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A comparison of bradykinin- and capsaicin-induced myocardial and coronary effects in isolated perfused heart of guinea-pig: involvement of substance P and calcitonin gene-related peptide release

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1 Bradykinin and capsaicin were compared for their ability to elicit functional effects and to release sensory neuropeptides from guinea-pig isolated perfused hearts.

2 Both bradykinin (10 μM) and capsaicin (1 μM) produced a marked increase in coronary flow, a large positive chronotropic effect and a significant reduction in contractile strength. These actions were associated with a marked release of substance P-like immunoreactivity (SP-LI) and calcitonin gene-related-like immunoreactivity (CGRP-LI). The percentage of the tissue content of SP-LI and CGRP-LI released by each agent was similar, although bradykinin was less effective than capsaicin. The ratio of SP-LI/CGRP-LI released by both agents was similar to that present in cardiac tissue.

3 Neuropeptide release could be evoked only once with capsaicin but at least four times with bradykinin. Also, functional responses to capsaicin underwent desensitization. After either *in vitro* or systemic capsaicin pretreatment, the release of SP-LI and CGRP-LI by bradykinin was reduced and the positive chronotropic effect of bradykinin was significantly reduced, while the increase in coronary flow and negative inotropic responses remained unchanged.

4 Pretreatment with indomethacin (10 μM) strongly antagonized the release of SP-LI and CGRP-LI by bradykinin and reduced the increase in heart rate.

5 These findings suggest that activation by bradykinin (probably through indirect mechanisms) of capsaicin-sensitive sensory nerves in the heart, leads to a local release of sensory neuropeptides. These neuropeptides, in turn, could participate in determining the complex functional effects of this kinin on cardiac performance.

Introduction

Peripheral release of neuropeptides from capsaicin-sensitive sensory nerve endings leads to various motor, inflammatory and trophic effects (Holzer, 1988; Maggi & Meli, 1988). In guinea-pig isolated hearts perfused with capsaicin, myocardial and coronary effects coupled to release of substance P-like immunoreactivity (SP-LI) have also been described (Hoover, 1987). Neuropeptide-containing, capsaicin-sensitive sensory fibres are widely distributed in cardiac tissues of various mammalian species, including the guinea-pig (Papka *et al.*, 1984; Lundberg *et al.*, 1985; Uddman *et al.*, 1986; Hougland *et al.*, 1986; Saito *et al.*, 1986a). In addition sensory neuropeptides, such as tachykinins and calcitonin gene-related peptide (CGRP) have also been found to produce powerful chronotropic, inotropic and vasodilator effects in cardiac preparations (Franco-Cereceda & Lundberg, 1985; Haas & Skofitsch, 1985; Holman *et al.*, 1986; Sigrist *et al.*, 1986; Saito *et al.*, 1986b).

Various data indicate that bradykinin, produced locally during inflammation, or following tissue damage, is capable of stimulating and/or sensitizing capsaicin-sensitive afferents (Juan & Lembeck, 1974;

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Kaufman et al., 1980). Some of the peripheral effects of bradykinin, especially on iris smooth muscle preparations, are at least partially attributable to release of tachykinins (Bynke et al., 1983; Ueda et al., 1984; Hakanson et al., 1987). However, thus far the potential involvement of neuropeptide release in the actions of bradykinin on heart tissues (for a review see Regoli & Barabè, 1980) has not been investigated. Recently, we have shown that infusion of bradykinin in guinea-pig isolated perfused hearts elicits a simultaneous release of SP-LI and CGRP-like immunoreactivity (CGRP-LI) from capsaicin-sensitive structures (Geppetti et al., 1988). The aim of the present work was to investigate and to compare functional and neurochemical studies in the characteristics of the actions of capsaicin and bradykinin in guinea-pig isolated perfused hearts.

Methods

Functional studies

Male albino guinea-pigs (250–350 g) were used. Animals were killed by cervical dislocation and the heart rapidly removed, a cannula was inserted into the aorta immediately distal to the aortic valves and the preparation was transferred to a perfusion apparatus. Hearts were perfused under constant pressure at 37°C with oxygenated (96% O_2 and 4% CO_2) physiological salt solution of the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgCl_2$ 0.54, NaH_2PO_4 1.06, $NaHCO_3$ 24.5 and glucose 10. Coronary flow was detected with an electronic device (MARB Gx-84) and the electrocardiogram was recorded. Contractile function of the whole heart was assessed by means of a hook inserted in the cardiac apex connected to an isometric transducer under a resting tension of 2 g.

Preparations were allowed to equilibrate for at least 60 min. Drug administration was by injection through a side arm of the perfusion apparatus immediately above the cannula. A volume of 0.5 ml of the required concentration of capsaicin or peptides was slowly injected over 2 min. Preliminary experiments indicated that no modification in cardiac and coronary parameters could be detected after administration of 0.5 ml of physiological salt solution. The injection of capsaicin vehicle (ethanol) produced a slight decrease in contractile force (about 10–15%) along with a slight and transient increase in coronary flow (less than 10%). In some experiments non-cumulative concentration-response curves were obtained by injecting increasing concentrations of neuropeptides at 30 min intervals. ECG, contractile function and coronary flow were continuously recorded on a three channel polygraph (MARB 3/50). Heart rate, at a fast speed of the recording system, was recorded at 2, 5, 10 and 15 min after drug injection.

Some experiments were performed in capsaicin-pretreated animals in which capsaicin desensitization was achieved by giving a total dose of 55 mg kg^{-1} s.c. over two days according to Maggi et al. (1987a); the experiments were carried out five days after the last injection of capsaicin.

Sample collection and extraction

Fractions of the heart perfusate (approximately 6 ml) were taken every minute. Perfusates, collected in tubes containing enough acetic acid to give a final concentration of 2 N were freeze-dried. Atria and ventricles were dissected from the whole heart and then homogenized in 2 N acetic acid (1/10; w/v at 95°C) kept in a boiling water bath for 10 min and cooled at 4°C for 10 min. After centrifugation at 20,000 *g* for 30 min at 4°C, the supernatant was freeze-dried. All samples were then reconstituted with the assay buffer (0.1 M, pH 7.4, phosphate buffer containing 0.9% NaCl, 0.01% NaN_3 and 0.1% bovine serum albumin) and peptide content measured by radioimmunoassay (RIA).

