in these experiments was $1.5 \pm 0.2 \times 10^{-3}$ M. The negative inotropic effect of higher concentrations of verapamil (10^{-5} M) was, however, only partially reversed by amrinone. Possible effects of amrinone on Ca^{2+} mobilisation from intracellular stores were tested using dantrolene (2×10^{-5} M), which is known to inhibit Ca^{2+} flux across the sarcoplasmic reticulum (Morgan & Bryant, 1977). In the presence of dantrolene there was a significant 3 fold rightward shift in the concentration-response curve to amrinone (Figure 3c) (the EC_{50} values before and after dantrolene were $2.2 \pm 0.2 \times 10^{-3}$ M and $7.2 \pm 0.6 \times 10^{-3}$ M, respectively). Dantrolene at the concentration used in these experiments had no apparent effect on developed tension.

Chronotropic effects in isolated right atria

Isoprenaline produced concentration-dependent increases in beating frequency (Figure 4) with an EC_{50} value of 2.2×10^{-8} M. Thus, on a molar basis, it was 16 times more potent when compared to its positive inotropic action. The chronotropic effect was fast in onset (10–15 s) and reached peak effect in 2–3 min. The effect of isoprenaline was blocked by propranolol (10^{-7} M) which caused an 18 fold rightward shift in the concentration-effect curve. IBMX and amrinone also produced concentration-related positive chronotropic responses (Figure 5). These drugs were less potent than isoprenaline and the largest responses produced were 91% and 85% of the isoprenaline maximum for IBMX and amrinone, respectively. The chronotropic responses to IBMX and amrinone were not affected by propranolol. In

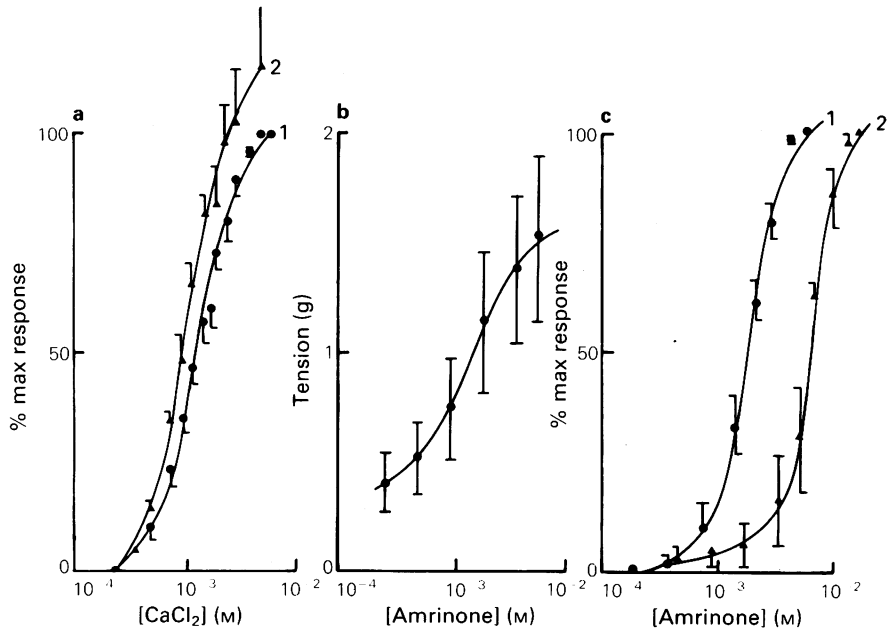


Figure 3 (a) Mean cumulative concentration-effect curves to $CaCl_2$ before (●, 1) and after (▲, 2) a 30 min incubation with amrinone (6×10^{-4} M) in papillary muscles stimulated at a frequency of 1 Hz. Mean ± s.e.mean basal tensions (at 2.5×10^{-4} M $CaCl_2$), before and after amrinone were 90 ± 19 mg and 100 ± 28 mg, respectively ($n = 5$). (b) Mean cumulative concentration-effect curve to amrinone in papillary muscles (0.4 Hz) in the presence of verapamil (10^{-6} M). Verapamil was allowed to act for 30 min before a concentration-response curve was obtained. Mean basal tensions before and after verapamil were 535 ± 160 mg and 335 ± 120 mg, respectively. The mean maximum tension induced by amrinone in the presence of verapamil was 1515 ± 385 mg ($n = 4$). (c) Mean cumulative concentration-effect curve to amrinone before (●, 1) and after (▲, 2) a 30 min incubation with dantrolene (2×10^{-5} M) in papillary muscles (stimulated at a frequency of 0.4 Hz). Mean basal tensions before and after dantrolene were 365 ± 85 mg and 285 ± 65 mg, respectively. The mean ± s.e.mean tension induced by amrinone in the presence of dantrolene was 1350 ± 325 mg ($n = 4$). Each point represents the mean and vertical lines show s.e.mean.

contrast, carbazeran had only a minor positive chronotropic action at low concentrations (23% increase at 3.6×10^{-5} M) and a negative chronotropic action at higher ($> 10^{-4}$ M) concentrations. The latter effect was not significantly modified by atropine (10^{-6} M) indicating the lack of involvement of acetylcholine release. Thus IBMX and amrinone show a clear selectivity for chronotropic activity (approximately the same order of magnitude as isoprenaline), whereas carbazeran was selective for inotropic activity at concentrations below 10^{-4} M (Figure 5, Table 1).

There was no significant parallel shift in the isoprenaline concentration-response curve in the presence of IBMX (2.3×10^{-6} M), carbazeran (3×10^{-5} M) or amrinone (3×10^{-5} M), but carbazeran produced a significant reduction (11%) in the maximum chronotropic response to isoprenaline (Figure 4).

Effects on cyclic nucleotide levels

From the experiments described above, concentrations of carbazeran, IBMX and amrinone producing approximately equieffective inotropic responses were selected for determining the effects of these drugs on cyclic nucleotides. Each concentration of drug was allowed to reach its peak effect (20 min) before papillary muscles were frozen rapidly in liquid nitrogen and analysed for cyclic nucleotide content. The tension and cyclic nucleotide results for these experiments are summarised in Table 2. It is clear that the positive inotropic effects of all three compounds, at the concentrations tested, were associated with concentration-dependent increases in both cyclic AMP and cyclic GMP levels. IBMX and carbazeran both produced similar elevations in cyclic AMP (~4 fold) and cyclic GMP (2–3 fold) content, whereas amrinone produced smaller increases in both cyclic

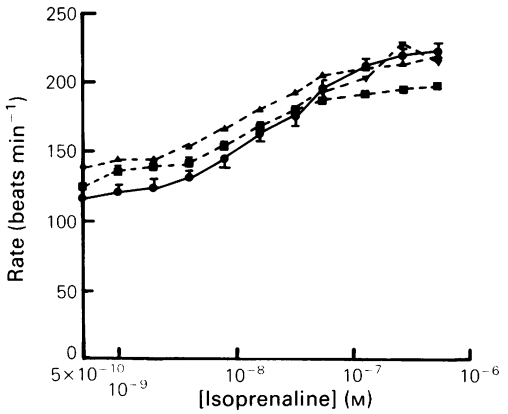


Figure 4 The chronotropic effects of isoprenaline, in rabbit isolated spontaneous right atria, in the absence (●) and presence of isobutylmethyl xanthine (2.3×10^{-6} M; ▲), amrinone (3×10^{-5} M; ▼) and carbazeran (3×10^{-5} M; ■). Each point represents the mean ($n = 4$–6) and vertical lines show s.e.mean.

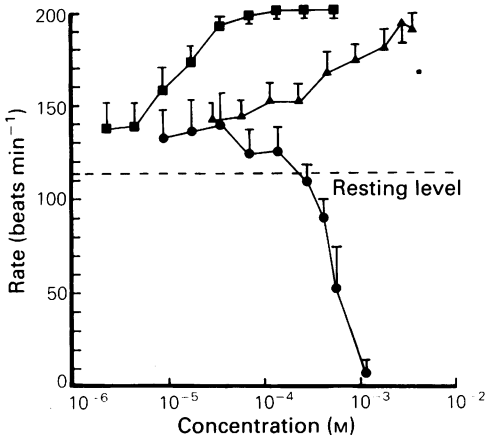


Figure 5 The chronotropic effects of isobutylmethyl xanthine (■), amrinone (▲) and carbazeran (●) in rabbit isolated spontaneously beating right atria. The basal rate was 113 ± 5 beats min^{-1} and each point is the mean of 4–6 determinations. Vertical lines show s.e.mean.

nucleotides. For all three compounds there was a good correlation between the tension response and the absolute values of both cyclic AMP and cyclic GMP level. However, there was no clear relationship between contractile force and the ratio of cyclic AMP to cyclic GMP. A supramaximal concentration of IBMX (4.5×10^{-3} M), which did not elicit any further increase in the maximum developed tension, produced a further increase in the levels of both cyclic AMP (6.66 ± 0.95 pmol mg^{-1}) and cyclic GMP (0.48 ± 0.05 pmol mg^{-1}).

Effects on phosphodiesterase activity

The distribution of phosphodiesterase activity in the rabbit ventricle is shown in Table 3. It is clear that a

Table 2 Effects of isobutylmethyl xanthine (IBMX), carbazeran and amrinone on tension responses and cyclic nucleotide levels in rabbit isolated papillary muscles

Drug	*Concentration* (mol l^{-1})	*Tension* (mg)	*Cyclic AMP* (pmol mg^{-1} tissue)	*Cyclic GMP* (pmol mg^{-1} tissue)	*Ratio Cyclic AMP: cyclic GMP*
IBMX	Solvent control	317 ± 56	0.63 ± 0.09	0.055 ± 0.008	11.5
	4.5×10^{-5}	517 ± 103	1.11 ± 0.10	0.082 ± 0.017	13.5
	2.3×10^{-4}	779 ± 208	1.42 ± 0.17	0.117 ± 0.018	12.1
	4.5×10^{-4}	1067 ± 152	2.36 ± 0.20	0.135 ± 0.021	17.5
			($r = 0.97$)	($r = 0.95$)	
Carbazeran	Solvent control	358 ± 26	0.60 ± 0.06	0.021 ± 0.002	28.6
	1.8×10^{-4}	565 ± 62	1.42 ± 0.20	0.049 ± 0.009	28.9
	9×10^{-4}	954 ± 141	2.03 ± 0.29	0.067 ± 0.010	30.3
	1.8×10^{-3}	1200 ± 110	2.47 ± 0.31	0.073 ± 0.008	33.8
			($r = 0.98$)	($r = 0.94$)	
Amrinone	Solvent control	303 ± 60	0.74 ± 0.06	0.039 ± 0.009	19
	6×10^{-4}	314 ± 90	0.77 ± 0.08	0.036 ± 0.007	21.4
	3×10^{-3}	882 ± 206	1.22 ± 0.09	0.079 ± 0.006	15.4
	6×10^{-3}	1250 ± 109	1.47 ± 0.14	0.102 ± 0.010	14.4
			($r = 0.98$)	($r = 0.98$)	

Each value is the mean ± s.e.mean, $n = 4$–5.
r values are for comparison between aboslute cyclic nucleotide and tension responses.

Table 3 Distribution of phosphodiesterase (PDE) activities in rabbit heart ventricle

Fraction	*Cyclic AMP-PDE activity* (pmol min^{-1} mg^{-1} protein) *Control*	*+ Ca/calmodulin*
Homogenate	265 ± 7.2 (100)	315 ± 2.4 (19)
Soluble (S1)	677 ± 11 (64)	948 ± 59 (40)
Membrane (P1)	112 ± 1 (32)	114 ± 0.8 (2)

* Values in parentheses represent % of total PDE activity.
† Values in parentheses represent % stimulation of PDE activity by addition of Ca^{2+} (20 μM) and calmodulin (0.3 μg).

large proportion (64%) of the total phosphodiesterase activity appears in the soluble fraction which is stimulated (by 40%) on the addition of Ca^{2+} (20 μM) and calmodulin (0.3 μg). However, there is a significant amount (32%) of phosphodiesterase activity which appears to be membrane bound. The membrane-bound phosphodiesterase (memb-PDE) was not activated by Ca/calmodulin. IBMX, amrinone and carbazeran produced concentration-dependent inhibition of cyclic AMP hydrolysis by ventricular phosphodiesterases (Figure 6) with mean (± s.e.mean) IC_{50} values of 4.1×10^{-6} M ($\pm 4 \times 10^{-7}$ M), 1.83×10^{-4} M ($\pm 3 \times 10^{-5}$ M) and 4.1×10^{-6} M ($\pm 1.2 \times 10^{-6}$ M), respectively. All three compounds displayed selectivity (2 to 43 fold) for inhibition of cyclic AMP hydrolysis when compared to their effects against cyclic GMP (mean IC_{50} values for IBMX, amrinone and carbazeran were 5.7×10^{-5} M ($\pm 1.3 \times 10^{-6}$ M), $>2.5 \times 10^{-4}$ M and 1.71×10^{-4} M ($\pm 2 \times 10^{-5}$ M) respectively). IBMX and carbazeran were approximately equipotent, and both compounds were 46 times more potent than amrinone at inhibiting cyclic AMP hydrolysis. IBMX was 3 times more potent than carbazeran at inhibiting cyclic GMP hydrolysis; amrinone was the least potent, producing 10–20% inhibition at the highest concentration tested. Thus the rank order of potency for inhibition of cyclic AMP hydrolysis was IBMX = carbazeran ≫ amrinone, and for cyclic GMP hydrolysis IBMX > carbazeran > amrinone. Although a low speed supernatant fraction was used as the source of the enzyme in these experiments, it is possible that the poor potency of amrinone may be due to missing memb-PDE. The results shown in Figure 7a indicate that IBMX, carbazeran and amrinone produced concentration-dependent inhibition of cyclic AMP hydrolysis by memb-PDE. Amrinone, however, was still the least potent (IC_{50}: $8.6 \pm 1.6 \times 10^{-5}$ M) when compared to IBMX (IC_{50}: $7.7 \pm 1.5 \times 10^{-6}$ M) and carbazeran (IC_{50}: $3.2 \pm 0.20 \times 10^{-6}$ M). Carbazeran was more potent than IBMX, giving a rank order potency of carbazeran > IBMX ≫ amrinone. The inhibitory effect of amrinone on memb-PDE was more marked than that obtained on the soluble phosphodiesterase.

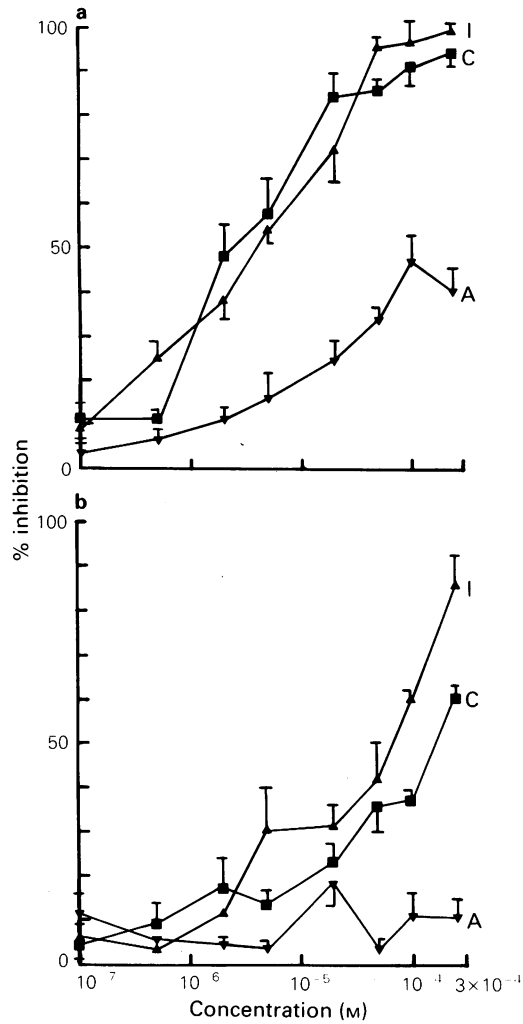


Figure 6 The inhibitory effects of carbazeran (■, C), isobutylmethyl xanthine (▲, I) and amrinone (▼, A) on (a) cyclic AMP hydrolysing and (b) cyclic GMP hydrolysing phosphodiesterase in a 12,000 *g* supernatant fraction from rabbit heart ventricles. Each point represents the mean from 4–6 different enzyme preparations. Vertical lines show s.e.mean.

To parallel the experiments examining the chronotropic actions of IBMX, amrinone and carbazeran, the effects of these compounds on right atrial phosphodiesterase were also analysed. All three drugs

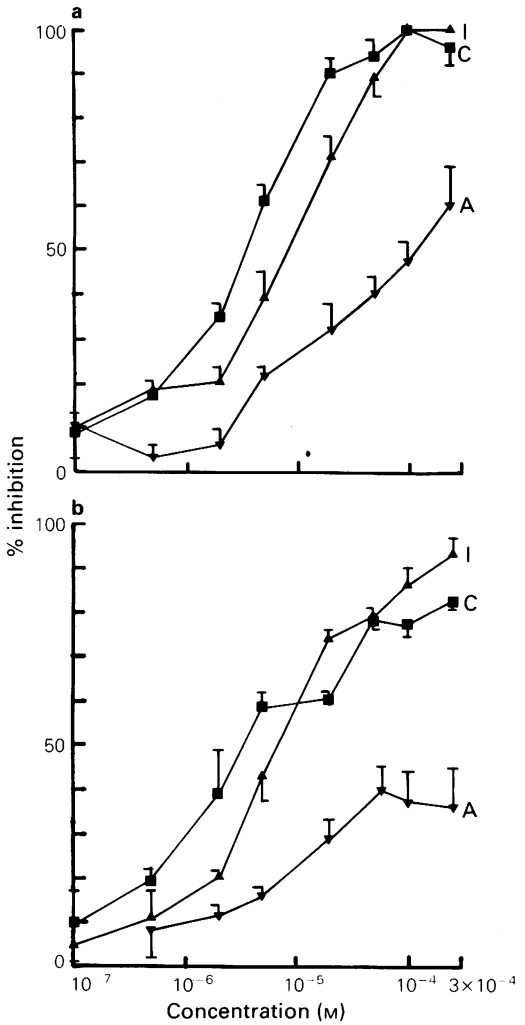


Figure 7 The inhibitory effects of carbazeran (■, C), isobutylmethyl xanthine (▲, I) and amrinone (▼, A) on cyclic AMP hydrolysis by (a) membrane-bound phosphodiesterase prepared from rabbit heart ventricles and (b) rabbit right atrial phosphodiesterase. See Methods for detailed description of the procedure for preparation of phosphodiesterases. Each point represents the mean of 4–6 experiments on different enzyme preparations. Vertical lines show s.e.mean.

produced concentration-dependent inhibition of cyclic AMP hydrolysis by right atrial phosphodiesterase (Figure 7b). Carbazeran (IC_{50}: $5.7 \pm 1.8 \times 10^{-6}$ M) and IBMX (IC_{50}: $7.6 \pm 0.4 \times 10^{-6}$ M) were approximately equipotent whilst amrinone was at least 30 times less potent, producing 35% inhibition at the top concentration tested. Thus, the rank order of potency was carbazeran = IBMX > amrinone.