CGRP-LI and SP-LI radioimmunoassays

After reconstitution in assay buffer, 100 µl of rat CGRP standard or samples were incubated with 100 µl of rabbit anti-human CGRP antiserum (RAS6012, Peninsula, CA, U.S.A.) for 48 h at 4°C; 100 µl of [^{125}I]-iodohistidyl-CGRP (Amersham, UK) was added and incubated for a further 48 h at 4°C. After the addition of 1 ml of buffer containing 7.5% polyethylene glycol, 1/200 goat anti-rabbit antiserum and 1/2000 normal rabbit serum-bound antigen was separated from free by centrifugation at 2000 *g* for 30 min at 4°C. The coefficient of variation was less than 10% for values between 20 and 300 pmol l^{-1}. The lower detection limit was 2.5 fmol per tube. Cross-reactivity of the antiserum was 100% for rat-CGRP I and human-CGRP.

SP-LI was measured as previously reported (Geppetti et al., 1987). Briefly, SP-standard or samples were incubated overnight at 4°C with rabbit anti-SP antiserum and ^{125}I-Bolton and Hunter conjugated SP (Amersham, UK). Bound and free antigen were separated by double antibody precipitation. The sensitivity of the assay was 1.1 fmol per tube. The antiserum cross-reacts to 1% with NKA, 0.5% with NKB and less than 0.1% with physalaemin and eledoisin. Both anti-SP and anti-CGRP antiserum did not cross-react with bradykinin up to 100 µM.

Statistical analysis

All data in the text, Figures and Tables are given as the mean ± s.e.mean. Statistical analysis of the data was performed by means of Student's t test for paired or unpaired data, analysis of variance and Newman-Keuls test, when appropriate. A P value of less than 0.05 was considered to be significant.

Drugs

Drugs used were: bradykinin (Serva), calcitonin gene-related peptide (CGRP rat) (Peninsula), capsaicin (Serva), indomethacin (Sigma) and substance P (SP) (Serva).

Results

General

After 60 min equilibration, the resting performance of guinea-pig isolated perfused hearts was as follows: frequency of beat 248 ± 3 beats min^{-1}, force of contraction 2.7 ± 0.006 g and mean coronary flow 5.1 ± 0.1 ml min^{-1} ($n = 51$). Preliminary experiments indicated that such values remained stable for at least 2 h.

Effects of bradykinin and capsaicin on chronotropism, inotropism and coronary flow of guinea-pig isolated perfused hearts

Either bradykinin (10 μM) or capsaicin (1 μM) exerted similar, marked effects on myocardial and coronary function in the guinea-pig isolated perfused hearts. These concentrations were selected since they were near maximally effective in producing release of sensory neuropeptides (Geppetti *et al.*, 1988). Bradykinin (10 μM, $n = 9$, Figure 1a) produced a prompt and rapid increase in coronary flow (from 5.8 ± 0.3 to 8.1 ± 0.5 ml min^{-1}, $P < 0.05$), and a marked positive chronotropic effect (from 242 ± 5 to 285 ± 7 beats min^{-1}, $P < 0.05$) coupled to a significant reduction in contractile strength (from 3.1 ± 0.2 to 2.3 ± 0.1 g, $P < 0.05$).

Administration of capsaicin (1 μM, $n = 15$, Figure 1b) elicited a rapid increase in heart rate (from 245 ± 7 to 303 ± 10 beats min^{-1}, $P < 0.05$) and coronary flow (from 5.3 ± 0.2 to 7.1 ± 0.3 ml min^{-1}, $P < 0.05$) and a concomitant negative inotropic effect (force of contraction was reduced from 2.6 ± 0.1 to 2.0 ± 0.15 g, $P < 0.05$).

The myocardial and coronary effects of the two drugs peaked at about the second min from administration and returned to resting values, within 5–7 min (Figure 1).

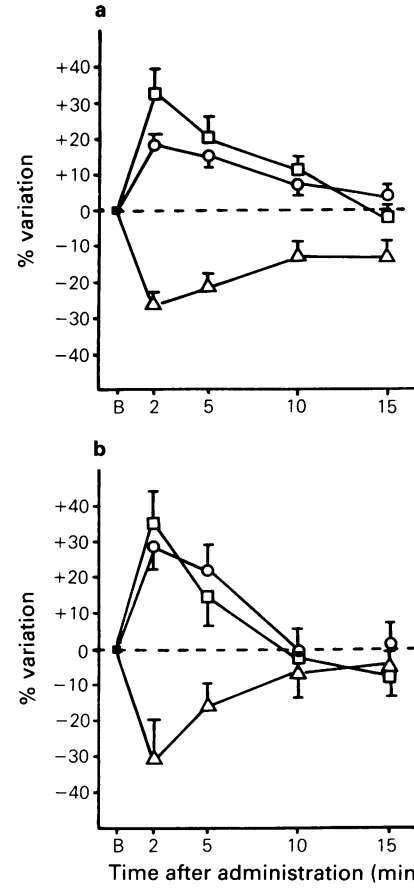


Figure 1 Time-dependency of the effects of bradykinin (a) and capsaicin (b) on heart rate (○), coronary flow (□) and contractile strength (△) in guinea-pig isolated perfused hearts. Each value is the mean of at least 9 experiments; vertical bars show s.e.mean.

In some experiments the response to bradykinin or capsaicin was assessed several times (at 30 min intervals) in the same heart. With bradykinin similar responses (positive chronotropic effect, increase in coronary flow and negative inotropic effect) were elicited for at least four times without significant modification ($n = 5$). On the other hand, a second administration of capsaicin had no positive chronotropic effect, although a consistent increase in coronary flow was still observed in 5 out of 8 preparations (the mean increase in coronary flow was 0.95 ± 0.40 ml min^{-1}, as compared to 2.02 ± 0.48 at the first challenge, $P < 0.05$). Also a negative inotropic response was still evident at the second challenge with capsaicin (from 2.4 ± 0.2 to 1.8 ± 0.4 g, NS as compared to the effect of the first

dose). A third challenge with capsaicin did not produce any detectable effect on heart rate or coronary flow ($n = 4$), while a slight decrease in force of contraction was still evident (0.3 ± 0.01 g, $n = 4$; not significantly different from the effect of the vehicle alone, see methods section).

Effect of substance P or calcitonin gene-related-peptide on chronotropism, inotropism and coronary flow of isolated perfused guinea-pig hearts

Substance P (0.1–10 μM, $n = 5$) in increasing non-cumulative concentrations produced a concentration-dependent increase in coronary flow without any significant inotropic or chronotropic effect (Figure 2, upper panel). At the highest concentration tested (10 μM) the increase in coronary flow was 2.07 ± 0.24 ml min^{-1} ($P < 0.01$); heart rate and force of contraction were 237 ± 10 and 240 ± 9 beats min^{-1} (NS) and 2.5 ± 0.1 and 2.3 ± 0.1 g (NS) before and after substance P, respectively.

On the other hand, CGRP produced a sharp and concentration-dependent (10 nM–1 μM; $n = 5$) increase in heart rate along with a marked increase in coronary flow (Figure 2, lower panel). A slight negative inotropic effect was also evident. At the highest concentration tested (1 μM), CGRP increased coronary flow from 4.1 ± 0.3 to 5.7 ± 0.4 ml min^{-1} ($P < 0.05$), reduced the force of contraction from 2.6 ± 0.2 to 2.2 ± 0.3 g (NS) and increased heart rate from 245 ± 8 to 326 ± 17 beats min^{-1} ($P < 0.05$).

Both the chronotropic and vasodilator-responses to substance P and CGRP had a time-course similar to that of capsaicin (1 μM) or bradykinin (10 μM), peaking about 2–3 min from administration and rapidly declining within 5–10 min.

In hearts from capsaicin-pretreated animals, both substance P (10 μM) and CGRP (1 μM) elicited myocardial and coronary responses similar to those observed in control hearts ($n = 4$; data not shown).

Effects of bradykinin after acute or chronic treatment with capsaicin

To investigate further whether activation of capsaicin-sensitive sensory fibres could be involved in the effects of bradykinin, experiments were performed in hearts obtained from animals desensitized to capsaicin five days earlier (see methods section for details) or in hearts pre-exposed *in vitro* to a bolus injection of capsaicin (1 μM), 30 min before the addition of bradykinin (10 μM).

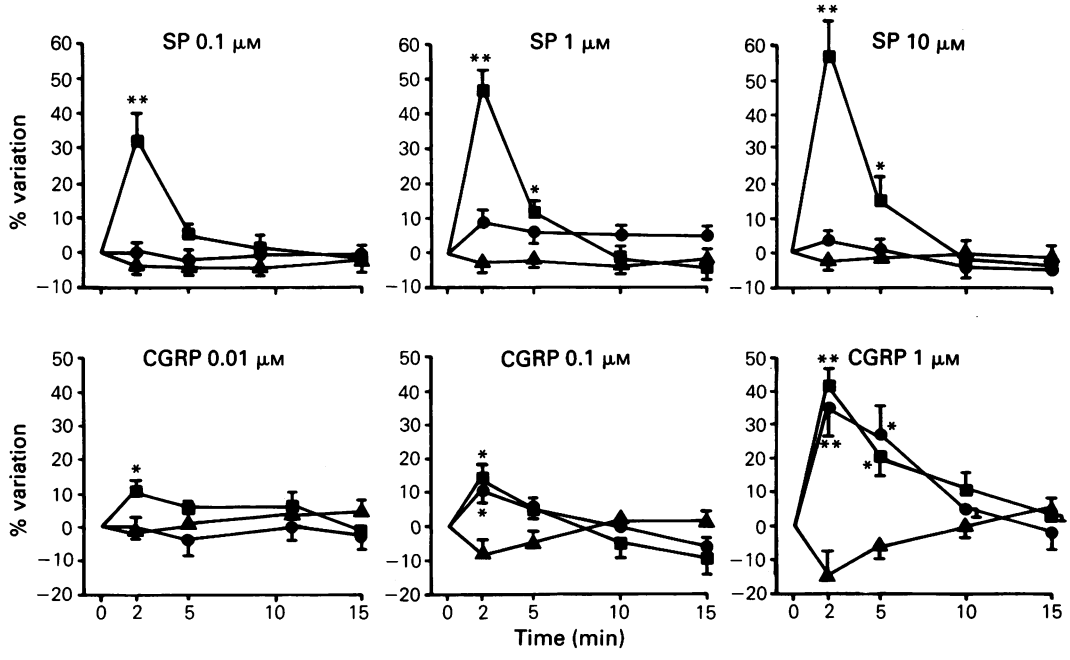


Figure 2 Concentration-dependent effect of substance P (SP, upper panels) and calcitonin gene-related peptide (CGRP, lower panels) on heart rate (●), coronary flow (■) and contractile strength (▲) in guinea-pig isolated perfused hearts. Each value is the mean of at least 5 experiments; vertical bars show s.e.mean.

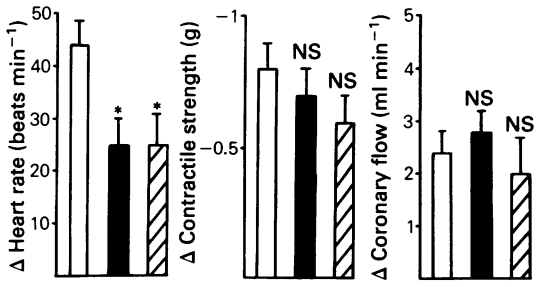


Figure 3 Effect of bradykinin on heart rate, contractile strength and coronary flow in hearts obtained from control animals (open column), animals systematically pretreated with capsaicin (55 mg kg^{-1}, s.c.) five days before experiment (solid columns), and in hearts previously (30 min) challenged *in vitro* with capsaicin (1 μM) (hatched column). Each point is the mean of at least 6 experiments; s.e.mean shown by vertical bars.
*$P < 0.05$ as compared to response obtained in hearts from control animals.

Both the *in vitro* and systemic capsaicin desensitization produced a significant reduction in the increase in heart rate by bradykinin (44 ± 6, 25 ± 6 and 25 ± 5 beats min^{-1} in control hearts, $n = 9$, and after *in vitro*, $n = 6$, or chronic pretreatment, $n = 9$, with capsaicin, respectively, $P < 0.05$). On the other hand the effect of bradykinin on coronary flow and cardiac inotropism were not significantly altered by capsaicin pretreatment (Figure 3).

In some experiments ($n = 4$) the effects of capsaicin (1 μM) were assessed in hearts previously challenged four times with bradykinin (10 μM). The amplitude of the responses to capsaicin on coronary flow and cardiac performance were not significantly different from those in control hearts (data not shown).

Bradykinin and capsaicin-induced release of SP-LI and CGRP-LI in guinea-pig isolated perfused hearts

SP-LI and CGRP-LI in the 6 hearts examined were 7.71 ± 0.88 and 19.2 ± 1.8 pmol per whole heart, respectively. The content of SP-LI and CGRP-LI in the atria and ventricles of control and capsaicin-pretreated guinea-pigs are shown in Table 1. In both atria and ventricles the ratio of SP-LI/CGRP-LI was approximately 0.4. The systemic capsaicin pretreatment depleted SP-LI by 94–80% and CGRP-LI by 87–65%.