Discussion

Amrinone, carbazeran and IBMX all produced positive inotropic responses in rabbit isolated heart preparations that were associated with increases in intracellular cyclic AMP and cyclic GMP concentrations. All three drugs were shown to inhibit cardiac phosphodiesterase activity, indicating that this may contribute to the mechanism underlying their positive inotropic effects.

The relatively slow onset of action of these drugs is indicative of an intracellular site of action. The effects of IBMX on ventricular twitch shape were qualitatively similar to those of isoprenaline, which is in agreement with results obtained in guinea-pig papillary muscles (Korth, 1978). The maximum positive inotropic effect, elicited by IBMX was smaller, despite a larger increase in cyclic AMP, when compared to published data for isoprenaline in rabbit papillary muscles (Schumann *et al.*, 1975; Rodger & Shahid, 1984). Amrinone, although not as potent as IBMX, mimicked the effects of the β-adrenoceptor agonists at low concentrations but prolonged relaxation time at higher concentrations, suggesting the involvement of other (cyclic AMP-independent) mechanisms. It is possible that amrinone may also directly enhance Ca^{2+} release from intracellular stores. This view is supported by the observation that the positive inotropic effects of amrinone were inhibited by dantrolene. A mixed profile of mechanism of action for amrinone has also been proposed on the basis of experiments in guinea-pig and canine cardiac tissues (Honerjager *et al.*, 1981; Endoh *et al.*, 1982).

Both IBMX and amrinone elicited positive chronotropic responses. These observations are consistent with the results of Kodama *et al.* (1983), who showed that amrinone increased the spontaneous activity of rabbit sinus node pacemaker cells, and are in accord with the hypothesis that cyclic AMP mediates positive chronotropic responses. In contrast to IBMX and amrinone, carbazeran displayed a substantially different profile in that it did not reduce ventricular relaxation time and it produced a negative chronotropic effect, despite producing generalised phosphodiesterase inhibition in both atrial and ventricular tissues. Thus, in contrast to IBMX and amrinone, carbazeran was force-selective but only at concentrations below 10^{-4} M. The bradycardic effects of carbazeran are unusual for a phosphodiesterase inhibitor but are in agreement with the results obtained by Smith *et al.* (1987). It is conceivable that at higher concentrations ($>10^{-4}$ M) carbazeran exerts direct depressant effects to reduce

activity of S-A nodal cells. Although the exact reasons for this difference are not clear, the pharmacological profile displayed by carbazeran may be more appropriate for the management of congestive heart failure. Another difference between atrial and ventricular effects of these compounds was the lack of potentiation of isoprenaline-induced positive chronotropy. This suggests that either cyclic AMP generation is irrelevant to the positive chronotropic effects of isoprenaline or that the phosphodiesterase predominant in atrial sinus-node cells is relatively insensitive to amrinone, carbazeran and IBMX at the concentrations tested. Elucidation of the differences in the sinus-node phosphodiesterases and those present in ventricular myocardium may aid the development of force-selective inotropic drugs, although microcompartmentalisation of the enzyme(s) may make this difficult.

Amrinone was markedly less potent than IBMX and carbazeran at inhibiting ventricular phosphodiesterase activity, the rank order of potency being the same as for the positive inotropic effect. Furthermore, the effects of the drugs on tissue cyclic nucleotide levels and on isoprenaline-induced positive inotropy are also consistent with their relative potency at inhibiting cardiac phosphodiesterase. The IC_{50} value for amrinone is in reasonable agreement with previously published data (Honerjager *et al.*, 1981; Endoh *et al.*, 1982). These data strongly suggest that phosphodiesterase inhibition and cyclic AMP mediate the positive inotropic effects of the compounds studied. However, there is a large difference between the IC_{50} and EC_{50} values for phosphodiesterase inhibition and positive inotropy. Concentrations of drugs required to elevate tissue cyclic nucleotide concentrations in intact cells are far in excess of those causing phosphodiesterase inhibition in broken cell preparations. These observations suggest that basal phosphodiesterase activity in intact active papillary muscles may be much higher than that obtained in broken cell preparations, so that larger drug concentrations are required for significant inhibition of phosphodiesterase. Poor penetration of drugs in intact cells is unlikely to be the cause of these differences since low, non-inotropic concentrations of the drugs potentiated the effects of isoprenaline. Thus, the phosphodiesterase inhibitory effect can be observed under conditions of increased cyclic AMP production by isoprenaline. This observation suggests that the phosphodiesterase inhibitory effects of these compounds (at low concentrations) in intact tissue preparations are only apparent under conditions of stimulated cyclic AMP production, which would also be associated with increased phosphodiesterase activity. Under non-stimulated conditions, there would be sufficient phosphodiesterase reserve to cancel any inhibitory effect of the compounds. There is also a lack of correlation between the effects of tissue cyclic GMP concentrations and the rather weak inhibition of cyclic GMP hydrolysis. The exact function of cyclic GMP in the cardiac effects of amrinone, carbazeran and IBMX remains undefined. Indeed, the effects of phosphodiesterase inhibitors on cardiac cyclic GMP metabolism have not in general been intensely studied. It is conceivable that increases in cyclic GMP may limit the inotropic/chronotropic effects of phosphodiesterase inhibitors since cyclic GMP has been shown to counteract the effects of cyclic AMP under certain circumstances (Nawrath *et al.*, 1981; Dickson *et al.*, 1986).

The mechanisms by which IBMX and carbazeran produced such large increases in cyclic AMP at equieffective inotropic concentrations when compared to isoprenaline (Rodger & Shahid, 1984) or other documented phosphodiesterase inhibitors (Farah *et al.*, 1984) are not clear. Cyclic nucleotide phosphodiesterases are known to exist in multiple molecular forms which differ in their sensitivities to inhibitors as well as in their physical, regulatory and kinetic properties (Beavo, 1988). Cardiac ventricular tissue contains at least four phosphodiesterase subtypes (Reeves *et al.*, 1987; Beavo, 1988). Thus it is possible that IBMX and carbazeran may inhibit ventricular phosphodiesterases in a non-selective fashion to produce an exaggerated cyclic AMP response. In contrast, the effects of selective phosphodiesterase inhibitors may be restricted to one type of phosphodiesterase involved in regulating cyclic AMP concentration, in a functionally important compartment such as the sarcoplasmic reticulum (Manganiello, 1987). Amrinone was slightly more potent at inhibiting memb-PDE, which may reflect differential effects on phosphodiesterase subtypes. Furthermore, the observation that a supramaximal concentration of IBMX which produced no increase in contractile force, but still further elevated cyclic nucleotide levels, suggests that the maxima for tension and cyclic nucleotide responses are different. This emphasises the view that there is no general relationship between gross tissue cyclic AMP concentration and contractile force. One suggestion proposed is that there may be distinct intracellular 'pools' of cyclic AMP mediating different responses (Brunton *et al.*, 1981; Rodger & Shahid, 1984; England & Shahid, 1987). Cyclic nucleotides mediate many cellular responses each of which may involve different phosphodiesterase isoenzymes and/or phosphodiesterases in different compartments of the cell.

In conclusion, the data indicate that amrinone possesses a similar cardiac rate/force selectivity to isoprenaline and IBMX. This is not the case for carbazeran which exerts both positive inotropic and negative chronotropic effects. Increases in intracellu-

lar cyclic AMP concentration caused by phosphodiesterase inhibition are clearly associated with the positive inotropic effects of amrinone, carbazeran and IBMX. However, the exact mechanisms underlying the chronotropic effects of these compounds are not clear.

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Vasodilator responses to dopamine in rat perfused mesentery are age-dependent

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1 Dose-dependent vasodilator responses to dopamine, isoprenaline, noradrenaline, 3-isobutyl-1-methylxanthine (IBMX) and sodium nitroprusside were obtained in isolated perfused mesentery preparations, taken from reserpine-treated rats of different ages. The preparations were pretreated with phenoxybenzamine (1 μM) and perfused with physiological salt solution containing cocaine (10 μM), additional KCl (20 mM) and vasopressin (0.1 μM).

2 Vasodilator responses to dopamine were abolished by the dopamine$_1$ (DA_1)-selective antagonist SCH 23390 (10 nM) and those to isoprenaline by propranolol (1 μM), but the vasodilator responses to noradrenaline were abolished only when SCH 23390 and propranolol were used together. This indicated that dopamine was acting via DA_1-receptors, isoprenaline via β-adrenoceptors and that noradrenaline could act via DA_1-receptors and β-adrenoceptors in this preparation.

3 Responses to all the vasodilator drugs decreased in magnitude between the ages of 1 and 2 months. Responses to dopamine declined further in 4 month-old rats and were negligible at 6 or 22–24 months of age. Responses to isoprenaline were well maintained up to 6 months of age, but were negligible at 22–24 months.

4 It is concluded that, in the rat mesenteric vasculature, there is a non-specific decline in responses to vasodilator drugs during development (1 to 2 months). Subsequently there is a specific decline in DA_1-receptor-mediated and β-adrenoceptor-mediated responses; the former are lost at an earlier age than the latter. This different time course suggests that age influences receptor numbers, or their coupling to adenylate cyclase, rather than a post-receptor event in the adenylate cyclase/cyclic AMP pathway.

Introduction

It is well known that β-adrenoceptor-mediated relaxation of isolated arterial preparations declines as the age of the animal increases (Fleisch, 1981). In a previous study on rat isolated pulmonary artery and aorta, relaxant responses to several β-adrenoceptor agonists were reduced in aged rats, whereas responses to the adenylate cyclase activator, forskolin, and to the phosphodiesterase inhibitors, 3-isobutyl-1-methylxanthine (IBMX) and enprofylline, were unchanged (O'Donnell & Wanstall, 1986). These observations suggested that age influenced a β-adrenoceptor-linked event rather than a post-receptor event in the adenylate cyclase/adenosine 3':5'-cyclic monophosphate (cyclic AMP) pathway. The aim of the present study was to determine whether vascular relaxation mediated by the vascular dopamine receptor (DA_1 subtype) was also age-dependent and, if so, to compare the time course of this age-dependence with that for β-adrenoceptors. The vascular DA_1-receptor was chosen because, like the β-adrenoceptor, it is linked to adenylate cyclase (Amenta *et al.*, 1984: Alkadhi *et al.*, 1986).

The preparation used in the study was rat isolated perfused mesentery, because dopamine receptors have previously been identified in the mesenteric vasculature of this species (Nichols & Hiley, 1985; Dupont *et al.*, 1987). Preparations were taken from rats ranging in age from 1 month to 2 years enabling the effects both of maturation and of ageing to be assessed. A preliminary account of these data has been presented to the American Society for Pharmacology and Experimental Therapeutics (Wanstall &

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O'Donnell, 1988a) and to the Australasian Society of Clinical and Experimental Pharmacologists (Wanstall & O'Donnell, 1988b).

Methods

Perfused mesentery preparations

Male Wistar rats of the following ages and weights were used: 1 month (60–120 g), 2 months (200–310 g), 4 months (430–570 g), 6 months (570–640 g), and 22 to 24 months (340–550 g). The rats were pretreated with reserpine (1 mg kg^{-1} i.p. 18–24 h before the experiment) and deprived of food overnight. On the day of the experiment they were anaesthetized with pentobarbitone (60 mg kg^{-1} i.p.) and injected with heparin (250 i.u. i.p.). The abdominal cavity was opened, the superior mesenteric artery was cannulated, and the intestine and mesentery removed to a petri dish where the mesentery was carefully teased away from the intestine. The mesenteric vascular bed was then perfused, at 4.5 ml min^{-1}, with physiological salt solution (PSS; 95% O_2/5% CO_2; 37°C; pH 7.4). The composition of the PSS was (mM): NaCl 118, KCl 5.9, $CaCl_2$ 1.5, $MgSO_4$ 0.72, $NaHCO_3$ 25, glucose 11.7, ascorbic acid 1.14, and it contained 10 μM cocaine to block neuronal uptake. In preliminary experiments it was shown that it was unnecessary to include an extraneuronal uptake inhibitor. Perfusion pressure was recorded with a Statham pressure transducer (P23AC) and displayed on a Grass polygraph.

Bolus injections of drugs, in volumes not greater than 15 μl, were given at 5 min intervals into the perfusing solution immediately proximal to the cannula, by Agla micrometer syringes.

Experimental protocols

At the commencement of each experiment, constrictor responses to noradrenaline (10 and 30 nmol) were obtained. The preparations were then perfused with 1 μM phenoxybenzamine for 15 min to block α-adrenoceptors irreversibly. After reverting to phenoxybenzamine-free PSS, the above doses of noradrenaline were retested to check that the α-adrenoceptors had been successfully blocked.

The preparations were perfused thereafter with PSS containing 25.9 mM KCl (i.e. an increase of 20 mM, with a corresponding reduction in the NaCl concentration) plus 0.1 μM vasopressin, to elevate the perfusion pressure so that vasodilator responses could be recorded (Figure 1). In preliminary experiments, a sustained rise in perfusion pressure of sufficient magnitude was not obtained with either vasopressin alone or with KCl alone, unless KCl was used in concentrations greater than 40 mM. These higher concentrations of KCl markedly attenuated the vasodilator responses, as noted in other blood vessel preparations (O'Donnell & Wanstall, 1987).

Table 1 Resting perfusion pressure, and increases in perfusion pressure produced by 20 mM KCl plus 0.1 μM vasopressin, in isolated mesenteric preparations from rats of different ages

Age of rats (months)	n	*Resting perfusion pressure* (mmHg)	*Increase in perfusion pressure* (mmHg)
1	16	19 ± 1.6	49 ± 3.7
2	8	17 ± 2.4	38 ± 5.1
4	5	17 ± 2.0	35 ± 3.5
6	4	13 ± 3.4	35 ± 4.6
22–24	9	12 ± 1.2	83 ± 9.0*

* Value significantly greater than the value obtained in 2 month-old rats, $P < 0.001$ (Student's t test).

The KCl/vasopressin perfusate caused an initial large increase in perfusion pressure (>125 mmHg). The pressure subsequently declined, but remained at a level which was above the resting perfusion pressure (Table 1). There were minor fluctuations in the elevated perfusion pressure for the time required to complete an experiment (2–4 h from the commencement of the KCl/vasopressin perfusion). Therefore, for the values quoted in Table 1, the increase in perfusion pressure for any one preparation was taken as the mean of individual values determined immediately before the administration of each dose of vasodilator drug. A mean value ± s.e. was then calculated for the total number of preparations (Table 1). Vasodilator responses obtained during the first hour after the commencement of the KCl/vasopressin perfusion were erratic, and were not used.

Dose-response curves were obtained, on preparations from rats of different ages (*vide supra*), to the following dilator drugs: dopamine (5 to 150 nmol), noradrenaline (5 to 75 nmol), isoprenaline (0.1 to 1.5 nmol), IBMX (5 to 75 nmol) and sodium nitroprusside (0.5 to 4 nmol). Two to five of these drugs (3 to 4 doses per drug) were examined on any one preparation (with IBMX included in every experiment), and responses to the various drugs used on a particular preparation were alternated (Figure 1).

In addition, on preparations from 1 month-old rats, dose-response curves to dopamine, isoprenaline, noradrenaline and IBMX (3 doses per drug) were

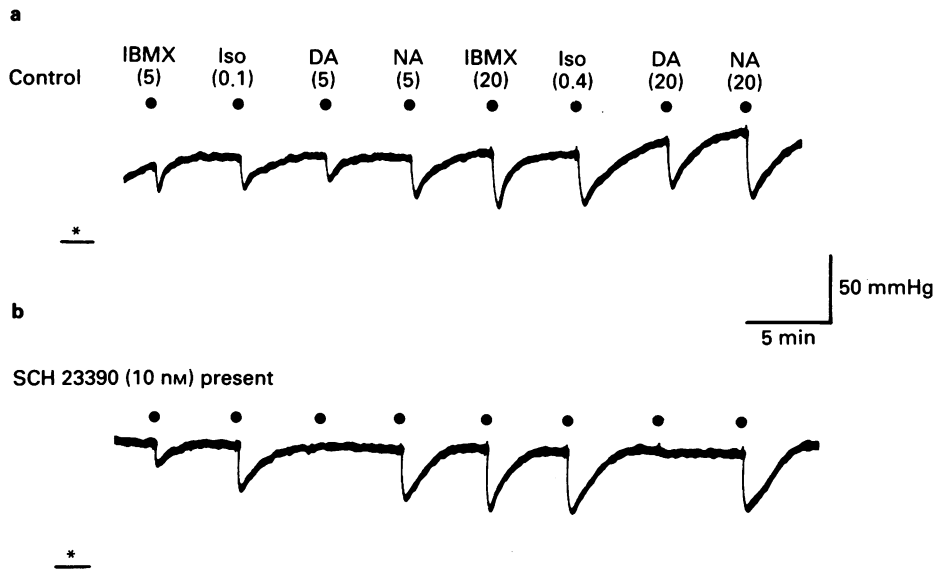


Figure 1 Responses of a rat isolated perfused mesentery preparation to isobutyl methylxanthine (IBMX), isoprenaline (Iso), dopamine (DA) and noradrenaline (NA). Doses, in nmol, are shown in parentheses. Data in the absence of any antagonist (control, a) and in the presence of 10 nM SCH 23390 (b) are shown. The preparation was treated with phenoxybenzamine (1 μM) for 15 min and perfused with PSS containing cocaine (10 μM), KCl (25.9 mM) and vasopressin (0.1 μM). The level of the perfusion pressure before changing to the KCl/vasopressin perfusate is shown (*).

obtained first in the absence of any antagonist, then in the presence of either propranolol (1 μM), SCH 23390 (10 nM) or propranolol (1 μM) plus SCH 23390 (10 nM). The antagonists were present in the perfusate 15 min before, and then during, the agonist dose-response curves, and all four agonists were examined on each preparation. Sometimes propranolol or SCH 23390 caused slight increases in perfusion pressure (5–20 mmHg), but these increases were short-lived (< 10 min).

Analysis of data

Vasodilator responses were determined as decreases in perfusion pressure, and were expressed as a percentage of the KCl/vasopressin-induced increase in perfusion pressure measured immediately before the administration of each dose of vasodilator drug. Responses, when normalized as described above, were highly reproducible both within and between experiments, regardless of the magnitudes of the increases in pressure. Mean dose-response curves were obtained by calculating mean vasodilator responses (%, as defined above) to each dose of vasodilator drug used.