The infusion of either capsaicin or bradykinin evoked a marked and concentration-related increase of both SP-LI and CGRP-LI in the perfusate (Table 2). On a molar basis, the outflow of SP-LI induced by bradykinin (10 μM) or capsaicin (1 μM) was about 32% and 41% of the CGRP-LI released by the same concentrations of both drugs, i.e. very close to the SP-LI/CGRP-LI ratio in heart (see above). Furthermore, both capsaicin and bradykinin released a similar amount of either SP-LI or CGRP-LI as a percentage of their respective tissue content (Table 2). When bradykinin (10 μM) or capsaicin (1 μM) was perfused 30 min after a challenge *in vitro* with capsaicin (1 μM), neither agent produced a measurable release of SP-LI or CGRP-LI. Likewise, neither capsaicin nor bradykinin released SP-LI or CGRP-LI in hearts excised from animals pretreated *in vivo* with capsaicin (Table 2).

Table 1 Substance P-like immunoreactivity (SP-LI) and calcitonin gene-related peptide-like immunoreactivity (CGRP-LI) (pmol g^{-1}) in hearts pretreated *in vitro* or *in vivo* with capsaicin

		Right atrium	*Left atrium*	*Right ventricle*	*Left ventricle*
In vitro pretreatment					
SP-LI	Vehicle	14.7 ± 1.8	11.7 ± 0.9	5.1 ± 0.9	3.4 ± 0.4
	Capsaicin	12.9 ± 1.3	10.4 ± 1.7	5.1 ± 0.9	2.9 ± 0.5
In vivo pretreatment					
SP-LI	Vehicle	15.1 ± 1.6	12.9 ± 0.9	6.2 ± 1.0	4.8 ± 0.9
	Capsaicin	0.9 ± 0.2*	0.7 ± 0.1*	0.9 ± 0.1*	1.0 ± 0.2*
CGRP-LI	Vehicle	34.5 ± 3.1	28.2 ± 1.9	16.8 ± 1.2	12.1 ± 1.3
	Capsaicin	4.5 ± 1.2*	5.2 ± 0.9*	5.1 ± 0.7*	4.3 ± 1.0*

In vitro pretreatment: hearts excised from control guinea-pigs mounted in the perfusion apparatus and challenged with injection of capsaicin (1 μM) or vehicle.
In vivo pretreatment: hearts excised from animals pretreated, five days before, with capsaicin (55 mg kg^{-1} s.c. on two consecutive days) or vehicle.
Each value is the mean ± s.e.mean from at least 4 experiments.
*$P < 0.01$.

Table 2 Substance P-like immunoreactivity (SP-LI) and calcitonin gene-related peptide-like immunoreactivity (CGRP-LI) outflow induced by bradykinin or capsaicin from guinea-pig isolated perfused hearts: experiments were performed in hearts excised from (A) control animals; (B) animals pretreated *in vivo* with capsaicin (55 mg kg^{-1} s.c. on two consecutive days) and (C) control animals challenged *in vitro* with capsaicin (1 μM) 30 min before drug administration

	SP-LI (fmol 4 min^{-1})	*% of tissue content*	*CGRP-LI* (fmol 4 min^{-1})	*% of tissue content*
A				
Basal	19 ± 1	—	37 ± 3	—
Bradykinin 1 μM	50 ± 5*	0.6	161 ± 19*	0.8
Bradykinin 10 μM	181 ± 21**	2.3	565 ± 98**	2.7
Capsaicin 0.1 μM	524 ± 59**	6.8	n.m.	n.m.
Capsaicin 1 μM	1152 ± 156**	15.1	2761 ± 263**	14.2
B				
Bradykinin 10 μM	12 ± 2	—	15 ± 2	—
Capsaicin 1 μM	16 ± 2	—	19 ± 3	—
C				
Bradykinin 10 μM	14 ± 1	—	23 ± 3	—
Capsaicin 1 μM	18 ± 1	—	27 ± 4	—

Each value is the mean ± s.e.mean from at least 4 experiments.
n.m. = not measured.
*$P < 0.05$ as compared to basal values; **$P < 0.01$ as compared to basal values.

At its second administration, bradykinin (10 μM) evoked a release, although this was much reduced, of both SP-LI and CGRP-LI, and at the third and fourth injection of bradykinin, release of SP-LI and CGRP-LI was still detectable (Figure 4). After four challenges with bradykinin (10 μM), capsaicin (1 μM) was still able to evoke a marked increase of SP-LI and CGRP-LI outflow (Figure 4).

Effect of indomethacin on functional and neurochemical actions of bradykinin

Since many of the biological actions of bradykinin are mediated by prostaglandin release (Regoli & Barabè, 1980) we investigated whether blockade of tissue prostaglandin synthesis by indomethacin could influence either the release of neuropeptides by bradykinin or its functional effects.

As shown in Figure 5, in the presence of indomethacin (10 μM) the release of both SP-LI and CGRP-LI by bradykinin was markedly reduced (by 83% and 88% respectively, $n = 5$). Indomethacin alone did not evoke neuropeptide release.

Perfusion with indomethacin produced significant reduction in heart rate (from 222 ± 17 to 200 ± 16 beats min^{-1}, $P < 0.05$) while coronary flow and contractile strength were unaffected ($n = 6$). In the presence of indomethacin, functional responses to bradykinin were greatly changed as compared to those observed in control hearts: the increase in heart rate was significantly reduced (22 ± 6 compared to 44 ± 6 beats min^{-1} in controls, $P < 0.05$); the negative inotropic effect was virtually abolished (0.2 ± 0.07 g compared to 0.8 ± 0.1 g in controls, $P < 0.05$) and the increase in coronary flow was almost doubled (4.3 ± 0.5 ml min^{-1} compared to 2.4 ± 0.4 ml min^{-1} in controls, $P < 0.01$).

Discussion

In guinea-pig isolated perfused hearts both bradykinin and capsaicin produced qualitatively similar functional and neurochemical effects. Both agents evoked a marked increase in coronary flow and heart rate, at the same time as reducing contractile force. These effects were paralleled by the release of sensory neuropeptides (SP-LI and CGRP-LI) suggesting that a local release from sensory nerve endings could be involved in mediating the functional responses to these agents.