Drugs and solutions

Dopamine hydrochloride (3-hydroxy-tyramine, Sigma); heparin sodium (Weddel Pharmaceuticals); 3-isobutyl-1-methylxanthine (IBMX, Sigma); (−)-isoprenaline acid tartrate (Sigma); (−)-noradrenaline acid tartrate (Sigma); pentobarbitone sodium (Nembutal, Ceva Chemicals); propranolol hydrochloride (ICI); reserpine (Serpasil ampoules, Ciba); SCH 23390 ((**R**)-(+)-8-chloro-2,3,4,5-tetrahydro-3-methyl-5 phenyl-1H-3-benzazepine, gift from Schering Corporation); sodium nitroprusside (Sigma).

Solutions of dopamine (100 mM), isoprenaline (10 mM) and noradrenaline (100 mM) were prepared in 10 mM HCl. IBMX (5 mM) was prepared in 10 mM NaOH, SCH 23390 (1 mM) in 90% ethanol, and propranolol (10 mM) and sodium nitroprusside (10 mM) in deionized water. Dilutions of drugs were prepared in PSS and kept on ice for the duration of the experiment.

Statistical analyses

Mean values are quoted together with their standard errors (s.e.). Data from rats aged 1, 4, 6 and 22–24 months have been compared with those from 2

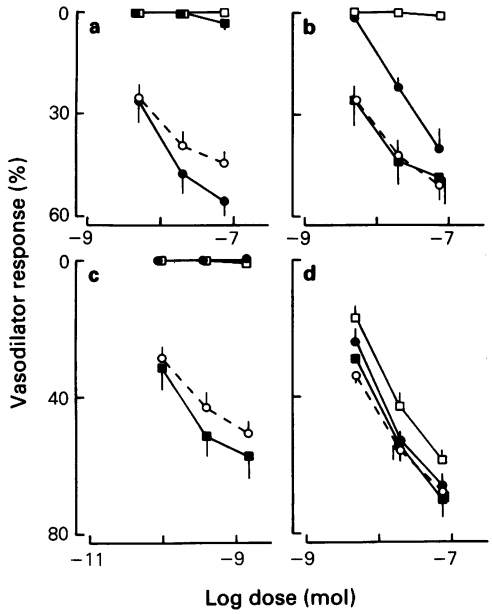


Figure 2 Mean dose-response curves to (a) dopamine, (b) noradrenaline, (c) isoprenaline and (d) isobutyl methylxanthine (IBMX) on rat isolated perfused mesentery preparations. Data are shown in the absence of antagonist (○---○, $n = 8$) and in the presence of 1 μM propranolol (●—●, $n = 4$), 10 nM SCH 23390 (■—■, $n = 4$) or 1 μM propranolol plus 10 nM SCH 23390 (□—□, $n = 4$–8). Vasodilator responses (decreases in perfusion pressure) are expressed as a percentage of the increase in perfusion pressure induced by KCl (20 mM) plus vasopressin (0.1 μM). The s.e. of mean responses are shown by the vertical lines, except when smaller than the symbols.

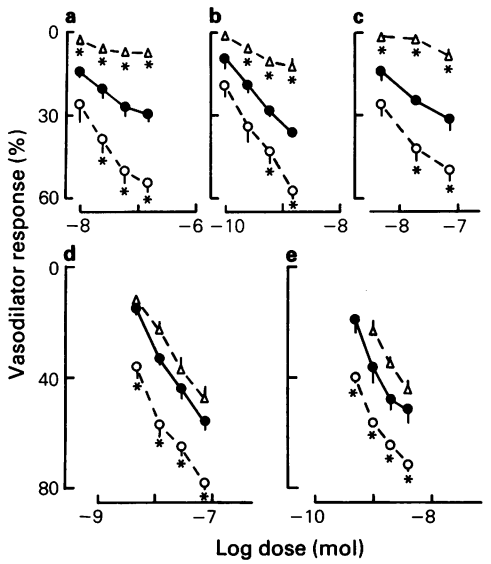


Figure 3 Mean dose-response curves to (a) dopamine ($n = 4$–5), (b) isoprenaline ($n = 4$–5), (c) noradrenaline ($n = 4$–8), (d) isobutyl methylxanthine (IBMX) ($n = 8$–9) and (e) sodium nitroprusside ($n = 4$) on isolated perfused mesentery preparations from rats aged 1 month (○---○), 2 months (●—●) or 22–24 months (△---△). Vasodilator responses (decreases in perfusion pressure) are expressed as a percentage of the increase in perfusion pressure induced by KCl (20 mM)) plus vasopressin (0.1 μM). The s.e. of mean responses are shown by the vertical lines, except when smaller than the symbols. Significant differences ($P < 0.05$, Mann-Whitney U test) between responses in 1 or 22–24 month-old rats and those in 2 month-old rats are indicated by an asterisk.

month-old rats by Student's t test (for values expressed as mmHg) or by Mann-Whitney U test (for values expressed as %).

Results

Establishment of receptor type(s) involved in vasodilator responses to agonists

Dopamine, isoprenaline and noradrenaline all gave dose-related vasodilator responses in rat perfused mesentery preparations. Responses to isoprenaline were abolished by propranolol (1 μM) but were unaffected by SCH 23390 (10 nM), whereas those to dopamine were abolished by SCH 23390 but were unaffected by propranolol (Figures 1, 2a and c). Responses to noradrenaline were abolished only when propranolol and SCH 23390 were both present; SCH 23390 alone had no effect while propranolol alone slightly attenuated the responses (Figure 2b). Neither propranolol nor SCH 23390, used singly or together, blocked the dilator responses to IBMX (Figures 1 and 2d), confirming that the action of these antagonists was specific for their respective receptors.

Effects of age of rats on vasodilator responses

Mean dose-response curves for dopamine, isoprenaline, noradrenaline, IBMX and sodium nitroprusside in preparations from rats of different ages are shown in Figures 3 and 4. For each of these drugs, vasodilator responses in preparations from 2 month old rats were significantly smaller than those in preparations from 1 month old rats (Figure 3). Between 2 months and 2 years of age there was a further decline in responses to the catecholamines dopamine, isoprenaline and noradrenaline, but not to IBMX or sodium nitroprusside (Figure 3).

Dopamine (DA_1-receptor agonist) and isoprena-

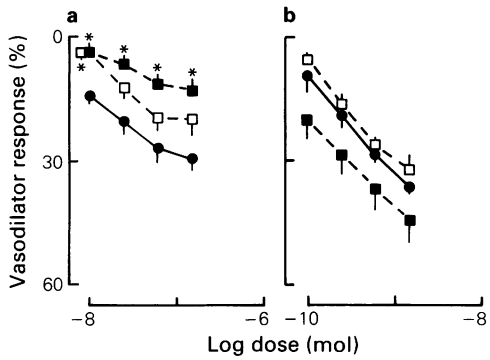


Figure 4 Mean dose-response curves for (a) dopamine and (b) isoprenaline on isolated perfused mesentery preparations from rats aged 2 months (●—●, $n = 4$), 4 months (□---□, $n = 5$) and 6 months (■---■, $n = 4$). Vasodilator responses (decreases in perfusion pressure) are expressed as a percentage of the increase in perfusion pressure induced by KCl (20 mM) plus vasopressin (0.1 μM). The s.e. of mean responses are shown by the vertical lines, except when smaller than the symbols. Significant differences ($P < 0.05$, Mann-Whitney U test) between responses in 4 or 6 month-old rats and those in 2 month-old rats are indicated by an asterisk.

line (β-adrenoceptor agonist) were then examined on preparations from rats at two intermediate ages (4 and 6 months). Vasodilator responses to dopamine were reduced at 4 and 6 months of age, when compared with data from 2 month old rats (Figure 4a). There was no reduction in responses to isoprenaline (Figure 4b) on the same preparations.

Discussion

This study has shown that, on rat perfused mesentery, dilator responses mediated by DA_1-receptors, as well as those mediated by β-adrenoceptors, declined in magnitude as the age of the rats increased. However the time course for this decline differed for these two receptor types. Other workers have shown a decrease in dopamine-stimulated adenylate cyclase activity in the central nervous system with increasing age (Cimino *et al.*, 1984; Nomura *et al.*, 1985), but an effect of age on dopamine receptor-mediated responses in the periphery has not previously been described.

In the vasculature, dopamine can cause contraction of vascular smooth muscle via α-adrenoceptors (Hughes *et al.*, 1988), relaxation of vascular smooth muscle via β-adrenoceptors (Kohli, 1968; Cohen & Berkowitz, 1975) and/or DA_1-receptors (Schmidt & Imbs, 1980; Nichols & Hiley, 1985), inhibition of the neuronal release of noradrenaline via prejunctional DA_2-receptors (Cavero *et al.*, 1982) and, *in vivo*, inhibition of ganglionic transmission via ganglionic DA_1- and/or DA_2-receptors (Cavero *et al.*, 1982; Alkadhi *et al.*, 1986; Van der Niepen *et al.*, 1987). Under the experimental conditions used in the present study (reserpine pretreated rats; preparations treated with phenoxybenzamine and cocaine), it can be assumed that the vasodilator responses to dopamine represented only the direct effects of this amine on receptors in the vascular smooth muscle. These responses appeared to involve DA_1-receptors, but not β-adrenoceptors, since they were blocked by a low concentration (10 nM) of the selective DA_1-receptor antagonist, SCH 23390 (Hilditch *et al.*, 1984), but not by propranolol, in a concentration (1 μM) which abolished responses to the β-adrenoceptor agonist, isoprenaline.

The studies with rats of different ages showed that this DA_1-receptor-mediated vasodilatation was age-dependent, in that it occurred only in the first few months of the life span of the rat. Responses to dopamine were most pronounced in 1 and 2 month-old rats, were reduced in 4 month-old rats, and by 6 months of age were negligible. Responses mediated by β-adrenoceptors in rat perfused mesentery were also age-dependent (confirming a preliminary observation of Fleisch & Spaethe, 1981) but, interestingly, the time course for the decline in responses to β-adrenoceptor-agonists differed from that for responses to dopamine. At 6 months of age, in preparations in which responses to dopamine were negligible, responses to isoprenaline were well maintained. Thus the decline in β-adrenoceptor-mediated responses did not occur until after the rats reached maturity. In this respect, the β-adrenoceptor-mechanisms of rat perfused mesentery resembled those of rat pulmonary artery (O'Donnell & Wanstall, 1984), rather than those of rat aorta, in which responses to isoprenaline are lost during maturation (Hyland *et al.*, 1987).

The small decline in vasodilator responses during development (between 1 and 2 months), seen for both dopamine receptor-mediated and β-adrenoceptor-mediated responses, appeared to be non-specific since it was also seen for the vasodilator responses to the phosphodiesterase inhibitor, IBMX, and to sodium nitroprusside. However, from two months onwards, the effect of age was specific for the receptor-mediated responses, since there was no further decline in responses to IBMX or sodium nitroprusside.

The different time courses for the decline in responses mediated by dopamine receptors and by β-adrenoceptors (*vide supra*) suggest that the influence of age is mainly at the receptor level i.e. age could affect receptor numbers and/or the coupling of the receptors to adenylate cyclase. Any effect of age

on a post-receptor event common to both receptor types, e.g. cyclic AMP-dependent protein kinase activation (Tsujimoto *et al.*, 1986), must be of minor importance or occur after the age of 6 months.

It was noted that the increase in perfusion pressure induced by the combination of 20 mM potassium and 0.1 μM vasopressin was the same in all age groups except the aged (22–24 month-old) rats, where it was significantly greater. The reason for this increased pressor response in aged rats remains to be established. Nevertheless, the vasodilator responses (when expressed as a percentage of the increases in pressure, see Methods) did not appear to be related to the absolute magnitude of the induced pressor response in that: (i) the reduction in vasodilator responses in the aged rats occurred for only some of the vasodilator drugs *viz.* the catecholamines; (ii) responses to all the vasodilator drugs were reduced between 1 and 2 months of age, when there was no change in the increase in perfusion pressure; (iii) responses to dopamine were already negligible in 6 month-old rats, when there was no change in the increase in perfusion pressure.

An additional finding from these studies was that, in the dose range used, noradrenaline could act as a DA_1-receptor agonist, as well as a β-adrenoceptor agonist, in rat mesentery. The results of previous studies with this amine on vascular dopamine receptors have been conflicting. Toda & Goldberg (1975) and Goldberg *et al.* (1978) concluded that noradrenaline was inactive on dopamine receptors in canine coronary arteries and renal vascular bed, whereas the data of Crooks & Martin (1979) and Toda (1976) indicated a possible action of noradrenaline on dopamine receptors in rabbit splenic artery and canine cerebral arteries, respectively. An action of noradrenaline on dopamine receptors in rat mesentery is supported by the observation that, in the presence of propranolol (1 μM), the isomeric potency ratio for (−)-noradrenaline: (+)-noradrenaline was only 5:1 (compared with >50:1 on β-adrenoceptors) (Wanstall & O'Donnell, 1988a). A low isomeric potency ratio is predicted for activation of dopamine receptors, but not β-adrenoceptors (Patil *et al.*, 1975).

Agonists for DA_1-receptors have been proposed as a new class of antihypertensive agents (Cavero *et al.*, 1982), but one can only speculate as to the relevance of the present findings to the clinical use of these drugs. It could be argued that, if dopamine receptor-mediated vasodilatation is age-dependent in the renal vasculature (where the effects of dopamine receptor agonists are particularly important with respect to hypertension), and the age-dependence should extend to man, then the use of these drugs as antihypertensive agents may be most beneficial in younger patients. The above possibilities cannot be ascertained from the clinical trials carried out so far with dopamine and the DA_1-receptor agonist, fenoldopam, since these have largely been carried out on relatively young patients (19–40 years, Hughes *et al.*, 1986; mean 30.6 years, Harvey *et al.*, 1985; mean 32 years, Mousdale *et al.*, 1988; mean 49 years, Caruana *et al.*, 1987) and the data have not been analysed with respect to age.

In summary, this study has shown that, in the rat mesenteric vasculature, the age-dependence of receptor-mediated vasodilatation is not restricted to β-adrenoceptors, but extends to at least one other receptor type linked to adenylate cyclase, namely DA_1-receptors albeit with a different time course. Noradrenaline could act via dopamine receptors as well as β-adrenoceptors in this preparation. Since dopamine receptor-mediated responses were virtually lost by the age of 6 months, whereas those mediated by β-adrenoceptors were not, any involvement of DA_1-receptors in the dilator responses to noradrenaline would presumably be confined to young rats.

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Functional and binding studies with muscarinic M_2-subtype selective antagonists

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1 The potency of a series of selective muscarinic antagonists has been measured on two functional isolated tissue preparations (rat ileum and atria) and these compared with their potency on a range of binding preparations in order to determine whether the subtypes of M_2 receptor measured functionally are the same as those measured in binding studies.

2 On the functional preparations pirenzepine, hexahydrosiladiphenidol (HSD) and 4-diphenylacetoxy-N-methylpiperidine (4-DAMP) were more potent on the ileum than on the atrium (3 fold, 29 fold and 5 fold respectively), whereas himbacine, AF-DX 116 and methoctramine showed the opposite selectivity (5 fold, 3 fold and 56 fold respectively). Atropine had a similar potency on the ileum and atrium.

3 [^{3}H]-N-methyl scopolamine was used to study M_2 binding sites on membranes from rat heart and rat submandibular gland. Each preparation appeared to contain a homogeneous binding site population. The potencies of the five M_2 selective antagonists (and pirenzepine) in binding studies to heart membranes were very similar to those observed in functional studies of rat atria (correlation coefficient = 0.98). Similarly the binding to submandibular gland membranes was very similar to that observed in functional studies on rat ileum (correlation coefficient = 0.97).

4 [^{3}H]-pirenzepine was used to examine the binding of these antagonists to M_1 binding sites on membranes from rat cerebral cortex. The affinities of 4-DAMP, HSD, AF-DX116 and himbacine at M_1 sites were similar to their affinities on the gland. Only pirenzepine and methoctramine had higher affinity on M_1 sites than on the gland.

5 Himbacine had a 20 fold lower affinity at M_1 binding sites than at heart sites, and it should therefore be an important tool in identifying M_1 sites.

6 Inhibition of [^{3}H]-N-methyl scopolamine binding to rat ileum and rat brainstem by M_2-selective antagonists was best described by a two-site model. In both cases the major population of sites (70—90%) appeared to be similar to sites found on the heart (correlation coefficients = 0.95 and 0.97). The other site appeared to be similar to that on the submandibular gland (correlation coefficients = 0.96 and 1.00).

7 The correlations observed in these studies in which a range of selective muscarinic antagonists was used lend weight to previous studies indicating the presence of three functionally important muscarinic receptor subtypes, typified by the binding sites studied in the cerebral cortex, submandibular gland and heart.

8 We propose that the sub-classification of the M_2 muscarinic receptor into M_2 and M_3 subtypes on the basis of ligand binding studies should be extended to cover functionally-defined receptors as well.

Introduction

Although it is generally agreed on the basis of pharmacological studies that muscarinic receptors exist in a variety of subtypes (Mitchelson, 1988) it is not clear yet how many there are and in which tissues they occur. Traditionally receptors with a high affinity for pirenzepine, such as those in the cerebral cortex, are referred to as M_1 and those with a lower affinity for pirenzepine are referred to as M_2 (Goyal & Rattan, 1978). M_2-type receptors have been described in functional preparations such as the guinea-pig isolated atrium and ileum.

Early attempts to distinguish between M_2 receptors used such drugs as 4-diphenyl-acetoxy-N-methylpiperidine (4-DAMP) (Barlow *et al.*, 1976)

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and hexahydrosiladiphenidol (HSD) (Mutschler & Lambrecht, 1984). Both these antagonists were more potent on ileum than on atrium. Only gallamine had the reverse selectivity but results obtained with this compound are difficult to interpret due to its allosteric activity (Stockton *et al.*, 1983). Recently three apparently competitive heart selective antagonists have been described: AF-DX 116 (11-[[2-[(diethylamino)methyl]-1-piperidinyl]-acetyl]-5,11-dihydro-6H-pyrido[2,3-b][1,4]-benzodiazepine-6-one) (Giachetti *et al.*, 1986), methoctramine (N,N'-bis[6-[(2-methoxybenzyl)amino]-hexyl]-1,8-octane-diamine tetrahydrochloride) (Melchiorre *et al.*, 1987) and himbacine (Anwar-ul *et al.*, 1986).

In the present study we have used all of the five selective antagonists mentioned above, together with pirenzepine and the non selective antagonist atropine, in binding and functional studies to a range of tissues. This number of antagonists has enabled quantitative rather than qualitative comparisons between binding and functional studies in a range of preparations and clarifies some of the discrepancies previously described.

Methods

In all experiments tissue from male Hooded Lister rats weighing 150–250 g was used.

Functional studies

Isolated tissue preparations Rat ileum or rat atria preparations were set up in 5 ml organ baths as described by Edinburgh Staff (1970) in a bicarbonate buffered solution at 37°C containing (mM): NaCl 120, KCl 4.7, $MgSO_4H_2O$ 1.3, KH_2PO_4 1.2, $NaHCO_3$ 2.5, $CaCl_2$ 2.5, glucose 10, and gassed with 95% O_2 and 5% CO_2. Concentration-effect curves to bethanechol were obtained every hour by use of either serial dosing (atria) or cumulative dosing (ileum). Responses were recorded either with a T3 Bioscience isotonic transducer (ileum contractions) or a Grass FT03 isometric transducer (atria rate of beating).