It has already been established that stimulation by bradykinin of sensory afferents of cardiac origin is relevant to the initiation of reflex cardiovascular responses (Kaufmann *et al.*, 1980). Our data indicate that activation of these fibres by bradykinin also evokes effects *in situ* due to a local release of neuropeptides. Thus the dual 'sensory-efferent' function of these fibres, as defined by Szolcsanyi (1984) and con-

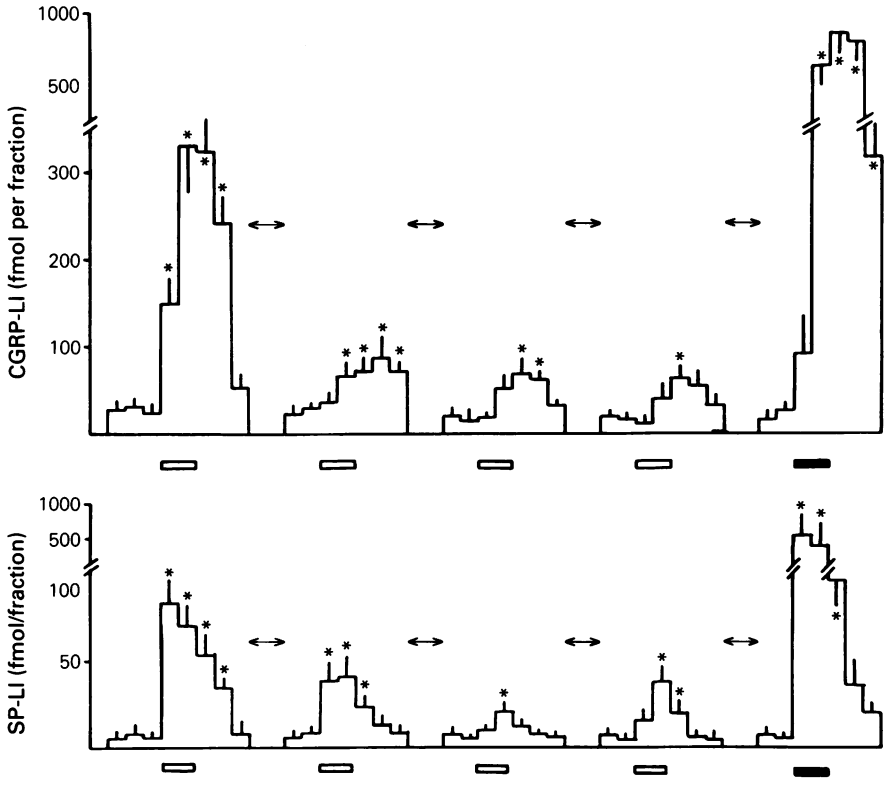


Figure 4 Substance P-like immunoreactivity (SP-LI) and calcitonin gene-related peptide-like immunoreactivity (CGRP-LI) outflow from guinea-pig isolated perfused heart, elicited by four consecutive challenges with bradykinin (10 μM) (open bar, □). At the end capsaicin (1 μM) (solid bars, ■) was administered. Each challenge was performed at 30 min interval. Horizontal arrows (↔) indicate an interval of 25 min. Each bar represent a 1 min fraction, and is the mean of at least 5 vertical lines show s.e.mean experiments; Drugs were slowly infused over 2 min.
*$P < 0.05$ as compared to respective predrug values.

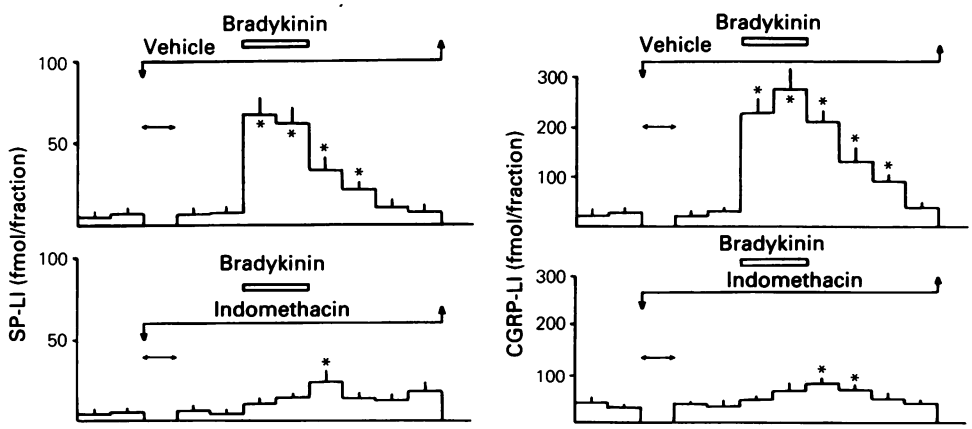


Figure 5 Influence of indomethacin (10 μM) on bradykinin (10 μM)-induced substance P-like immunoreactivity (SP-LI) and calcitonin gene-related peptide-like immunoreactivity (CGRP-LI) outflow from guinea-pig isolated perfused hearts. Indomethacin was perfused 15 min before, during bradykinin infusion and in the following 15 min. Horizontal arrows (←——→) indicate an interval of 13 min. Indomethacin itself did not elicit neuropeptide release. Each bar represents a 1 min fraction, and is the mean of at least 5 experiments: vertical lines show s.e.mean. Bradykinin was slowly infused over 2 min.
*$P < 0.05$ as compared to respective predrug values.

firmed in various tissues and test systems mainly by the use of capsaicin (Maggi & Meli, 1988; Holzer, 1988) appears to be a specific feature of these fibres which follows their activation either by exogenous or endogenous agents.

However some important differences may exist in the mechanisms for the release of neuropeptides by bradykinin or capsaicin. Thus at the maximal dose used, the amount of SP-LI and CGRP-LI released is about five times greater with capsaicin than with bradykinin. Second, neuropeptide release can be evoked repeatedly with bradykinin but only once with capsaicin and finally, after challenge with capsaicin, bradykinin is no longer able to produce release of neuropeptide. These data can be explained on the basis of the hypothesis advanced by Hakanson *et al.* (1987) that at least two releasable neuropeptide pools are present in sensory nerve endings, i.e.: a minor one that can be rapidly released and refilled and which is sensitive both to bradykinin and capsaicin, and a second pool that is capsaicin-sensitive but bradykinin-resistant, which refills the former pool and is not replenishable on a short term basis. However, an additional factor that might be involved is that exposure to capsaicin may produce certain irreversible modifications in the membrane and/or intraneuronal structures of sensory nerve endings that render the nerve refractory to successive stimulations. The observation that acute exposure to capsaicin produces a slight, and not significant, reduction in tissue content of SP-LI confirms that the bulk of neuropeptides contained in the nerve endings are in a non-releasable store (at least by capsaicin), as previously suggested in other preparations (Maggi *et al.*, 1987b; Hakanson *et al.*, 1987).

Much evidence has been presented for a co-localization of both peptides in central and peripheral sensory nerve endings and a dense network of fibres containing both SP-LI and CGRP-LI, has been demonstrated in cardiac tissues (Papka *et al.*, 1984; Hougland *et al.*, 1986; Lundberg *et al.*, 1985; Mulderry *et al.*, 1985; Saito *et al.*, 1986a).

It is interesting to note, that the percentage of tissue SP-LI or CGRP-LI released by each agent is fairly similar (14% and 2.5% for capsaicin and bradykinin, respectively). Further, the ratio of SP-LI and CGRP-LI released by capsaicin (0.41) or bradykinin (0.32) is similar to that found in atrial and ventricular tissues (range 0.36–0.45). Taken together, these findings strongly suggest that SP-LI and CGRP-LI are co-released from sensory nerve endings. In addition, it is interesting to note that the effects of capsaicin and bradykinin on heart could be mimicked by substance P (increase in coronary flow) and CGRP (positive chronotropic effect and increase in coronary flow). The possibility that other neuropeptides may also be released from sensory nerve endings to participate in mediating functional responses must also be considered. For example, neurokinin A has been reported to be colocalized with SP and CGRP in sensory structures and to exert a negative inotropic effect in the isolated perfused heart (Lundberg *et al.*, 1985). This latter effect, which is observed with both bradykinin and capsaicin, is not mimicked by SP and CGRP. Regarding the positive chronotropic response, our data with capsaicin, substance P and CGRP confirm results obtained in other cardiac preparations (Franco-Cereceda & Lundberg, 1985; Lundberg *et al.*, 1985) and further substantiate the hypothesis that CGRP is a major neurotransmitter in non-adrenergic non-cholinergic cardioaccelerator nerves (Saito *et al.*, 1986a).

An important difference between the functional effects of bradykinin and of capsaicin is that after previous exposure to capsaicin, while all functional effects of capsaicin undergo desensitization, those of bradykinin do not, with the exception of part of its positive chronotropic effect. Thus it is likely that in the isolated perfused heart the overall action of bradykinin will be the resultant not only of the stimulation of sensory nerve endings but also of a direct effect on its own receptor(s) on the coronary vasculature (Toda, 1977; Regoli & Barabè, 1980; Beny *et al.*, 1987), and release of prostaglandin (Goldberg *et al.*, 1976; Warren *et al.*, 1987) and/or histamine (Johnson & Erdos, 1973). It is important to emphasize that, to obtain detectable release of sensory neuropeptides we used a high concentration of bradykinin. In addition, since the peptide was injected into the coronary vasculature, all structures of the heart would be exposed. In view of the above, it is likely that in our experimental conditions there was activation of all bradykinin-dependent mechanisms. Further studies are necessary to investigate whether localized application of low concentrations of bradykinin could selectively act by releasing neuropeptides from sensory nerve endings.

It is interesting that most of the bradykinin-induced release of SP-LI and CGRP-LI is strongly reduced in the presence of indomethacin and this release may therefore be due to an indirect activation of sensory fibres through the action of prostanoids. Indeed, in various tissues prostaglandins have been reported to be endogenous activators of afferent fibres (Yanagisawa *et al.*, 1986; Longhurst & Dittman, 1987; Maggi *et al.*, 1988). In this context, it is worth noting that the effect of indomethacin on the positive chronotropic effect of bradykinin is similar to that of capsaicin desensitization.

In conclusion our findings indicate that in heart tissues, bradykinin can activate (mostly through prostaglandin release) capsaicin-sensitive sensory nerve fibres. The consequent release of neuropeptides

may produce at least part of the complex functional effects of this kinin on cardiac performance. Thus bradykinin might activate both the sensory (Kaufman *et al.*, 1980) and the local peripheral (this study) functions of the sensory nerves, giving rise to local and systemic cardiovascular responses. It is interesting to note that during myocardial ischaemia in addition to bradykinin, other chemical or physical agents, such as anoxia, hypoxia or prostaglandins (Yanagisawa *et al.*, 1986; Longhurst & Dittman, 1987), could produce further activation of sensory nerve fibres. Therefore the 'sensory-efferent' function of these fibres might play an important role in the pathophysiology of ischaemic cardiac diseases.

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Effect of aminophylline on cisplatin nephrotoxicity in the rat

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1 The effect of the methylxanthine aminophylline on cisplatin (5 mg kg^{-1} i.v.)-induced acute renal failure was investigated in the rat. Renal function was measured 5 days after cisplatin administration.

2 Cisplatin caused a polyuric acute renal failure. The creatinine clearance was significantly reduced.

3 Aminophylline (24 mg kg^{-1} 12 h^{-1}) ameliorated the cisplatin nephrotoxicity when administered during the maintenance phase of acute tubular necrosis. However, it had no effect when only administered prophylactically before the cisplatin application.

4 Enprofylline (20 mg kg^{-1} 4 h^{-1} with dose adjustment), a methylxanthine lacking adenosine receptor antagonism in comparison to aminophylline, had no protective effect on cisplatin nephrotoxicity.

5 Adenosine is a renal vasoconstrictor and decreases glomerular filtration rate. Endogenous adenosine in the kidney is formed by degradation of ATP and is thought to be involved in various forms of acute renal failure. The results suggest that adenosine may be involved in the haemodynamic changes in the kidney induced by cisplatin.

Introduction

Cisplatin is a potent antineoplastic drug whose optimal clinical use is limited because of its ability to induce nephrotoxicity (Goldstein & Mayor, 1983; Loehrer & Einhorn, 1984). The nephrotoxicity which can be observed in man as well as in animals is dose-dependent (Ward *et al.*, 1977; Loehrer & Einhorn, 1984). Cisplatin-induced acute renal failure follows a typical sequence. For the first 48 h the renal haemodynamics are normal; thereafter, a decrease of both renal blood flow and glomerular filtration can be observed with a maximum change occurring between the 3rd and 6th day and a subsequent slow recovery (Safirstein *et al.*, 1981; Chopra *et al.*, 1982; Winston & Safirstein, 1985).

It has been proposed that adenosine modulates renal haemodynamics under physiological and pathophysiological conditions (Osswald *et al.*, 1980; Osswald, 1983). Adenosine is a potent vasoconstrictor in the renal circulation (Thurau, 1964; Osswald, 1983). Intra-arterial infusion of adenosine causes a reduction in glomerular filtration rate (GFR) in rats and dogs (Osswald, 1975; Osswald *et al.*, 1978; Miller *et al.*, 1978). Osswald and co-workers (1980) demonstrated an inverse correlation between renal tissue content of adenosine 5'-triphosphate (ATP) and that of adenosine when the kidney was challenged with intra-arterial infusion of hypertonic saline. The ATP tissue content was reduced as the tubular sodium absorption was increased. Since an intrarenal vasoconstrictive metabolite was postulated to mediate vasoconstriction in acute renal failure, it was proposed that adenosine was involved in the pathophysiology of acute renal failure (Osswald *et al.*, 1977; 1980; Churchill & Bidani, 1982). In fact, theophylline improves kidney function following 60 min of ischaemia (Osswald *et al.*, 1977). Aminophylline and 8-phenyltheophylline ameliorate glycerol-induced renal failure (Bidani & Churchill, 1983; Bowmer *et al.*, 1986) and aminophylline has also been shown to inhibit the vasoconstrictor response to acute i.v. infusions of amphotericin B in the rat (Heidemann *et al.*, 1983) and in the dog (Gerkens *et al.*, 1983). All these studies can be interpreted as providing confirmation for the role of

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adenosine in mediating the renal response; however, aminophylline is a renal vasodilator and known to have pharmacological activity independent of adenosine receptor inhibition, for example the inhibition of phosphodiesterase.