Experimental design Each antagonist was examined over at least a 30 fold concentration-range, the highest concentration producing a concentration-ratio greater than 30. Each concentration was examined at least in triplicate in independent experiments as described below.

In a typical experiment on rat ileum four preparations were examined in parallel. Two control concentration-effect curves to bethanechol were obtained on each and then each was equilibrated with a different concentration of antagonist. Two concentration-effect curves were then obtained in the presence of the antagonist. Data from preparations in which the latter two curves, or the two control curves, were not superimposable were excluded from the analysis. Each antagonist was examined in at least three experiments.

A somewhat different experimental design was used with atria as only one preparation can be obtained from each animal. Again four preparations were examined on each day, but with this preparation a different antagonist was examined on each. Two control concentration-effect curves to bethanechol were obtained on each before the preparation was exposed to the first concentration of the antagonist. A further concentration-effect curve to bethanechol was then obtained (i.e. corresponding to approximately 40 min of equilibration) before exposure to the next concentration of the antagonist. Three concentrations of antagonist could be examined on each preparation in this way. Preparations in which the atria were arrhythmic or the two control curves were not superimposable were discarded from the analysis. Control experiments established that there was no consistent sensitivity change of such preparations over the normal experiment duration.

Data analysis In order to estimate the concentration-ratio produced by each concentration of antagonist, on ileum preparations the peak contractions during exposure to bethanechol were recorded and plotted against the concentration of bethanechol. Such preparations show a slight reduction of basal tone with time and so strict concentration-ratios were measured corresponding to the effect produced by the EC_{50} concentration of the control curve. With atrial preparations the peak rates of beating during exposure to each concentration of bethanechol were recorded and plotted against the concentration of bethanechol. With atrial preparations no consistent changes in basal rate were seen and EC_{50} values were recorded for the control and subsequent curves. All antagonists appeared to produce parallel shifts of the log concentration-effect curve for bethanechol and none of the slopes of the Schild plots were significantly different from unity. pK_B values for each concentration-ratio were therefore obtained using the Gaddum equation (Schild, 1949):

$$(r - 1) = [B]/K_B$$

where r is the concentration ratio, [B] is the concentration of antagonist and K_B is its apparent dissociation constant, assuming that it is acting competitively. $pK_B = -\log K_B$. The arithmetic mean of these values and their standard errors were calculated.

Binding

Membrane preparations Rats were stunned and decapitated. The cerebral cortices and brainstems, including the colliculi, pons and medulla, were dissected on ice. Hearts and submandibular glands were cleared of fat and cut into small pieces. Ileum longitudinal muscle was obtained as described by Paton & Vizi (1969). Tissues were homogenized in 20 volumes of homogenizing buffer (20 mM NaHEPES plus 10 mM Na_2 EDTA, pH 7.2) with an Ultraturrax homogenizer (three or four 5 s bursts) and incubated on ice for 30 min. The homogenates of peripheral tissues were strained through two layers of gauze. The homogenates were spun at 40,000 *g* for 10 min, resuspended in assay buffer (20 mM NaHEPES + 100 mM NaCl + 0.5 mM Na_2 EDTA, pH 7.4) in a concentration of 200 mg wet weight per ml and either used fresh or stored in 1 ml aliquots at −70°C.

Assay conditions Fresh or thawed homogenate was diluted in assay buffer (5 mg ml^{-1} for cortex, ileum and heart, 3 mg ml^{-1} for submandibular gland or 2 mg ml^{-1} for brainstem) and incubated in duplicate (heart, gland, ileum and brainstem) or triplicate (cortex) with radioligand and competing agents in a final volume of 1.02 ml in polypropylene microfuge tubes at 23°C for 90 min, preliminary studies having shown that equilibrium was attained by this time. The assay was terminated by centrifugation for 2 min at 13,000 *g* in a Burkard Koolspin centrifuge. The pellets were washed twice with iced 0.9% saline, solubilised with 50 μl 1 N NaOH, neutralised and counted for radioactivity at an efficiency of 30–40%. Some assays were terminated by filtration with a Brandel cell harvester and Whatman GF/B filter strips previously soaked in 0.1% polyethylenimine and dried before use: in these cases 5 ml test tubes were used for the assay. The use of filtration had no effect on any pharmacological parameters of test drugs, but it did lead to an increase in the apparent B_{max} of submandibular gland, suggesting that the viscosity of homogenates of this tissue led to incomplete recovery of binding sites in the centrifugation assay.

Experimental design Each assay used a single type of tissue. In assays with [^{3}H]-pirenzepine a fixed concentration of 0.5–0.7 nM was used, and 5–9 concentrations of competing drug. Assays with [^{3}H]-NMS contained a saturation curve with 22 radioligand concentrations between 15 pM and 3 nM, and 5 or 6 inhibition curves against a fixed concentration of 0.2–0.3 nM [^{3}H]-NMS, each consisting usually of 13 or 16 concentrations spanning 4 or 5 log units. Non-specific binding of the fixed radioligand concentration was measured in the presence of 1 μM atropine. No more than 10% of the fixed radioligand concentration was bound to the membranes.

Data analysis Saturation curves were analysed with the multi-site curve fitting programme LIGAND (Munson & Rodbard, 1980). Non-specific binding was estimated by the programme, preliminary experiments having shown that the estimates agreed well with binding measured in the presence of 1 μM atropine. Saturation curves were always best fitted to a one-site model.

The inhibition curves from a single experiment were analysed simultaneously with the logistic curve fitting programme ALLFIT (de Lean *et al.*, 1978). The curves with cortex, heart and submandibular gland had slope factors close to 1 and were fitted adequately with a one-site model using LIGAND. IC_{50} values obtained with [^{3}H]-NMS in heart and submandibular gland were converted to K_i values using the K_d of [^{3}H]-NMS measured in the same experiment and the equation of Cheng & Prusoff (1973); the IC_{50} values obtained with [^{3}H]-PZ in cortex are equivalent to K_i values since the fixed concentration of [^{3}H]-PZ was much lower than its K_d.

Experiments in ileum muscle and brainstem were further analysed by use of LIGAND. Initially a 1-site model was simulated by selecting a 2-site model with all drugs constrained to be non-selective. Then the two K (affinity) values of the flattest curve (usually methoctramine) were allowed to float, and the fit was always significantly improved, indicating that the data were always better fitted to a 2-site model. In subsequent fits the K value of the flattest remaining constrained curve was allowed to float. [^{3}H]-NMS was always constrained to be non-selective since its K values influence the K values of the displacers and any selectivity of [^{3}H]-NMS was too small to measure accurately. If [^{3}H]-NMS did have different affinities for the two sites, constraining them to be the same would result in small systematic errors in the K values of the displacers. Each experiment therefore yielded a single K_d and B_{max} for [^{3}H]-NMS, the proportion of each of the two sites, and K values for each antagonist at each site.

The values reported are the mean and s.e.mean of pK_i, pK_d or B_{max} from 3–6 independent experiments.

Materials

[^{3}H]-N-methyl scopolamine (specific activity 72 Ci $mmol^{-1}$) was obtained from Amersham International. [^{3}H]-pirenzepine (specific activity 87 Ci $mmol^{-1}$) was obtained from New England Nuclear. Atropine sulphate and bethanechol chloride were from Sigma. The following compounds were generous gifts: 4-DAMP (Dr R.B. Barlow, Bristol),

Table 1 pK_B and Schild slope values for inhibiting bethanechol-stimulated responses in atria and ileum

	Atria		*Ileum*	
	pK_B	*Slope* (*n*)	pK_B	*Slope* (*n*)
Atropine	9.16 ± 0.04	1.07 ± 0.08 (9)	9.25 ± 0.05	0.94 ± 0.09 (14)
Pirenzepine	6.31 ± 0.04	0.98 ± 0.10 (15)	6.73 ± 0.05	1.02 ± 0.10 (13)
4-DAMP	8.20 ± 0.02	1.02 ± 0.08 (12)	8.92 ± 0.05	1.05 ± 0.09 (12)
HSD	6.76 ± 0.09	1.36 ± 0.22 (11)	8.22 ± 0.04	0.93 ± 0.10 (9)
AF-DX 116	7.07 ± 0.03	0.98 ± 0.05 (5)	6.59 ± 0.07	0.91 ± 0.06 (3)
Himbacine	8.13 ± 0.05	0.95 ± 0.12 (9)	7.41 ± 0.09	1.03 ± 0.13 (10)
Methoctramine	8.00 ± 0.02	1.01 ± 0.04 (8)	6.25 ± 0.05	1.05 ± 0.10 (12)

The slopes were calculated by linear regression of log (concentration-ratio −1) against the log of the antagonist concentration, and the standard error of the slope was derived from the linear regression analysis. pK_B values were calculated from each concentration-ratio by the Gaddum Equation. Mean ± s.e.mean values are shown. *n* is the number of values. These were obtained from >3 independent experiments except in the case of AF-DX 116 where there was only sufficient drug for 1–2 independent experiments.
For abbreviations see text.

pirenzepine (Dr A. Donetti, de Angeli), hexahydrosiladiphenidol (Dr R. Tacke, Braunschweig), himbacine (Prof. W.C. Taylor, Sydney), methoctramine (Prof. C. Melchiorre, Camerino).

Results

Functional studies

Antagonist potencies at muscarinic receptors in atria and ileum are shown in Table 1. The most selective compounds were HSD (29 fold ileum selective) and methoctramine (56 fold atria selective). 4-DAMP, AF-DX 116 and himbacine had selectivities of 5 fold or less.

Binding studies

Saturation analysis [^{3}H]-NMS bound to apparently homogeneous populations of sites in all four tissues. The saturation parameters are shown in the legends to Tables 2 and 3.

Single site analysis The potencies of drugs for inhibition of [^{3}H]-PZ binding in cortex (M_1 sites) and [^{3}H]-NMS binding in submandibular gland and heart are shown in Table 2. In agreement with previous reports, pirenzepine was most potent in cortex, 4-DAMP and HSD were about 10 fold more potent in gland than in heart, AF-DX 116 was weakly heart-selective, and himbacine and methoctramine were 20 to 30 fold more potent in heart than in gland. Methoctramine had an intermediate potency

Table 2 pK_i and slope factor values for inhibiting [^{3}H]-pirenzepine in cortex (M_1) or [^{3}H]-N-methyl scopolamine ([^{3}H]-NMS) in heart and submandibular gland

	Cortex		*Heart*		*Gland*	
	pK_i	*Slope*	pK_i	*Slope*	pK_i	*Slope*
Atropine	9.17 ± 0.05	1.29 ± 0.03	8.99 ± 0.03	0.98 ± 0.01	9.40 ± 0.02	1.20 ± 0.06
Pirenzepine	7.97 ± 0.02	0.91 ± 0.03	6.42 ± 0.02	0.90 ± 0.03	6.99 ± 0.03	0.85 ± 0.06
4-DAMP	8.85 ± 0.11	0.96 ± 0.07	8.19 ± 0.04	0.99 ± 0.03	9.27 ± 0.03	0.97 ± 0.02
HSD	7.88 ± 0.06	1.10 ± 0.05	6.84 ± 0.09	0.91 ± 0.02	8.30 ± 0.02	0.91 ± 0.08
AF-DX 116	6.73 ± 0.06	0.92 ± 0.04	7.15 ± 0.15	0.98 ± 0.12	6.48 ± 0.04	0.94 ± 0.04
Himbacine	7.06 ± 0.05	0.84 ± 0.02	8.43 ± 0.07	0.85 ± 0.06	7.07 ± 0.03	0.88 ± 0.02
Methoctramine	7.30 ± 0.04	0.89 ± 0.05	8.07 ± 0.04	1.07 ± 0.04	6.56 ± 0.02	0.84 ± 0.03

IC_{50} values were estimated with the logistic curve fitting programme ALLFIT, and those obtained with [^{3}H]-NMS were converted to K_i values using the K_d value for [^{3}H]-NMS measured in the same experiment. IC_{50} values obtained with [^{3}H]-PZ were assumed to be equivalent to K_i values. Values are the mean and standard error of at least three observations. K_d and B_{max} values for [^{3}H]-NMS were: heart, 264 ± 22 pM and 9.6 ± 0.6 pmol g^{-1} tissue; submandibular gland, 120 ± 16 pM and 6.7 ± 1.5 pmol g^{-1} tissue ($n \geq 4$).
For abbreviations see text.

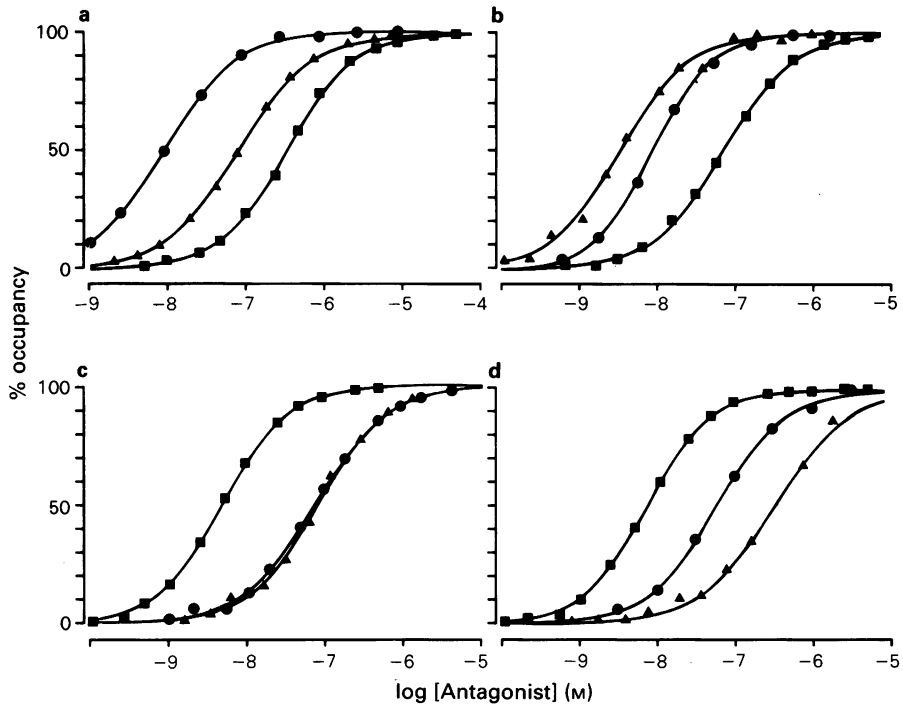


Figure 1 Occupancy curves for four antagonists (a) pirenzepine, (b) hexahydrosiladiphenidol, (c) himbacine, (d) methoctramine at three populations of sites: inhibition curves were constructed against [^{3}H]-pirenzepine ([^{3}H]-PZ) in cortex (●) and [^{3}H]-N-methyl scopolamine ([^{3}H]-NMS) in heart (■) and submandibular gland (▲) and analysed with ALLFIT as described in Methods. In each experiment with [^{3}H]-NMS its K_d was measured and used to adjust the concentrations and IC_{50} of competing drugs according to the formula: Adjusted concentration = true concentration/([^{3}H]-NMS]/K_d + 1). Total and non-specific binding were estimated by ALLFIT and used to calculate % inhibition values. These values and the computer derived fits are plotted against the adjusted concentration of competing drug to yield % occupancy curves, i.e. they indicate the predicted occupancy of muscarinic sites by the unlabelled drug had [^{3}H]-NMS not been present. The inhibition curves obtained with [^{3}H]-PZ are equivalent to occupancy curves because the fixed concentration of [^{3}H]-PZ was less than a tenth its K_d. Each curve represents data obtained in a single experiment.

Table 3 Antagonist pK_i at muscarinic receptor subtypes in brainstem and ileum

	Brainstem		*Ileum*	
	R_1	R_2	R_1	R_2
% total sites	84 ± 2		69 ± 9	
4-DAMP	8.4 ± 0.1	9.2 ± 0.1	8.1 ± 0.2	8.8 ± 0.1
HSD	7.1 ± 0.1	8.2 ± 0.1	6.6 ± 0.2	7.7 ± 0.3
AF-DX 116	7.4 ± 0.1	6.3 ± 0.1	7.5 ± 0.2	6.4 ± 0.2
Himbacine	8.3 ± 0.1	6.6 ± 0.1	8.6 ± 0.1	7.3 ± 0.2
Methoctramine	8.4 ± 0.1	6.4 ± 0.1	8.4 ± 0.1	6.8 ± 0.1

Each experiment contained a saturation curve for [^{3}H]-NMS and inhibition curves with four or five of the above drugs. The curves were analysed simultaneously by use of the 2-site curve-fitting programme LIGAND as described in Methods. The most numerous population of sites was designated R_1. Results are the mean and standard error of at least three observations. K_d and B_{max} values for [^{3}H]-NMS were: brainstem, 171 ± 21 pM and 20.1 ± 1.8 pmol g^{-1} tissue; ileum muscle, 240 ± 40 pM and 8.5 ± 1.6 pmol g^{-1} tissue ($n \geqslant 4$).
Abbreviations as in text.

at M_1 sites, while himbacine had a similar low potency in cortex and gland. Drug potencies in heart (in the presence of Mg^{2+}) were not modified by 0.1 mM GTP (data not shown). Occupancy curves for the four most selective compounds are shown in Figure 1.

Two-site analysis No high affinity pirenzepine binding could be detected in ileum or brainstem. The potencies of the five 'M_2-selective' antagonists were determined for a 2-site model as described in Methods, and the values are shown in Table 3. The major site, designated R_1, comprised 84 ± 2% of binding in brainstem and 69 ± 9% in ileum muscle. In both tissues this site was 'heart-like'. Methoctramine was particularly discriminating in these assays (Figure 2).