Enprofylline, a novel bronchodilator xanthine, in comparison to theophylline is not a significant adenosine receptor antagonist (Lunell *et al.*, 1982; 1983). Hermes & Osswald (1985) show that enprofylline does not change renal function and conclude that enprofylline has no adenosine antagonising action in the rat kidney.

In the present study we have therefore investigated the effect of aminophylline on the outcome of cisplatin-induced acute renal failure. Aminophylline was given either prophylactically before the cisplatin dose or in a curative manner after the administration of cisplatin. In addition, the effect of aminophylline on cisplatin nephrotoxicity was compared with enprofylline, to test the hypothesis that the effects of methylxanthines on drug-induced acute renal failure may be mediated by adenosine. Since the cisplatin nephrotoxicity can be enhanced by a low salt diet (Heidemann *et al.*, 1985) and an inverse correlation exists between the intrarenal adenosine concentration and the sodium chloride administration (Osswald *et al.*, 1980), the experiments were done with animals on a normal as well as a low salt diet.

Methods

Induction of acute renal failure

Male Wistar rats (Institut für Versuchstierzucht, Hannover) weighing 200–280 g received cisplatin, 5 mg kg^{-1} intravenously. Vehicle-cisplatin rats were injected with an equal amount of normal saline. Kidney function was measured 5 days after drug administration.

Experimental protocol

Chronic aminophylline administration and cisplatin nephrotoxicity The effect of chronic administration of aminophylline (24 mg kg^{-1} i.p., 12 hourly) on cisplatin nephrotoxicity was investigated in rats on a control diet (Natrium: 2.5 g kg^{-1}) ($n = 11$) or a low salt diet (Natrium: 0.15 g kg^{-1}) ($n = 12$) (Altromine, Lage). Cisplatin was administered 30 min after the first dose of aminophylline. Aminophylline was administered for 5 days. Vehicle-treated rats (control diet $n = 8$, low salt diet $n = 12$) received in addition to cisplatin an equal amount of ethylenediammine (4.5 mg kg^{-1} i.p.), the vehicle for aminophylline. Plasma creatinine concentrations were measured 5 days later using a standard spectrophotometric assay (reaction with alkaline picrate solution).

Prophylactic aminophylline treatment In the second part of the experiment, the effect of a single dose of aminophylline (24 mg kg^{-1} i.p.) on cisplatin-induced acute renal failure was studied in rats ($n = 8$).

Aminophylline or vehicle was injected 30 min before the cisplatin administration. The plasma creatinine concentrations were measured on the 5th day.

'Curative' aminophylline treatment In the third part of the experiment, the effect of a 'curative' aminophylline treatment regimen was investigated. Aminophylline (24 mg kg^{-1} i.p.) was injected 12 hourly for 5 days ($n = 6$). However, the first dose was given 30 min after cisplatin administration. On the 4th day all animals were housed in metabolic cages to collect the 24 h urine. After 12 h a blood sample (500 μl) was taken to measure haematocrit, plasma sodium, potassium (by flame photometer) and creatinine. Sodium, potassium and creatinine were also measured in urine. Total electrolyte excretion, filtration fraction for sodium and creatinine clearance were calculated using standard formulae. These results were compared with rats which had received vehicle (saline) plus aminophylline ($n = 6$) and rats which had received vehicle (ethylenediammine) in addition to the cisplatin ($n = 6$).

Enprofylline in comparison to aminophylline treatment In the fourth part of the experiment, the effect of enprofylline (20 mg kg^{-1} i.p.) ($n = 10$) on cisplatin nephrotoxicity was compared with aminophylline (24 mg kg^{-1} i.p.) ($n = 10$). Since enprofylline is mainly excreted through the kidneys a dose adjustment was necessary. On days 1 and 2 the drug was given every 4 h, on days 3 to 5 every 12 h. The plasma creatinine concentrations were measured in all rats on the 5th day. Before and at the end of all experiments the animals were weighed.

Materials

Cisplatin (Platinex, Bristol Meyers), aminophylline (Aminophyllin, Injektionslösung; Promonta), enprofylline (Draco, Lund), ethylenediammine (Fisher Scientific).

Statistics

Mean values ± s.d. were calculated. Two treatment groups were compared by use of Student's one-tailed t test for unpaired data. Three treatment groups were compared by use of analysis of variance. In the case of differences the t test was then used to assess

Table 1 The effect of cisplatin + vehicle (CP + Veh_{Am}) and cisplatin + aminophylline (CP + Amino) on plasma creatinine concentration (P_{Cr}), plasma sodium concentration (P_{Na}), plasma potassium concentration (P_K), haematocrit (Hct) and the development of body weight (BW)

	P_{Cr} (mg dl^{-1})	P_{Na} (mmol l^{-1})	P_K (mmol l^{-1})	*Hct* (%)	*BW* (g)
Veh_{CP} + Amino	0.4 ± 0.1	145 ± 3	4.0 ± 0.8	43 ± 2	+16.0 ± 5.5
CP + Veh_{Am}	#4.0 ± 2.3	153 ± 8	4.0 ± 0.8	40 ± 4	# −10.0 ± 7.9
CP + Amino	*1.3 ± 0.4	*173 ± 7	3.9 ± 0.4	42 ± 1	# −14.0 ± 7.4

The results shown are means ±s.d. *$P < 0.05$ in comparison to CP + Veh_{Am}; #$P < 0.05$ in comparison to Veh_{CP} + Amino.

the significance of any differences noted. A P value of <0.05 was considered as being significant.

Results

Effect of cisplatin on kidney function

Intravenous cisplatin administration caused acute renal failure in all rats. The serum creatinine concentration was significantly increased in comparison to vehicle controls and the body weight was significantly reduced (Table 1). The serum electrolyte concentrations showed no change (Table 1). The creatinine clearance was significantly reduced in comparison to the animals serving as vehicle controls. The renal failure had no effect on sodium excretion and potassium excretion in the urine (Table 2). The fractional sodium excretion was unchanged, the urine/plasma creatinine ratio was significantly decreased in comparison to the vehicle rats (Table 2).