Correlations

In Figure 3 the potencies of the five 'M_2-selective' antagonists at heart and gland binding sites are plotted against their functional potencies in atria and ileum, and against the two sites detected in brainstem and ileum muscle. Binding potencies in heart were found to be highly correlated ($P < 0.001$) with functional potencies in atria ($r = 0.98$) and with binding potencies at R_1 in ileum muscle ($r = 0.95$) and brainstem ($r = 0.97$), but uncorrelated with functional potencies in ileum ($r = -0.01$) and binding potencies at R_2 ($r = 0.29$ and -0.02). In a complementary way binding potencies in submandibular gland were highly correlated with functional potencies in ileum ($r = 0.97$) and with binding potencies at R_2 in ileum muscle ($r = 0.96$) and brainstem ($r = 1.00$), but uncorrelated with functional potencies in atria ($r = 0.12$) and binding potencies at

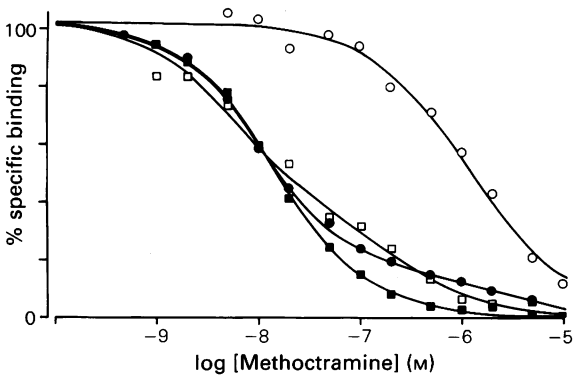


Figure 2 Representative inhibition curves obtained with methoctramine against [^{3}H]-N-methylscopolamine ([^{3}H]-NMS) in heart (■), submandibular gland (○), brainstem (●) and ileum muscle (□). The K_d of [^{3}H]-NMS was measured in each experiment and used to calculate K_i values as described in Methods. The curves in heart and gland were analysed with ALLFIT and yielded pK_is of 8.1 and 6.6 respectively, and slope factors of 0.96 and 0.87 respectively. The curves in brainstem and ileum were analysed with LIGAND to yield the following values: in brainstem pK_1 8.4, pK_2 6.1, R_1 85%; in ileum pK_1 8.6, pK_2 6.9, R_1 64%. The lines were drawn according to these parameters.

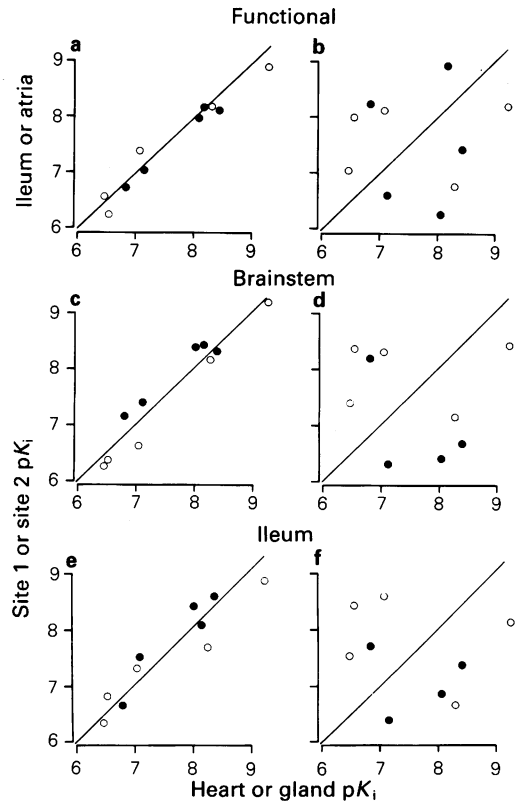


Figure 3 Relationship of mean −log dissociation constants (pK_B or pK_i) from Tables 1, 2 and 3 of 4-diphenyl-acetoxy-N-methylpiperidine, hexahydrosiladiphenidol, AF-DX 116, himbacine and methoctramine measured in functional studies (using atria and ileum) (graphs a and b), and at the two populations of sites found in both brainstem (graphs c and d) and ileum (graphs e and f) (the proportionally larger population of sites in each tissue is designated site 1) and the mean pK_i values found in binding studies in heart (●) and submandibular gland (○). In the left hand graphs (a, c and e) the drug potencies in the atria and at site 1 are plotted against their binding potency in heart (●), and the potencies in ileum and at site 2 are plotted against their binding potency in gland (○). The reverse relationships are plotted in the right hand graphs (b, d and f). The diagonal is the line of unity.

R_1 ($r = -0.28$ and 0.07). Linear regression analysis of the highly correlated measures revealed slopes not significantly different from unity and intercepts not significantly different from zero (results not shown).

Discussion

There are at least four structurally distinct muscarinic receptors expressed in mammalian tissues (Bonner *et al.*, 1987; Peralta *et al.*, 1987). A fifth muscarinic receptor gene (Bonner *et al.*, 1988) has not so far been found to be expressed. A number of recent pharmacological classifications have described three subtypes of muscarinic receptor, but it is not clear whether the classifications refer to the same three subtypes and, in particular, whether the binding site in glands is the same as the functional receptor in smooth muscle (Mitchelson, 1988).

In confirmation of previous reports (Barlow *et al.*, 1976; Mutschler & Lambrecht, 1984; Giachetti *et al.*, 1986; Hammer *et al.*, 1986; de Jonge *et al.*, 1986; Anwar-ul *et al.*, 1986; Melchiorre *et al.*, 1987; Michel & Whiting, 1988a; Wang *et al.*, 1988), we found 4-DAMP, HSD, AF-DX 116, himbacine and methoctramine to discriminate between functional muscarinic responses in atria and ileum, and between apparently homogeneous populations of [^{3}H]-NMS binding sites in heart and submandibular gland. The correlations we have found between these measures support suggestions that the binding sites in heart (mainly ventricle) are equivalent to the receptors studied in the functional atrial preparation, and that the binding sites in gland are equivalent to the receptor subtype mediating the functional response recorded from ileum. We propose that the 'M_1 M_2 M_3' classification (de Jonge *et al.*, 1986; Doods *et al.*, 1987), where M_2 receptors predominate in heart, be applied to muscarinic receptor subtypes studied in both binding and functional studies. These receptors probably correspond to the m1, m2 and m3 genes of Bonner *et al.* (1987) (HM_1, HM_2 and HM_4 genes of Peralta *et al.*, 1987).

In our binding studies, himbacine and methoctramine were 20 to 30 fold selective for heart over gland sites. Methoctramine had intermediate potency for M_1 sites, while himbacine had a similar low potency at M_1 and gland sites. This result suggests that himbacine will be an important tool in the classification of 'M_1' binding sites and receptors, which have hitherto been defined exclusively with the use of pirenzepine. In our functional studies with atria and ileum, however, himbacine was less selective than methoctramine.

The inhibition constants we obtained for methoctramine and AF-DX116 are consistent with the literature, but a disconcertingly wide range of values has been reported for both compounds (e.g. Micheletti *et al.*, 1987; Eglen & Whiting, 1987; Michel & Whiting, 1988a,b; Giraldo *et al.*, 1988). Batink *et al.* (1987) obtained values for AF-DX 116 similar to ours using functional ileum and atria preparations and binding to heart, but a much weaker value with binding to submandibular gland, leading the authors to propose four subtypes of muscarinic receptor. The reason for these discrepancies is unclear.

The inhibition curves we obtained in binding studies with brainstem and ileum muscle were more complex than those obtained in heart and submandibular gland, and were consistent with the presence of two populations of sites. The correlations we obtained, between drug potencies at these two populations of sites and at sites in heart and gland, indicate that in both tissues the major population of sites had a heart-like pharmacology, while the minor population was gland-like.

Heterogeneity of non-M_1 sites in brain has been elegantly demonstrated by Waelbroeck *et al.* (1986; 1987), who found that [^{3}H]-NMS had different kinetics at the subtypes, and took advantage of this fact to restrict heterogeneity in the populations of sites studied with selective antagonists. Flat inhibition curves with AF-DX 116 in various brain regions, suggesting heterogeneity, were also found by Giraldo *et al.* (1987a). In previous studies, binding sites in smooth muscle studied with gland-selective drugs appeared similar to sites in heart (Choo & Mitchelson, 1985; Nilvebrant, 1986). Recently a number of workers have detected heterogeneity in binding to smooth muscle membranes with AF-DX116 (Roffel *et al.*, 1987; Giraldo *et al.*, 1987b; Michel & Whiting, 1987; Monferini *et al.*, 1988; Ladinski *et al.*, 1988) and methoctramine (Michel & Whiting, 1988b). The inability of gland-selective drugs to reveal heterogeneity in smooth muscle membranes may be explained by the small selectivity of available compounds and the fact that the radioligand often used, [^{3}H]-NMS, itself has some selectivity for 'gland-like' sites which will reduce the apparent selectivity of gland-selective drugs (Waelbroeck *et al.*, 1986; 1987). We are able to estimate affinities for gland-selective compounds at the two sites in brainstem and ileum muscle because each experiment, containing both heart- and gland-selective drugs, was analysed as a whole. Our method of analysis caused the most discriminating drugs, methoctramine and himbacine, to constrain the relative proportions of sites in a particular experiment, allowing two-site analysis of 4-DAMP and HSD, which by themselves were not selective enough to define the minor populations of sites.

It is surprising that the 'gland-like' subtype corresponding to functional effects in ileum should comprise only a small fraction of the muscarinic sites.

Our estimates do not take account of any selectivity of [^{3}H]-NMS itself, since [^{3}H]-NMS was always constrained to be non-selective (see Methods). If the correlations obtained with the selective drugs extend to [^{3}H]-NMS, its proportional occupancy at 0.2–0.3 nM will be greater at R_2 than at R_1 sites, and our values for the sizes of R_2, the 'gland-like' component, will be overestimates. It is unlikely that the 'heart-like' sites derive by proteolysis from the 'gland-like' sites since inclusion of protease inhibitors in the membrane preparation and assay did not affect the relative proportions of sites (Michel & Whiting, 1988b; Lazareno, unpublished observations). The recent report that smooth muscle expresses m2 RNA together with m3 RNA (Maeda *et al.*, 1988) supports the suggestion that ileum muscle contains both 'heart-like' (M_2) and 'gland-like' (M_3) receptors. The functional significance of the 'heart-like' sites in smooth muscle is at present unclear.

The 'M_1, M_2, M_3' pharmacological classification of muscarinic receptors is probably incomplete: a fourth muscarinic receptor gene (m4) is expressed in rat brain (Buckley *et al.*, 1988), gene products may undergo post-transcriptional modification, and the pharmacology of a receptor protein may be modified by its membrane environment and effector mechanisms. If further subtypes are contained among the populations we have studied, their presence may be hinted at by some of the anomalies in our data. For example, the selectivity of himbacine was at least four times greater in binding than in functional studies, and its slope factors in 'single site' assays were slightly shallow. These and other anomalies are small and may be related to allosteric effects shown by himbacine (and methoctramine) at concentrations greater than 1 μM (Giraldo *et al.*, 1988; Lazareno, unpublished observations).

In conclusion, our results with both functional and binding studies are consistent with the pharmacological subdivision of muscarinic receptors into M_1, M_2 and M_3 subtypes. M_2 receptors occur in heart, ileum muscle and brainstem, and mediate the negative chronotrophic effect in atria; M_3 receptors occur in submandibular gland, ileum muscle and brainstem and mediate the contractile effect in ileum. Our results also indicate that the cardioselective compounds himbacine and methoctramine are useful new tools in the study of muscarinic mechanisms and receptor subtyes.

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Comparison of the effects of isobutylmethylxanthine and milrinone on ischaemia-induced arrhythmias and platelet aggregation in anaesthetized rabbits

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1 The aim of this study was to compare the effects of the non-selective phosphodiesterase (PDE) inhibitor, isobutylmethylxanthine (IBMX) and the selective PDE III inhibitor, milrinone, in a rabbit model of acute myocardial ischaemia.

2 Coronary artery occlusion caused changes in the ST-segment of the ECG and ectopic activity in all control rabbits. Ventricular fibrillation occurred in 10 out of 14 (71%) of these animals. Pretreatment with IBMX $100\,\mu g\,kg^{-1}$ plus $10\,\mu g\,kg^{-1}\,min^{-1}$, starting 10 min before coronary artery occlusion, reduced ischaemia-induced ST-segment changes and ventricular fibrillation occurred in only 10% of this group ($n = 10$). A similar dose of milrinone had no antiarrhythmic activity, whereas with a lower dose of milrinone, $30\,\mu g\,kg^{-1}$ plus $3\,\mu g\,kg^{-1}\,min^{-1}$ ($n = 10$), only 30% of rabbits fibrillated and ST-segment changes were attenuated.

3 Acute administration of both IBMX and milrinone reduced arterial blood pressure. With the higher dose of milrinone a significant effect was still present after 10 min of drug infusion. A greater hypotensive response to the higher dose of milrinone was observed in the rabbits which subsequently fibrillated during ischaemia. A marked tachycardia was also observed after administration of the higher dose of milrinone.

4 At the end of the experiment platelet aggregation was studied *ex vivo*. ADP-induced aggregation was reduced by pretreatment of the rabbits with milrinone but not IBMX. Both PDE inhibitors enhanced the ability of isoprenaline to inhibit ADP-induced platelet aggregation but milrinone was more effective, particularly at the higher dose.

5 The results demonstrate that IBMX was antiarrhythmic but that this activity was not directly related to inhibition of platelet aggregation. Adverse haemodynamic effects may explain the failure of milrinone to have similar activity during myocardial ischaemia.

Introduction

Several novel phosphodiesterase (PDE) inhibitors are being developed currently for use in the treatment of heart failure. Drugs such as milrinone (Alousi *et al.*, 1983) have both positive inotropic and vasodilator activity which may contribute to their therapeutic effects. However, patients with heart failure may also have or be at risk of developing myocardial ischaemia. It is possible that because of their mechanism of action, PDE inhibitors may have a significant influence on the development of myocardial ischaemia. For example, increasing adenosine 3′:5′-cyclic monophosphate (cyclic AMP) in the myocardium may precipitate arrhythmias (Podzuweit, 1982) thus exacerbating ischaemia. In contrast, elevated cyclic AMP in platelets would inhibit their aggregation (Packham & Mustard, 1980) and this may protect against ischaemia since inappropriate platelet aggregation has been implicated in the genesis of sudden cardiac death (Haerem, 1972).

Most of the novel PDE inhibitors have selective activity on PDE III, an isoenzyme found in cardiac muscle which hydrolyses only cyclic AMP (Weishaar *et al.*, 1986). Isobutylmethylxanthine (IBMX) is a non-selective PDE inhibitor which will prevent the breakdown of cyclic GMP as well as cyclic AMP (Mushlin *et al.*, 1981). Inhibition of the hydrolysis of cyclic GMP may have additional effects during acute myocardial ischaemia. For example, the activity of endothelium-derived relaxing factor (EDRF) will be

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prolonged thus enhancing vasodilatation (Martin *et al.*, 1986) and inhibiting platelet aggregation (Hogan *et al.*, 1988). Thus the aim of the present study was to compare the effects of selective and non-selective PDE inhibitors on arrhythmogenesis and platelet aggregation in anaesthetized rabbits subject to acute myocardial ischaemia.

Methods

Animal preparation

Male New Zealand White rabbits (1.9 to 3.1 kg) purchased from Hylyne, Cheshire, were prepared for coronary artery occlusion by techniques that have been described in detail previously (Coker, 1989). Anaesthesia was induced by i.m. injection of diazepam 2.5 mg kg^{-1} followed by Hypnorm 0.4 ml kg^{-1}. A Lead II ECG was recorded along with arterial blood pressure, left ventricular pressure and its first derivative with time. A catheter was also placed in the vena cava (via the right femoral vein) for drug administration. After cannulation of the trachea, sodium pentobarbitone was administered i.v. (24 to 48 mg kg^{-1}) to maintain anaesthesia. A left thoracotomy was performed at the fourth intercostal space and the rabbits were ventilated with room air at 38 strokes per min, 12 to 18 ml per stroke, with a positive end expiratory pressure of 1 to 2 cmH_2O. Arterial blood gases were measured with a Corning 158 blood gas/pH analyser and stroke volume was adjusted to maintain P_{CO_2} within normal limits. The pericardium was incised and a fine silk ligature was placed around the major anterolateral branch of the left circumflex coronary artery which supplies the majority of the free left ventricular wall in the rabbit.

Experimental protocol

After completion of the surgical preparation a stabilisation period of at least 15 min was allowed. Any rabbits which had arrhythmias or ST-segment changes at this time were excluded from the study (4 out of a total of 48). Drugs were then administered i.v. as an initial bolus dose followed immediately by a continuous infusion. IBMX ($n = 10$) was given at a dose of 100 $\mu g\,kg^{-1}$ plus 10 $\mu g\,kg^{-1}\,min^{-1}$ for the duration of the experiment. Milrinone ($n = 10$) was also administered at this dose with a further group ($n = 10$) receiving a lower dose of 30 $\mu g\,kg^{-1}$ plus 3 $\mu g\,kg^{-1}\,min^{-1}$. Rabbits in the control group ($n = 14$) received equivalent volumes of 0.9% w/v NaCl solution. Ten min after starting drug administration the coronary artery was occluded. After 20 min of myocardial ischaemia the ligature around the coronary artery was released to allow reperfusion. At the end of each experiment i.e. after 10 min of reperfusion or after 3 min of continuous ventricular fibrillation, blood was removed from the right ventricle via a 21G needle so that platelet aggregation could be studied *ex vivo*. The heart was also removed, the aorta cannulated and after retying the ligature around the coronary artery, a 1% w/v solution of Coomassie Blue was injected retrogradely into the aorta to stain the non-ischaemic myocardium. The unstained tissue was dissected from the rest of the ventricles and both ischaemic and normal regions were weighed. The 'area at risk' was calculated as a percentage of the total ventricular mass. Ischaemia- and reperfusion-induced arrhythmias were analysed as described previously (Coker, 1989).

Platelet aggregation

Blood samples were placed in plastic tubes containing 3.8% w/v sodium citrate solution (1 ml to 9 ml blood) and centrifuged at 220 *g* for 10 min. The supernatant, platelet rich plasma (PRP) was removed and the remnants were centrifuged at 2000 *g* to give platelet poor plasma (PPP). After performing a platelet count, PRP was diluted with PPP to give a final platelet count of 2.5 to 3.0 $\times$ 10^5 μl^{-1}. Aliquots of 100 μl of PRP were placed in cuvettes in a Payton dual channel aggregometer and stirred at 900 r.p.m. at 37°C. After an equilibration period of 3 min, aggregating agents were added and platelet aggregation was measured as the change in light transmission.

Drugs

IBMX and milrinone were gifts from Organon Laboratories Ltd., Newhouse and were made up fresh in saline each day. Adenosine diphosphate (ADP), isoprenaline, collagen, arachidonic acid and thrombin were purchased from Sigma, Poole; sodium pentobarbitone (Sagatal) from May and Baker, Dagenham, diazepam from the Royal Liverpool Hospital Pharmacy and Hypnorm (which contains 0.315 mg fentanyl citrate and 10 mg fluanisone per ml) from Janssen, Wantage.

Statistics

Where appropriate, values have been expressed as the mean $\pm$ s.e.mean of n experiments. Comparisons within groups were made with a paired t test and between groups with an unpaired t test. A probability of $P < 0.05$ was considered to be significant. The incidence of events was compared by Fisher's exact test.

Results

Arrhythmias and ST-segment changes

Ischaemia-induced ventricular premature beats (VPBs) occurred in all of the rabbits in the control and high dose milrinone groups, 80% of the IBMX group and only 60% of the group that received the lower dose of milrinone. In this model ventricular tachycardia (VT, defined as 4 or more consecutive VPBs) is a rare event. It was observed in only 2 control rabbits and in 2 rabbits that had been pretreated with the higher dose of milrinone. In contrast, ventricular fibrillation occurred in more than half of the control animals, usually with little or no warning and commonly between 8 and 12 min post-occlusion. Spontaneous reversal of ventricular fibrillation to normal sinus rhythm was observed in 6 rabbits (4 controls, 1 IBMX and 1 low dose milrinone). Figure 1 illustrates clearly that pretreatment of rabbits with IBMX before coronary artery occlusion reduced ventricular fibrillation and mortality during the first 20 min of myocardial ischaemia. Milrinone given at the same dose, $100\,\mu g\,kg^{-1}$ plus $10\,\mu g\,kg^{-1}\,min^{-1}$ was devoid of antiarrhythmic activity, however, ventricular fibrillation and mortality were observed less frequently in the group that received the lower dose of milrinone, $30\,\mu g\,kg^{-1}$ plus $3\,\mu g\,kg^{-1}\,min^{-1}$ (Figure 1).

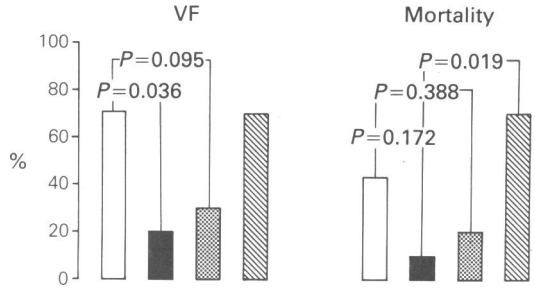


Figure 1 The incidence of ventricular fibrillation (VF) and mortality resulting from coronary artery occlusion in control rabbits (open columns, $n = 14$) and in those which received isobutylmethylxanthine (IBMX) $100\,\mu g\,kg^{-1}$ plus $10\,\mu g\,kg^{-1}\,min^{-1}$ (solid columns, $n = 10$), milrinone $30\,\mu g\,kg^{-1}$ plus $3\,\mu g\,kg^{-1}\,min^{-1}$ (stippled columns, $n = 10$) or milrinone $100\,\mu g\,kg^{-1}$ plus $10\,\mu g\,kg^{-1}\,min^{-1}$ (hatched columns, $n = 10$). *P* values were determined by Fisher's exact test.

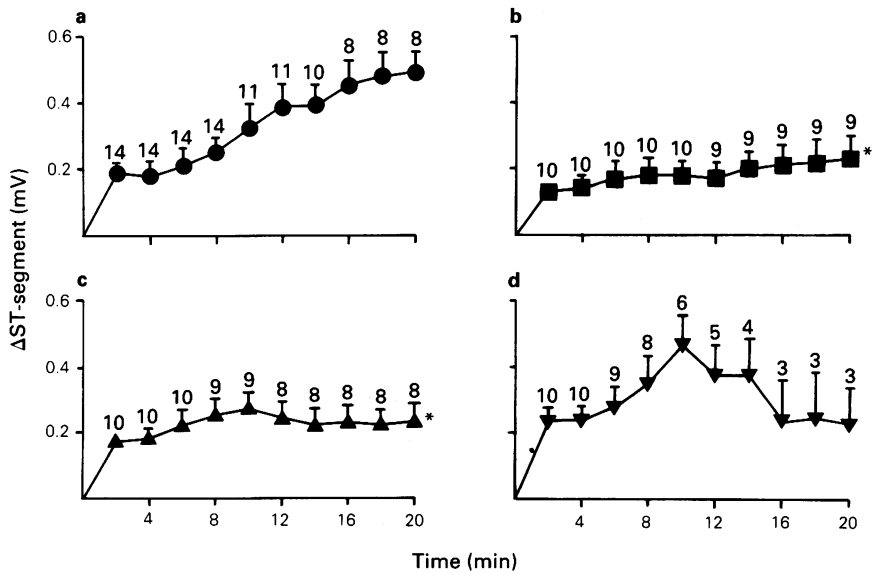


Figure 2 ST-segment changes during the first 20 min of coronary artery occlusion in control rabbits (a; ●) and in those which received isobutylmethylxanthine (b; ■), low dose milrinone (c; ▲) or high dose milrinone (d; ▼). Each value is the mean with vertical bars indicating the s.e.mean. Numbers above each point indicate the number of survivors at that time.
*$P < 0.05$ compared with the control value at 20 min. Comparisons were also made at the 8 min time point, which is before the onset of arrhythmias in the majority of rabbits, but no significant differences were found, independent *t* test.

Table 1 The effects of drug (or vehicle) administration at −10 min, coronary artery occlusion at time 0 and reperfusion at 20 min on heart rate (HR), systolic and diastolic blood pressures (SBP and DBP), left ventricular end-diastolic pressure (LVEDP) and left ventricular (LV) dP/dt_{max}

Time (min)	n	*HR* (beats min^{-1})	*SBP* (mmHg)	*DBP* (mmHg)	*LVEDP* (mmHg)	*LV* dP/dt_{max} (mmHg s^{-1})
Control						
−11	14	277 ± 9	75 ± 4	45 ± 3	2.2 ± 0.2	3290 ± 160
−1	14	278 ± 10	75 ± 3	45 ± 3	2.3 ± 0.3	3250 ± 160
1	14	272 ± 9	68 ± 3†††	40 ± 3†		2730 ± 180††
19	8	282 ± 15	78 ± 4	48 ± 4	7.8 ± 2.0†	3280 ± 210
30	8	289 ± 15	79 ± 3	48 ± 3	3.9 ± 0.7	3310 ± 180
IBMX (100 μg kg^{-1} plus 10 μg kg^{-1} min^{-1})						
−11	10	280 ± 14	78 ± 5	45 ± 3	3.4 ± 0.4	3300 ± 240
−1	10	290 ± 15**	76 ± 3	42 ± 2	3.3 ± 0.5	3310 ± 230
1	10	285 ± 14	67 ± 4††	38 ± 3†		2790 ± 240††
19	9	300 ± 10	74 ± 4	44 ± 3	5.7 ± 1.8	2940 ± 280
30	9	311 ± 11	73 ± 5	41 ± 3	4.7 ± 1.8	2860 ± 310
Milrinone (30 μg kg^{-1} plus 3 μg kg^{-1} min^{-1})						
−11	10	276 ± 6	76 ± 4	48 ± 3	2.3 ± 0.3	3480 ± 100
−1	10	290 ± 6***	72 ± 4*	46 ± 3	2.0 ± 0.3	3530 ± 70
1	10	283 ± 5	65 ± 3	42 ± 4		3230 ± 110††
19	8	283 ± 6	69 ± 3	47 ± 3	3.6 ± 0.4†	3360 ± 140
30	8	283 ± 7	66 ± 4	45 ± 4	2.3 ± 0.3	3410 ± 150
Milrinone (100 μg kg^{-1} plus 10 μg kg^{-1} min^{-1})						
−11	10	279 ± 12	79 ± 6	50 ± 6	3.2 ± 0.7	3140 ± 180
−1	10	322 ± 13***	63 ± 6***	41 ± 5*	3.4 ± 0.5	3240 ± 210
1	10	316 ± 12	54 ± 7††	38 ± 6		2700 ± 200††
19	3	338 ± 33	71 ± 14	49 ± 10	2.8 ± 0.6	2670 ± 580
30	3	335 ± 33	68 ± 16	48 ± 9	2.0 ± 0.9	2960 ± 570

Each value is the mean ± s.e.mean. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with pre-drug (−11 min) value; †$P < 0.05$, ††$P < 0.01$, †††$P < 0.001$ compared with pre-occlusion (−1 min) value; paired t test.

Coronary artery occlusion caused changes in the ST-segment of the ECG in all rabbits, except one that had been pretreated with IBMX. The magnitude of the ischaemia induced ST-segment changes was reduced by IBMX and the lower dose of milrinone but not by the higher dose of milrinone (Figure 2). The reduction in the magnitude of the ST-segment changes was confined to the latter half of the occlusion period.

These effects were not due to variations in the size of the occluded zone. The areas at risk were 39 ± 3% of total ventricular mass in the controls; 37 ± 1% in the IBMX group; 43 ± 3% in the low dose milrinone group and 41 ± 2% in the high dose milrinone group.

In all surviving rabbits release of the ligature around the coronary artery after 20 min of ischaemia resulted in immediate reduction, but not complete reversal, of the ischaemia-induced ST-segment changes. This was accompanied by reperfusion-induced VPBs in 88% of the control group ($n = 8$), 55% of the IBMX group ($n = 9$), 50% of the low dose milrinone group ($n = 8$) and 100% of the high dose milrinone group ($n = 3$). Only one rabbit (which had received the lower dose of milrinone) had VT following reperfusion. The reperfusion-induced arrhythmias were rapid in onset and of short duration, normally terminating within 2 to 3 min. In this study none of the rabbits fibrillated after reperfusion.

Haemodynamics and blood gases

Administration of the initial bolus dose of the PDE inhibitors reduced arterial blood pressure. As the subsequent infusion of drug continued, arterial blood pressure returned toward pre-drug values and the heart rate gradually increased. After 10 min of administration of the higher dose of milrinone there was still a significant reduction in both systolic and diastolic pressure (Table 1). In addition, subdivision of this group into those which fibrillated during coronary artery occlusion and those which survived revealed that those which fibrillated had lower arterial pressures, after drug administration but before coronary artery occlusion, than the survivors; 52 ± 6/36 ± 5 mmHg, $n = 7$ and 79 ± 13/56 ± 6 mmHg, $n = 3$, respectively. No such difference was observed in the control group where those that survived ($n = 8$) had pre-occlusion arterial pressures of 76 ± 3/45 ± 3 mmHg and the values in those that

died ($n = 6$) were 74 ± 6/45 ± 4 mmHg. The higher dose of milrinone also caused a marked tachycardia which was sustained for the duration of the experiment. In the control group, coronary artery occlusion reduced arterial pressure and left ventricular *dP/dt max* transiently and caused a sustained increase in left ventricular end-diastolic pressure (LVEDP). Neither IBMX nor milrinone prevented the initial reduction in blood pressure but both drugs attenuated the ischaemia-induced increase in LVEDP (Table 1).

In control rabbits, before administration of saline the arterial blood gas values were; P_{O_2} 88 ± 3 mmHg; P_{CO_2} 38 ± 1 mmHg; pH 7.44 ± 0.03 units ($n = 14$). Similar values were observed in the other groups. Administration of IBMX or either dose of milrinone did not alter blood gases or pH.

Platelet aggregation

Platelets taken from all of the rabbits aggregated in response to ADP and to arachidonic acid (10^{-3} M). Thrombin (2 units ml^{-1}) caused platelet aggregation in 70 to 80% of the samples in each group whereas the number of rabbits whose platelets responded to collagen (0.2 mg ml^{-1}) varied; 64% in controls, 40% IBMX, 60% low dose milrinone and 30% of the group that received the higher dose of milrinone. Although all platelets responded to ADP the magnitude of this response was reduced significantly by the higher dose of milrinone whereas IBMX had no effect (Figure 3).

Addition of isoprenaline to the platelets prior to ADP (10^{-5} M) did not reduce the response of platelets from the control rabbits and with high concentrations of isoprenaline, enhancement of ADP-induced aggregation was observed presumably due to stimulation of α-adrenoceptors by isoprenaline. In the platelets from the rabbits that had received a PDE inhibitor, however, isoprenaline reduced ADP-induced platelet aggregation (Figure 4). Milrinone was more effective than IBMX in this situation.

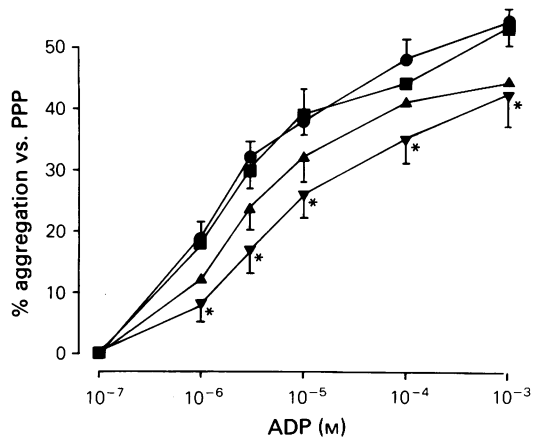


Figure 3 ADP-induced aggregation of platelets obtained from control rabbits (●) and from those which had received isobutylmethylxanthine (■), low dose milrinone (▲) or high dose milrinone (▼). Each value is the mean with vertical bars indicating the s.e.mean. Some error bars have been omitted for clarity. *$P < 0.05$ compared with the corresponding control value, independent t test.

Discussion

The results of this study indicate that the non-selective PDE inhibitor IBMX had significant anti-arrhythmic activity in anaesthetized rabbits subject to acute myocardial ischaemia. In contrast, a dose of the PDE III inhibitor milrinone, with similar haemodynamic actions appeared to be less effective and a higher dose of milrinone was completely devoid of benefit during ischaemia. A different profile of activity was observed, however, when platelet aggregation was studied *ex vivo*. In these experiments milrinone reduced platelet aggregation induced by ADP and revealed the β-adrenoceptor-mediated ability of isoprenaline to inhibit platelet aggregation. The higher dose of milrinone had greater activity than the lower dose, whereas IBMX had little effect. Consideration of these facts suggests that the antiarrhythmic activity of IBMX is not related to the ability of PDE inhibitors to reduce platelet aggregation. However, in view of the limited range of drugs and doses studied, firm conclusions cannot be drawn at this stage.

It is also possible that other adverse effects of milrinone may have counteracted any benefit resulting from its antiplatelet activity. The higher dose of milrinone produced a marked tachycardia and it has been shown that there is a positive correlation between heart rate and the prevalence of ischaemia-induced ventricular fibrillation (Bolli *et al.*, 1986). A significant reduction in arterial blood pressure was also observed after administration of the higher dose of milrinone and the rabbits which subsequently fibrillated had lower pressures than those which survived. Systemic vasodilatation would have reduced arterial driving pressure and thus reduced blood flow to the ischaemic myocardium (Coker & Parratt, 1981). If milrinone also caused direct dilatation of coronary blood vessels supplying the normal region of the myocardium this could result in a 'coronary steal' effect (Parratt *et al.*, 1980) and further exacerbation of ischaemia. A related compound, amrinone, has been reported to exacerbate ischaemia in dogs

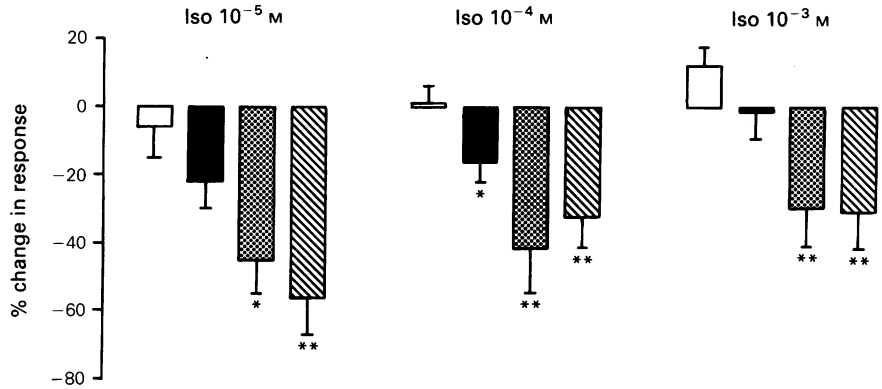


Figure 4 The effect of isoprenaline (Iso) on platelet aggregation induced by ADP (10^{-5} M) in platelet-rich plasma (PRP) from control rabbits (open columns, $n = 14$) and those which were pretreated with isobutylmethylxanthine (solid columns, $n = 10$), low dose milrinone (stippled columns, $n = 10$) or high dose milrinone (hatched columns, $n = 10$).
Each value is the mean with vertical bars indicating the s.e.mean.
$*P < 0.05$, $**P < 0.01$ compared with control, independent t test.

subject to acute coronary artery occlusion (Rude *et al.*, 1980). These arguments may therefore explain why the higher dose of milrinone did not protect against ischaemia-induced ST-segment changes and ventricular fibrillation. The lower dose of milrinone, however, had very similar haemodynamic effects to IBMX, greater antiplatelet activity but less antiarrhythmic effect. This suggests that other factors may need to be considered to explain the different effects of these two drugs observed in the present study.

Electrophysiological studies have suggested that milrinone and amrinone sensitize isolated cardiac muscle to the arrhythmogenic effects of reperfusion following ischaemia (Lukas & Ferrier, 1988). A review of clinical studies also indicates that milrinone and amrinone exacerbate ventricular arrhythmias (Colucci *et al.*, 1986). Since considerable experimental evidence exists to support the theory that cyclic AMP precipitates arrhythmias (Podzuweit, 1982) it is possible that the arrhythmogenic activity of PDE III inhibitors is related to their ability to increase cyclic AMP. However, non-selective PDE inhibitors such as IBMX will also increase cyclic AMP and yet antiarrhythmic activity was observed in the present study. IBMX has been reported to exacerbate ischaemia-induced arrhythmias in anaesthetized rats (Kane *et al.*, 1985) but the dose of IBMX which had this effect was much higher (approximately 10 fold) than that used in the present study and it caused a marked tachycardia. Kane *et al.* (1985) also showed that pretreatment with IBMX increased cyclic AMP in the ischaemic myocardium by approximately 33% whereas cyclic GMP concentrations were increased by more than 50%. Thus it is possible that the dose of IBMX used in the present study was insufficient to increase cyclic AMP to arrhythmogenic concentrations. Alternatively, a concomitant increase in cyclic GMP may have had some influence.

Although IBMX did not cause marked systemic vasodilatation it may have had a beneficial effect on blood flow to the ischaemic myocardium via its ability to increase cyclic GMP. A recent study (Nichols *et al.*, 1988) has demonstrated that coronary vasodilator reserve is impaired in regions of the myocardium that had previously been subject to ischaemia for a period of 1 hour. It was concluded that this effect may be related to loss of EDRF. If this EDRF was released during the ischaemic period, IBMX would potentiate its vasodilator effect (Martin *et al.*, 1986). Thus, unlike milrinone, IBMX could preferentially increase blood flow to the ischaemic myocardium rather than causing marked systemic vasodilatation and possible coronary steal.

The effects of cyclic GMP on cardiac muscle have received far less attention than those of cyclic AMP. The results of Kane *et al.* (1985) show that dibutyryl cyclic GMP increased arrhythmias in rats subject to coronary artery occlusion but dibutyryl cyclic AMP had greater effects. In contrast, cyclic GMP and cyclic AMP have been claimed to have opposite effects on the slow inward calcium current in single heart cells (Hartzell & Fischmeister, 1986). Obviously further work is required to determine whether increases in cyclic GMP are an important factor in the antiarrhythmic activity of IBMX.

Recently, two subclasses of PDE III have been identified in cardiac muscle, only one of which is inhibited by selective PDE inhibitors like milrinone

(Weishaar *et al.*, 1987). It has been shown in other tissues that IBMX inhibits both of these subclasses of PDE III (Elks & Manganiello, 1984). Thus it is possible that the beneficial effects of IBMX during myocardial ischaemia may be related to its effect on the milrinone-insensitive subclass of PDE III. Another important point that has emerged from the work on subclasses of PDE III in cardiac muscle is that there are significant species differences (Weishaar *et al.*, 1987). In view of this it would be sensible to exercise caution when comparing the present study with results obtained in other species.

One final point that should be considered is that the antiarrhythmic activity of IBMX may be completely independent of its ability to inhibit the breakdown of cyclic nucleotides. Although the precise mechanism(s) underlying the beneficial effects of IBMX remain to be clarified the results of the present study suggest that inhibition of platelet aggregation alone is not a major contributory factor.

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Effect of hydrogen peroxide on guinea-pig tracheal smooth muscle *in vitro*: role of cyclo-oxygenase and airway epithelium

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1 Hydrogen peroxide (H_2O_2) (0.1 μM–3 mM) induced variable contractions of guinea-pig isolated trachea which were attenuated by catalase (100 u ml^{-1}) and mannitol (15 mM) suggesting that contractions were induced by H_2O_2 and/or the hydroxyl anion.

2 Epithelial removal potentiated contractile responses of tracheal preparations to H_2O_2 with a leftward shift of the concentration-response curve and an increase in the maximal response.

3 Indomethacin (3 μM) inhibited contractions to H_2O_2 of intact preparations and preparations without epithelium suggesting that contractions may be mediated by cyclo-oxygenase products. Intact preparations (but not preparations without epithelium) contracted in response to high concentrations (>0.1 mM) of H_2O_2 in the presence of indomethacin suggesting that other excitatory factor(s) released by the epithelium may induce contraction.

4 Preincubation of intact tracheal preparations with H_2O_2 (1 mM) for 1 h had no effect on responses to histamine or isoprenaline.

5 These results suggest that hydrogen peroxide generated during the inflammatory process may play a role in bronchoconstriction.

Introduction

Oxygen-derived free radicals are important mediators of cell and tissue injury during inflammatory processes. In the lung, activation of alveolar macrophages (Drath & Karnovsky, 1975), neutrophils (Babior *et al.*, 1973) and eosinophils (De Chatelet *et al.*, 1977) leads to the release of highly reactive oxygen metabolites including the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($OH^\cdot$). These have been implicated in the pathogenesis of experimental lung injury in animals (Brigham, 1986) and *in vitro* are cytotoxic to a variety of isolated cells (Sacks *et al.*, 1978; Burman & Martin, 1986).

Effects of oxygen-derived free radicals on airway smooth muscle have been reported, including direct effects on muscle tone and alterations of receptor-mediated responses. H_2O_2 contracts canine parenchyma and bovine tracheal smooth muscle (Stewart *et al.*, 1981) and $OH^\cdot$ contracts guinea-pig tracheal smooth muscle (Nishida *et al.*, 1985). Macrophages induce a deterioration of β-adrenoceptor function in guinea-pig tracheal smooth muscle which can be blocked by free radical scavengers (Engels *et al.*, 1985). *In vivo*, inhalation of xanthine/xanthine oxidase (a free radical generating system) leads to an increase in airway responsiveness of anaesthetized cats to inhaled acetylcholine (Katsumata *et al.*, 1988).

In this study we have assessed the direct effect of H_2O_2 on guinea-pig tracheal smooth muscle tone and its pharmacological modulation. We have also examined the effect of H_2O_2 on relaxation induced by isoprenaline in order to determine whether this free radical species can induce a deterioration of β-adrenoceptor function *in vitro*.

Methods

Tissue preparation

Male Dunkin-Hartley guinea pigs (300–600 g) were killed by cervical dislocation and the tracheae were removed. Tracheae were dissected free of connective tissue and were slit longitudinally through the cartilage opposite the smooth muscle layer. Eight transverse segments, each comprising 3–4 opened

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cartilaginous rings, were cut and alternate segments were gently rubbed with a moistened cotton wool probe to remove the epithelium. Tracheal segments were suspended in 10 ml organ baths containing modified Krebs-Henseleit solution at 37°C and bubbled continuously with a 95% O_2 and 5% CO_2 mixture. The composition of the Krebs-Henseleit solution was as follows (mM): NaCl 118, KCl 5.9, $MgSO_4 \cdot 7H_2O$ 1.2, $CaCl_2 \cdot 6H_2O$ 2.5, $NaH_2PO_4 \cdot H_2O$ 1.2, $NaHCO_3$ 25.5 and glucose 5.6. The pH of the equilibrated solution was 7.4. Tension was measured isometrically by Grass FT.03 transducers (Grass Instrument Co., Quincy, Mass., U.S.A.) and responses were recorded on a Grass 7D Polygraph. An initial tension of 2 g, which was found to be optimal for measurement of changes in tension, was applied to the tissues. During an equilibration period of 1 h, tissues were washed 3–4 times with Krebs-Henseleit solution and tension readjusted to 2 g.

Effect of hydrogen peroxide on resting tone

Cumulative concentrations of H_2O_2 (0.1 μM–3 mM) were added to baths containing preparations with and without epithelium. Responses were allowed to plateau between successive additions of H_2O_2 or if no response was seen, 3 min contact time was allowed. To study the effect of free radical scavengers on responses to H_2O_2, intact preparations were incubated with either catalase 100 u ml^{-1}, which degrades H_2O_2, or 15 mM mannitol, an $OH^{\cdot}$ scavenger, for 10 min before the addition of H_2O_2. To study the effect of cyclo-oxygenase inhibition on responses to H_2O_2, preparations were incubated with 3 μM indomethacin for 30 min before the addition of H_2O_2.

Effect of hydrogen peroxide on responses to isoprenaline

In order to study relaxation of guinea-pig tracheal smooth muscle to isoprenaline, tissues were precontracted with histamine. Preliminary experiments were therefore carried out to determine the effect of H_2O_2 on contractile responses to histamine. Intact preparations were incubated with and without 1 mM H_2O_2 for 1 h and cumulative-concentration response curves were constructed for histamine. Increasing concentrations of histamine were added to the baths, responses being allowed to reach a plateau between successive additions of histamine. Concentrations of histamine eliciting 30% of maximal contraction (EC_{30}) were determined from these curves (EC_{30} = 1.8 μM untreated preparations, 1.0 μM pretreated preparations). In subsequent experiments intact preparations, incubated with and without 1 mM H_2O_2 for 1 h, were precontracted with the appropriate EC_{30} for histamine and cumulative concentration-response curves constructed to isoprenaline.

Drugs and solutions

The following compounds were used: catalase, histamine dihydrochloride, hydrogen peroxide, indomethacin, (−)-isoprenaline bitartrate (Sigma). Indomethacin was dissolved in phosphate buffer pH 7.8 (0.02 M KH_2PO_4 and 0.12 M Na_2HPO_4) by heating to 40°C for 30 min. Isoprenaline was dissolved in distilled water containing 200 $\mu g/ml^{-1}$ ascorbic acid. Stock solutions of all other agents were dissolved in distilled water and diluted to appropriate concentrations in Krebs-Henseleit solution.

Statistical analysis

Contractile responses to H_2O_2 and histamine were expressed in mg of tension. Relaxant responses to isoprenaline were expressed as % inhibition of tone induced by the EC_{30} of histamine used to precontract tissues. Contractile and relaxant responses under different conditions were compared by Student's t test for paired and unpaired data where appropriate. Probability levels of <0.05 were considered to be significant. pD_2 values were defined as $-\log EC_{50}$ values. In all experiments n represents the number of animals from which tissues were obtained.

Results

Effect of hydrogen peroxide on resting tone

In 10 out of 17 intact tracheal preparations (Figure 1a) 0.1 μM–1 mM H_2O_2 produced a concentration-dependent contraction ($pD_2 = 4.09 \pm 0.19$, maximal contraction 1167 ± 359 mg). Higher concentrations caused a variable relaxation although tension remained above the baseline level. In 5 out of 17 intact preparations (Figure 1b) H_2O_2 at concentrations greater than 10 μM only induced relaxation with a maximal magnitude of -371 ± 81 mg at 3 mM H_2O_2. In 2 out of 17 preparations H_2O_2 had no effect on resting tone at any concentration. An overall contractile response was apparent when changes in tension for all 17 preparations were averaged (Figure 1c), with a pD_2 of 3.90 and maximal contraction of 577 ± 266 mg. In all preparations there was no difference in the initial tone of the tissue and all preparations responded to a maximal concentration of histamine applied after washing out H_2O_2.

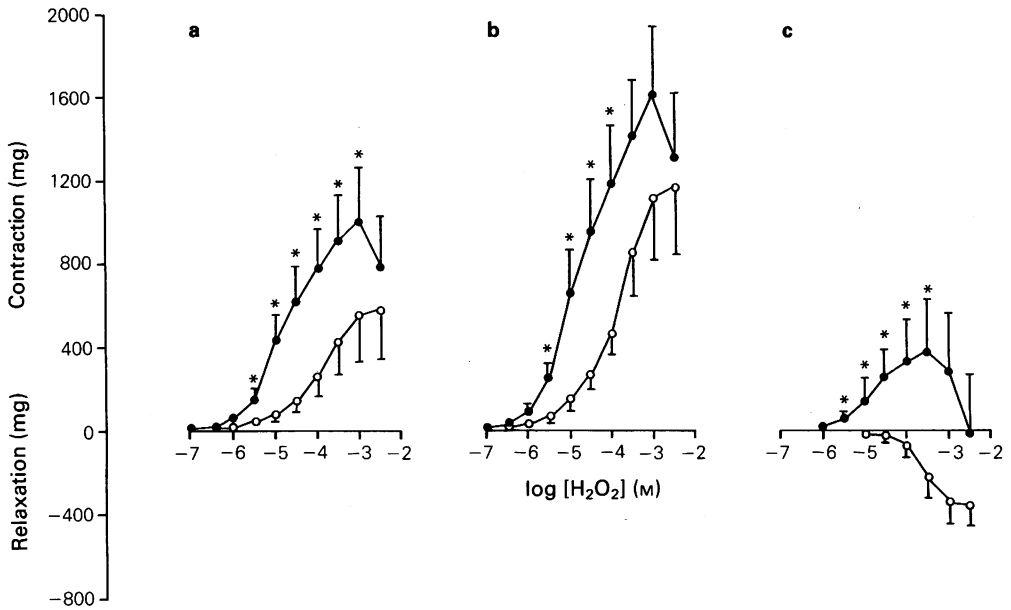


Figure 1. Changes in tension induced by H_2O_2 in intact tracheal preparations (○) and in preparations without epithelium (●). Figures show mean responses obtained from (a) preparations from all 17 tracheae studied, (b) preparations from 10 out of 17 tracheae where a contractile response was observed in intact preparations, and (c) preparations from 5 out of 17 tracheae where a relaxant response was observed in intact preparations. Points represent means with s.e. indicated by vertical bars. * $P < 0.05$ intact preparations versus those without epithelium.

Effect of epithelium Epithelial removal caused a potentiation of contractile responses to H_2O_2 manifested as a leftward shift of the concentration-response curve to H_2O_2. This was apparent in preparations from the 10 out of 17 tracheae which contracted to H_2O_2 (pD_2 4.67 ± 0.14, $P < 0.05$ intact versus preparations without epithelium) or when considering averaged responses of preparations from all 17 tracheae (pD_2 4.8), (Figures 1a and 1c). An increase in the maximal contraction induced by H_2O_2 was apparent only when averaged responses for all 17 tracheae were considered (993 ± 277 mg, $P < 0.05$ intact versus preparations without epithelium) but not when considering the 10 out of 17 tracheae which contracted to H_2O_2 (1604 ± 376 mg). Preparations with epithelium removed from the 5 out of 17 tracheae shown in Figure 1b also tended to contract in response to H_2O_2, with a maximum of 304 ± 275 mg at 0.3 mM H_2O_2. At higher concentrations tension tended to return to or relax below baseline.

Effect of free radical scavengers Catalase (100 u ml^{-1}) inhibited contractile responses of intact preparations to concentrations of up to 1 mM H_2O_2 ($n = 6$) (Figure 2). Mannitol (15 mM) reduced con-

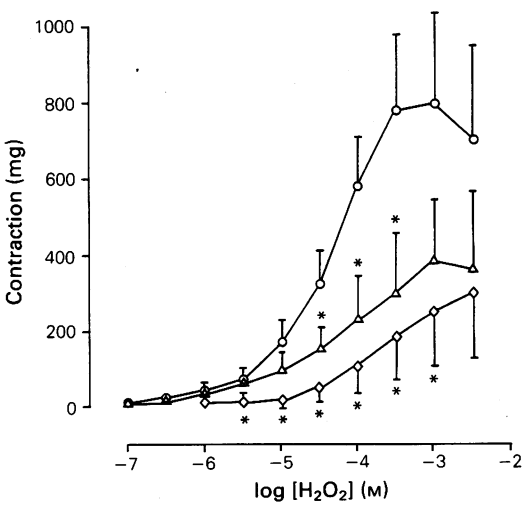


Figure 2 Contractile responses of intact tracheal preparations to H_2O_2 in the presence of catalase 100 u ml^{-1} (◇), mannitol 15 mM (△) or in the absence of free radical scavengers (○). Points represent mean with s.e. shown by vertical bars, $n = 6$. * $P < 0.05$ presence versus absence of free radical scavengers.

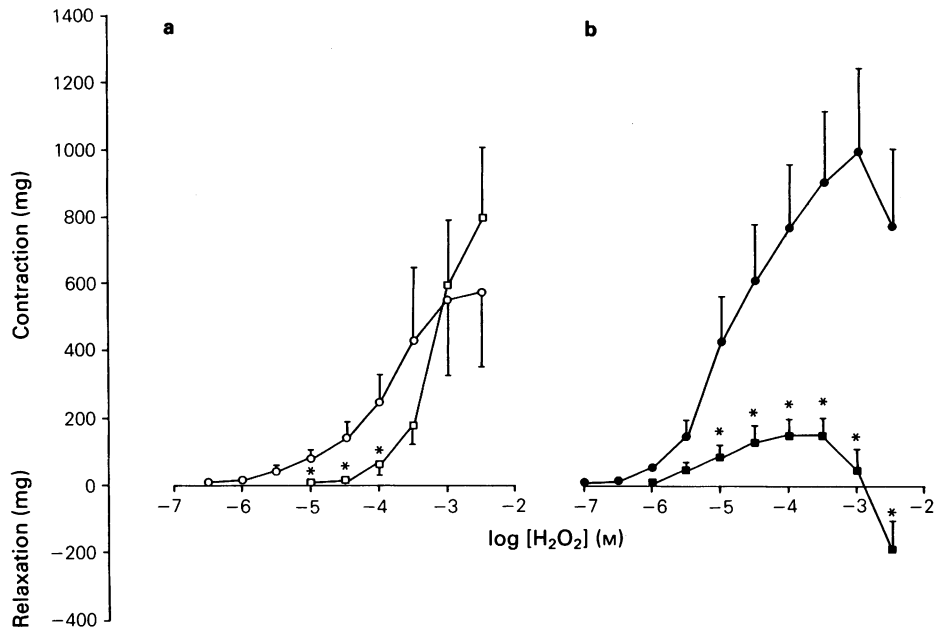


Figure 3 Contractile responses of (a) intact preparations and (b) preparations without epithelium to H_2O_2 in the presence (squares) and absence (circles) of 3 μM indomethacin. Points represent mean with s.e.mean shown by vertical bars, $n = 13$. * $P < 0.05$ presence versus absence of indomethacin.

tractions induced by 30–300 μM H_2O_2 ($n = 6$) (Figure 2). Catalse and mannitol had no effect on resting tone.

Effect of cyclo-oxygenase inhibition The effect of indomethacin on responses of tracheal preparations to H_2O_2 are shown in Figure 3. Indomethacin (3 μM) inhibited contractile responses of intact preparations to low concentrations (up to 0.1 mM) of H_2O_2 ($n = 13$). Contractile responses of preparations without epithelium were inhibited at all concentrations of H_2O_2 ($n = 13$). In the presence of indomethacin a small relaxation was seen in preparations without epithelium in response to 3 mM H_2O_2.

Effect of hydrogen peroxide on responses to histamine and isoprenaline

Contractile responses to histamine were unaffected by pretreatment with 1 mM H_2O_2 ($pA_2 = 5.36 \pm 0.17$ untreated preparations, $pA_2 = 5.54 \pm 0.13$ pretreated preparations; $n = 6$). Responses to isoprenaline were unaffected by pretreatment with 1 mM H_2O_2 ($pA_2 = 8.50 \pm 0.13$ untreated preparations, $pA_2 = 8.50 \pm 0.10$; $n = 6$). Contractions induced by H_2O_2 during the incubation period were transient and resting tension was at the baseline level prior to subsequent experimental procedures.

Discussion

This study demonstrates that H_2O_2 can induce concentration-dependent contraction and/or relaxation of guinea-pig tracheal smooth muscle. Similar concentrations of H_2O_2 contract canine parenchyma and bovine tracheal smooth muscle (Stewart *et al.*, 1981). However, in our study responses of intact tracheal preparations to H_2O_2 were variable with only 10/17 preparations contracting to H_2O_2. Of the remaining preparations, 2 did not respond at all to H_2O_2 and 5 relaxed but only at high concentrations of H_2O_2. The nature of this variability is unclear since all preparations were equilibrated to a constant tension and all tissues responded to a maximal concentration of histamine at the end of the experiment. Contractile responses to H_2O_2 were inhibited by catalase, which degrades H_2O_2, and to a lesser degree by mannitol, an $OH^{\cdot}$ scavenger, suggesting that both active oxygen metabolites may contribute to contractile responses.

Mechanical removal of the epithelium potentiates contractile responses to a variety of agonists (Flavahan *et al.*, 1985; Barnes *et al.*, 1985; Fedan *et al.*, 1988) and indeed contractile responses to H_2O_2 were also potentiated by epithelial removal. It is unlikely that the epithelium should represent a diffusional barrier to H_2O_2 reducing its access to the

underlying smooth muscle since molecules larger than H_2O_2, such as mannitol, are able to diffuse freely across the epithelium (Boucher & Gatzy, 1978). H_2O_2 is a highly reactive molecule and it is possible that it may be inactivated by the epithelium reducing its effective concentration at the smooth muscle layer. Alternatively, H_2O_2 may induce the release of a relaxant factor from the epithelium. By use of a modified 'sandwich' preparation, release of a relaxant factor from guinea pig tracheal epithelium has been demonstrated in response to ovalbumin (Hay *et al.*, 1987).

Contractile responses of intact preparations and preparations without epithelium to low concentrations of H_2O_2 were attenuated by indomethacin suggesting that they may be mediated at least in part by cyclo-oxygenase products. Contractions of canine parenchyma and bovine trachea induced by H_2O_2 were also inhibited by indomethacin (Stewart *et al.*, 1981). Vasoconstriction in isolated saline-perfused rabbit lungs induced by oxygen-derived free radicals is associated with thromboxane production (Tate *et al.*, 1984). In the presence of indomethacin high concentrations of H_2O_2 induced contractions of intact preparation but not preparations without epithelium suggesting that other excitatory factor(s) released by the epithelium may also mediate the response to H_2O_2. In the presence of indomethacin, high concentrations of H_2O_2 induced small relaxations of preparations without epithelium which may reflect release of a relaxant substance from smooth muscle cells or a direct effect on smooth muscle cells. The response of guinea-pig tracheal smooth muscle may therefore represent a composite of excitatory and inhibitory responses induced by cyclo-oxygenase products and other factors released by the epithelium and possibly smooth muscle cells.

Responses of tracheal smooth muscle to isoprenaline were unaffected by pretreatment with H_2O_2. Incubations with 1 mM cumene hydroperoxide for 1 h has been shown to cause a reduction in the number of β-adrenoceptors in lung membrane preparations (Kramer *et al.*, 1986). Pulmonary macrophages induce a reduction in β-adrenoceptor responses of guinea-pig tracheal smooth muscle which can be blocked by catalase and thiourea (an $OH^{\cdot}$ scavenger) suggesting that H_2O_2 and/or $OH^{\cdot}$ may mediate this effect (Engels *et al.*, 1985). In our study tissues were incubated over a similar period of time (1 h) with H_2O_2, however it is possible that the local concentration of oxygen-derived free radicals generated by macrophages may be greater than was possible in our study. Alternatively, other inflammatory mediators released by macrophages in addition to free radicals may be required to induce deterioration of β-adrenoceptor function.

In conclusion, H_2O_2 released by inflammatory cells may induce contraction and/or relaxation of airway smooth muscle which may be mediated partly by cyclo-oxygenase products and partly by factor(s) released by the epithelium and possibly smooth muscle cells.

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6. Discussion and conclusions
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8. List of references
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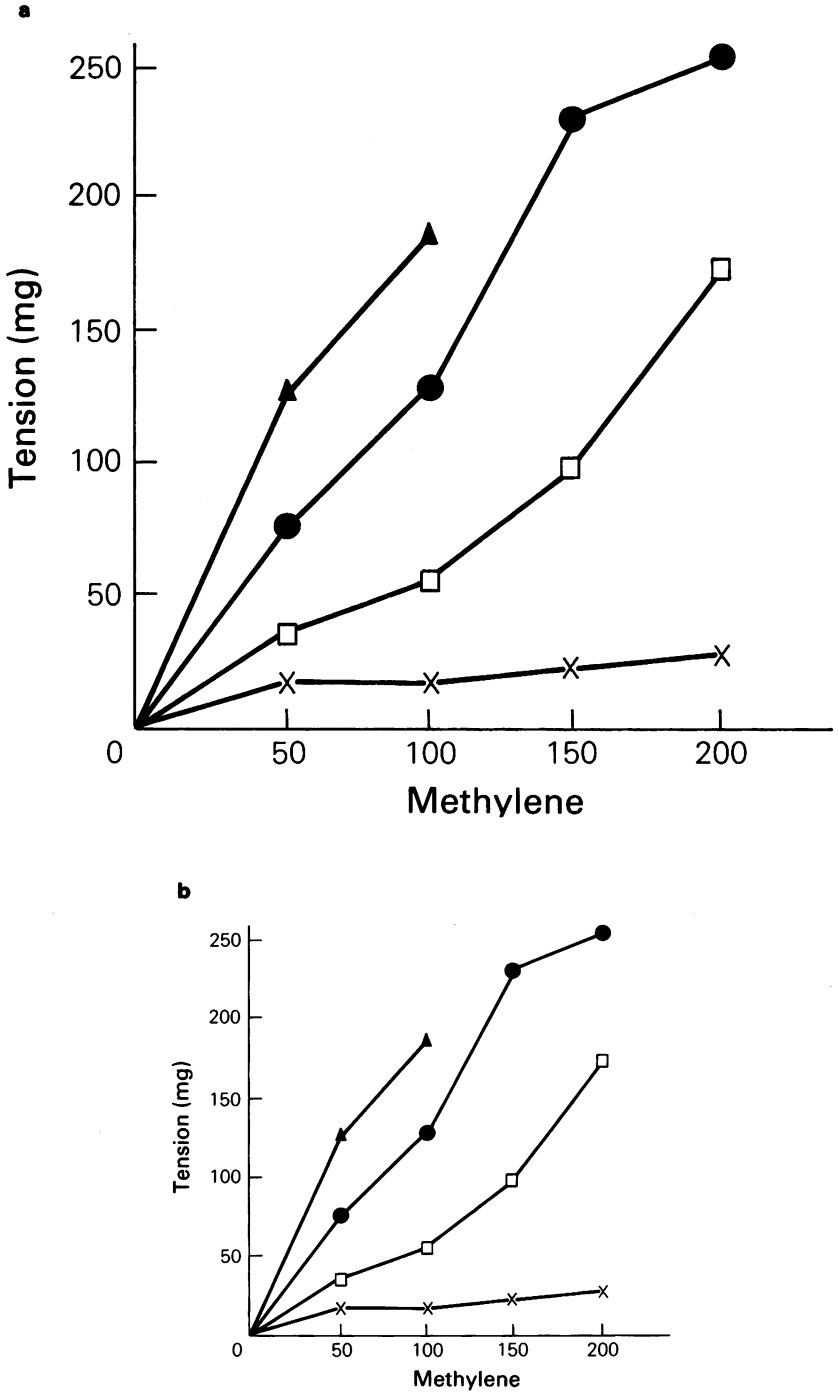


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10^{-3}	milli	m
10^{-6}	micro	μ
10^{-9}	nano	n
10^{-12}	pico	p
10^{-15}	femto	f
10^{-18}	atto	a

Multiplier	*Prefix*	*Symbol*
10^{3}	kilo	k
10^{6}	mega	M
19^{9}	giga	G
10^{12}	tera	T

Thus, micron = μm; ångstrom = 0.1 nm. Mixed prefixes are not permissible, thus mμg should be ng. The symbols d (10^{-1}) and c (10^{-2}) should be restricted to those occasions on which there is a strongly felt need for them (e.g. cm).

Use of the solidus

The solidus should be avoided as far as possible and the negative index substituted, e.g $mg\,kg^{-1}$ rather than mg/kg; $pmol\,mm^{-2}\,min^{-1}$ rather than pmol/mm^2/min.

Quantity	*Preferred unit*	*Symbol*
Amount (of substance)	mole	mol
Capacitance	farad	F
Concentration	moles per litre	M or mol l^{-1}
Current	ampere	A
Electrical conductance	siemens	S
Electromotive force	volt	V
Flow (blood or other liquid)	litres per second (or min)	$1\,s^{-1}$ or $1\,min^{-1}$
Flow (air or other gas)	litres per second (or min)	$1\,s^{-1}$ or $1\,min^{-1}$
Force	newton	N
Frequency of regular event	hertz	Hz
Length	metre	m
Mass	gram	g
Power	watt	W
Pressure (or partial pressure)	pascal*	Pa
Radioactivity	becquerel or curie	Bq (60 d.p.m.) or Ci (3.7×10^{10} Bq)
Resistance (electrical)	ohm	Ω
Temperature	degree celsius	°C
Time	second (preferred)	s
	minute	min
	hour	h
Volume (blood or other liquid)	litre	l
Volume (air or other gas)	litre	l
Work	joule	J

* mm of mercury (mmHg) are allowed if conventional, and if mercury manometer is used for calibration.

SYMBOLS

Symbols denoting physical quantities are usually printed as italic capitals (indicated by single underline in typescript). A dash over the symbol indicates a mean value; a dot over the symbol indicates a time derivative. Suffixes may be used to indicate 'where' and 'what'. They are printed as inferiors on the line. Multiple suffixes should be avoided if a simpler symbol adequately defined is unambiguous, but if necessary should be separated by commas e.g. P_{A,CO_2} denotes partial pressure of CO_2 alveolar air.

CHEMICAL AND BIOLOGICAL ABBREVIATIONS

The abbreviations listed may be used without definition *except* those for chemicals, drugs and enzymes which must be written in full at first mention in the title, summary and again in the text. At first mention they should be followed by the abbreviation in brackets. Subsequently, the abbreviation alone may be used.

The list of abbreviations for chemical, drug and enzyme names is clearly not comprehensive and includes only a few commonly used examples.

Use abbreviations sparingly as extensive use can make the text hard to follow.

acetylcholine	ACh
acetylcholinesterase	AChE
adenosine 3′ : 5′-cyclic monophosphate	cyclic AMP
adenosine 5′-phosphate	AMP
adenosine triphosphatase	ATPase
γ-aminobutyric acid	GABA
analysis of variants	F
adrenaline	Ad
analytical standard of reagent purity	A.R.
anhydrous	anhyd.
approximate(ly)	approx.
approximately equals	≈
aqueous	aq.
arg-vasopressin	AVP
boiling point	b.p.
bovine serum albumin	BSA

cardiovascular system	CVS
catechol-*O*-methyl transferase	COMT
central nervous system	CNS
cerebrospinal fluid	CSF
chi-squared (statistics)	χ^2
clearance	c
coenzyme A	CoA
concentrated	conc.
correlation coefficient	r
cubic	cu.
degree of freedom (statistics)	d.f.
deoxyribonucleic acid	DNA
deoxyribonuclease	DNase
dextro-(absolute configuration)	D-
dextro-(optical rotation)	(+)-
diameter	diam.
diameter, inside	i.d.
diameter, outside	o.d.
diffusion coefficient	D
3,4-dihydroxyphenylalanine	DOPA
3,4-dihydroxyphenylethylamine	dopamine
direct current	d.c.
disintegration per minute	d.p.m.
dissociation constant	K_D
dissociation constant, negative logarithm of	pK
distilled	dist.
dry ice	solid CO_2
edition	edn
editor(s)	ed.
effective concentration	EC_{50}
effective dose, median	ED_{50}
electrocardiogram	ECG
electrocorticogram	ECoG
electroconvulsive therapy	ECT
electroencephalogram	EEG
electromyogram	EMG
electron spin resonance	e.s.r.
endothelial-derived relaxing factor	EDRF
epithelial-derived relaxing factor	EpDRF
equilibrium constant	K
equivalent (general use)	equiv.
erythrocyte	r.b.c.
erythrocyte sedimentation rate	ESR
ethylenediaminetetracetic acid	EDTA
excitatory postsynaptic potential	e.p.s.p.
experiment	expt
experimental	exptl
fatty acids, nonesterified	NEFA
figure(s) (with reference number)	Figure(s)
figure (diagram)	figure
gas–liquid chromatography	g.l.c.
glomerular filtration rate	GFR
haemoglobin	Hb
half-life	$t_{1/2}$
high-frequency	h.f.
high performance liquid chromatography	h.p.l.c.
human serum albumin	HSA
hydrogen-ion concentration	$[H^+]$
hydrogen-ion activity, negative logarithm of (hydrogen-ion exponent)	pH
6-hydroxydopamine	6-OHDA
5-hydroxyindoleacetic acid	5-HIAA
5-hydroxytryptamine	5-HT
immunoglobulins	IgA, IgD, IgE, IgG, IgM
inhibitor constant	K_i
inhibitory concentration	IC_{50}
inhibitory postsynaptic potential	i.p.s.p.
insoluble	insol.
international unit	iu
intra-arterial	i.a.
intracellular fluid	ICF
intradermal	i.d.
intramuscular	i.m.
intraperitoneal	i.p.
intracerebroventricular	i.c.v.
intravenous	i.v.
isotope (atomic mass) e.g. iodine-131	^{131}I
isotopically substituted compounds e.g.	[^{14}C]-ethanol
laevo-(absolute configuration)	L-
laevo-(optical rotation)	(−)-
lethal dose, median	LD_{50}
leukotriene	LT
logarithm to base e	$\log_e$ or ln
logarithm to base 10	$\log_{10}$
maximum	max.
mean arterial pressure	MAP
mean value of (statistics)	$\bar{x}$
melting point	m.p.
meta	*m*-
Michaelis constant	K_M
minimum	min.
mobility (electrophoresis)	m
monoamine oxidase	MAO
noradrenaline	NA
nuclear magnetic resonance	n.m.r.
number	no. or No.
number of observations (statistics)	n
ortho	*o*-

packed cell volume	PCV
page/pages	p./pp.
para-	*p*-
paragraph	para. or ¶
parts per million	p.p.m.
per cent	%
posterior	post.
probability (significance level in a statistical test)	*P*
radioimmunoassay	RIA
rectus (configuration by the sequence rule)	**R**
red blood corpuscle	RBC
relative band speed to front (chromatography)	R_F
relative molecular mass	M_r
relative retention time (gas chromatography)	t_r
renal plasma flow	RPF
resistance (respiratory)	*R*
respiratory conductance	Sgaw
revolutions per minute	r.p.m.
ribonucleic acid	RNA
section	§
sedimentation coefficient (ultracentrifugation)	*s*
sinister (configuration by the sequence rule	**S**
soluble	sol.
solution	soln.
Spearman rank coefficient	r_s
standard deviation: (of observed sample)	s.d.
standard error (of estimate mean value)	s.e.mean
standard error (of sampling)	s.e.
standard temperature and pressure	STP
subcutaneous	s.c.
sum (statistical):	
of hypothetical population	Σ
of observed sample	S or Σ
temperature	temp.
thin layer chromatography	t.l.c.
time clock-24 h clock used e.g. 18 h 30 min	*t*
time constant	τ
2-amino-2-hydroxymethyl-propan-1,3-diol	Tris
ultraviolet	u.v.
unit	u
vacuum	vac.
valency	e.g. Fe^{2+}; Fe(II) protoporphyrin
volume by volume	v/v
wavelength	λ
weight	wt.
weight by volume	w/v

MEETINGS CALENDAR

10–15 September 1989

Biochemical Society, Harden Conference — Cellular Barriers and Drug Targetting, Ashford, U.K. (Meetings Officer, The Biochemical Society, 7 Warwick Court, London WC1R 5DP).

13–15 September 1989

British Pharmacological Society Autumn Meeting. Manchester, U.K. (Closed meeting for members and guests)

17–20 September 1989

1st International $GABA_B$ Symposium, Cambridge, U.K. (Professor N.G. Bowery, Department of Pharmacology, School of Pharmacy, 29/39 Brunswick Square, London WC1N 1AX.)

19–21 September 1989

Electrochemical Detection, HPLC and *in vivo* monitoring in the Biosciences, Nottingham, U.K. (Dr I.A. MacDonald, Department of Physiology & Pharmacology, Medical School, Queen's Medical Centre, Nottingham, U.K.)

20–21 September 1989

9th Annual Meeting of the Bayliss and Starling Society. Peptides: Physiology, Pharmacology and Therapeutics, Nottingham, U.K. (Professor T. Bennett, Dept. of Physiology & Pharmacology, Medical School, Queen's Medical Centre, Nottingham, U.K.)

28 September 1989

Leukotrienes. London, U.K. (Society for Drug Research, c/o Institute of Biology, 20 Queensbury Place, London SW7 2DZ, U.K.)

22–25 October 1989

Molecular and Cellular Biology of IL-1, TNF and Lipocortins in Inflammation and Differentiation. Siena, Italy. (L. Parente, Sclavo Research Centre, via Florentina 1, 53100 Siena, Italy).

8–11 November 1989

Tenth International Symposium on Drugs affecting Metabolism, Houston, USA. (Meeting Manager, 4550 Post Oak Place, Suite 248, Houston, Texas 77027, U.S.A.)

27–29 November 1989

Biological Actions of Extracellular ATP, Philadelphia, USA. (Conference Dept., New York Academy of Sciences, 2 East 63rd Street, New York, NY 10021, U.S.A.)

4–5 December 1989

Sensory Nerves and Neuropeptides in Gastroenterology, Florence, Italy. (Fondazione Internazionale Menarini, Piazza del Carmino, 4-20121 Milan, Italy).

6–8 December 1989

Presynaptic Receptors — an Examination of Different Views, New York City, USA. (Conference Department, New York Academy of Sciences, 2 East 63rd Street, New York, NY 10021, U.S.A.)

10–14 December 1989

Sino-Brazilian Symposium on Chemistry and Pharmacology of Natural Products, Rio de Janeiro, Brazil. (Secretariat, Fundacao Oswaldo Cruz, Departmento de Farmacodinamica, Aremida, Brasil, 6365, Rio de Janeiro, Brazil).

3–5 January 1990

British Pharmacological Society Winter Meeting. London, U.K. (Closed meeting for members and guests)

7 February 1990

Royal Society of Chemistry, Free Radicals in Biotechnology and Medicine, London, U.K. (Miss P.E. Hutchinson, The Analytical Division, Royal Society of Chemistry, Burlington House, London W1V 0BN).

26–28 March 1990

Brain Research Association National Meeting, Bristol, U.K. (Dr. I.C. Kilpatrick, Dept. of Pharmacology, The Medical School, University Walk, Bristol BS8 1TD).

1–6 April 1990

6th World Congress on Pain, Adelaide, Australia. (Int. Association for the Study of Pain, 909 NE 43rd Street, Suite 306, Seattle, WA 98105, U.S.A.)

2–4 April 1990

Central and Peripheral Significance of Neuropeptide Y and its Related Peptides, Baltimore, U.S.A. (Conference Department, New York Academy of Sciences, 2 East 63rd Street, New York, NY 10021, U.S.A.).

4–6 April 1990

New Concepts in Anxiety, Castres, France. (Martin Dehaye, Centre de Recherche Pierre Fabre, 17 avenue Jean Moulin, 81100 Castres, France).

18–20 April 1990

British Pharmacological Society Spring Meeting. Sheffield, U.K. (Closed meeting for members and guests)

27–30 May 1990

Heterocycles in Bio-organic Chemistry, Solbacka, Sweden (Secretariat, PO Box 1136, S-11181 Stockholm, Sweden).

29 May–1 June 1990

Fondazione Giovanni Lorenzini Conference on Prostaglandins and Related Compounds, Florence, Italy (Fondazione Giovanni Lorenzini, Via Monte Napoleone, 23, 20121 Milan, Italy).

28–30 June 1990

IUPHAR Satellite — Mediators in Airway Hyperreactivity, The Hague, The Netherlands. (F.P. Nimkamp, Department of Pharmacology, PO Box 80176, 3508 TD Utrecht, Netherlands).

30 June–4 July 1990

6th Southeast Asian/W. Pacific meeting of Pharmacologists, Hong Kong. (Secretary, Department of Pharmacology, Faculty of Medicine, University of Hong Kong, 5 Sassoon Road, Hong Kong).

27–30 June 1990

International Society for Heart Research XI European Section Meeting, Glasgow, UK. (Professor J.R. Parratt, Dept. of Physiology & Pharmacology, Royal College, University of Strathclyde, Glasgow G1 1XW).

1–6 July 1990

IUPHAR 11th International Congress of Pharmacology, Amsterdam, Netherlands (Contact to be advised).

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MEETINGS CALENDAR

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