The effect of aminophylline on cisplatin nephrotoxicity

In the first part of the experiment the effect of aminophylline on cisplatin nephrotoxicity was investigated in rats receiving a normal and low sodium chloride rat chow. In the chronic pretreatment study, intraperitoneal administration of aminophylline every 12 h for 5 days (the first dose given 30 min before the cisplatin administration) significantly reduced the plasma creatinine elevations due to cisplatin-induced renal failure in rats on both normal and low salt diets. The low salt diet *per se* enhanced the cisplatin nephrotoxicity, which could be seen by an increase in the serum creatinine concentration in comparison to the animals fed a normal salt diet. In the animals on a normal salt diet aminophylline reduced the serum creatinine concentrations from 2.23 ± 0.88 mg dl^{-1} to 1.16 ± 0.61 mg dl^{-1} and in the animals on a low salt diet from 4.43 ± 1.47 mg dl^{-1} to 2.78 ± 1.26 mg dl^{-1} (Figure 1).

In case the protective effect of aminophylline was mainly due to the first dose given before cisplatin, the effect of a single dose of aminophylline 30 min before cisplatin administration was investigated in an additional experiment. After 5 days no significant difference in the serum creatinine concentrations could be observed in the animals treated with vehicle

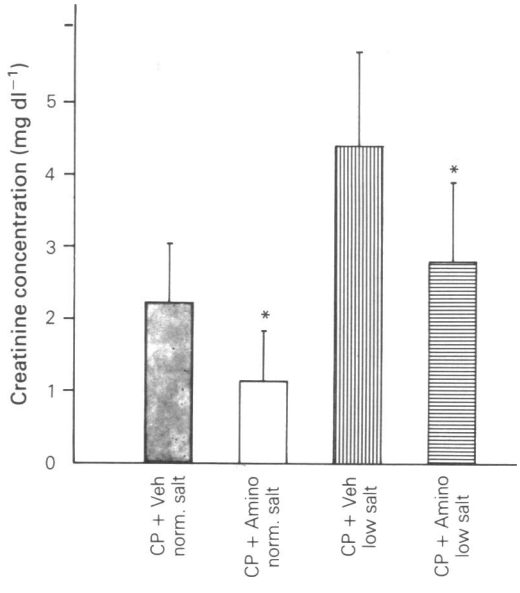


Figure 1 The effect of aminophylline (Amino) and vehicle (Veh) on cisplatin (CP)-induced nephrotoxicity in rats on control diet (norm. salt) and a low salt diet. Columns represent mean values and vertical lines show s.d. * Significant difference, $P < 0.005$).

Table 2 The effect of cisplatin + vehicle (CP + Veh_{Am}) and cisplatin + aminophylline (CP + Amino) on urine volume, electrolyte excretion, urine to plasma creatinine ratio (U/P_{Cr}), fractional sodium excretion (FE_{Na}) and creatinine clearance (Cl_{Cr})

	U_{Vol} (ml)	U_{Na} (mmol l^{-1})	U_K (mmol l^{-1})	$U_{Na} \times U_{Vol}$ (mmol)	$U_K \times U_{Vol}$ (mmol)	U/P_{Cr}	FE_{Na} (%)	Cl_{Cr} (ml min^{-1})	$Cl_{Cr} \times 100^{-1}$ g BW (ml min^{-1})
Veh_{CP} + Amino	21.6 ± 9.4	1.3 ± 0.3	106.5 ± 62.9	0.027 ± 10.8	1.830 ± 0.191	106.7 ± 62	0.01 ± 0.01	1.34 ± 0.23	0.57 ± 0.07
CP + Veh_{Am}	24.6 ± 8.2	15.8 ± 19.9	43.5 ± 19.5	0.290 ± 0.288	1.026 ± 0.526	#5.5 ± 2.5	4.35 ± 7.58	#0.12 ± 0.06	#0.05 ± 0.02
CP + Amino	42.4 ± 18.7	2.9 ± 1.7	33.7 ± 21.7	0.128 ± 0.086	1.142 ± 0.258	*12.5 ± 3.7	0.17 ± 0.09	*0.40 ± 0.14	*0.18 ± 0.06

The results shown are means ± s.d. *$P < 0.05$ in comparison to CP + Veh_{Am}; #$P < 0.05$ in comparison to Veh_{CP} + Amino.

plus cisplatin in comparison to aminophylline plus cisplatin (3.92 ± 2.87 versus 3.03 ± 1.72 mg dl^{-1}).

The curative effect of aminophylline treatment on cisplatin nephrotoxicity was investigated in a chronic post-treatment study. The methylxanthine was given 12 hourly for 5 days with the first dose 30 min after the cisplatin administration. In comparison to cisplatin plus vehicle-treated animals the serum creatinine concentrations were significantly reduced (4.0 ± 2.3 versus 1.3 ± 0.4 mg dl^{-1}) (Table 1). In the cisplatin plus aminophylline-treated rats the serum sodium concentration was significantly increased. However no differences were observed for the serum potassium concentration, the haematocrit and the change in body weight in comparison to the cisplatin plus vehicle group (Table 1). In comparison to the cisplatin plus vehicle group, the creatinine clearance was significantly elevated in the cisplatin plus aminophylline-treated rats (Table 2). The urine volume in the cisplatin plus aminophylline group was not different from that of the cisplatin plus vehicle group or the vehicle plus aminophylline group (Table 2). Although the urine volume was not different in the three treated groups, the urine to plasma creatinine ratio of the cisplatin plus aminophylline group was significantly elevated in comparison to the cisplatin plus vehicle group (Table 2). A beneficial effect of aminophylline was not evident from the fractional sodium excretion.

The effect of enprofylline

Daily injections of enprofylline, 12 hourly (dose adjustment day 3–5; the first dose after cisplatin administration) resulted in significantly higher serum creatinine concentrations than the concomitant therapy with aminophylline. The creatinine concentrations were 3.46 ± 1.43 mg dl^{-1} in comparison to 1.6 ± 0.9 mg dl^{-1}.

Discussion

A major side-effect of cisplatin treatment may be nephrotoxicity. We have previously shown that salt intake has a pronounced effect on cisplatin nephrotoxicity and that a single dose of the diuretic acetazolamide markedly reduces this deleterious side-effect (Heidemann *et al.*, 1983). To investigate further the mechanism of cisplatin nephrotoxicity we studied the effect of the methylxanthine aminophylline on cisplatin-induced acute renal failure. Aminophylline has diuretic properties and is a renal vasodilator, both effects most probably due to aden-

osine receptor blockade (Osswald, 1983). The rationale of this study was therefore to test the hypothesis that adenosine may be a mediator of cisplatin-induced renal impairment. We now present data that aminophylline reduces cisplatin nephrotoxicity and, more important, that this beneficial effect can be achieved by a 'curative' treatment regimen.

Cisplatin ($5\,mg\,kg^{-1}$, i.v.) caused acute renal failure in all rats five days after the drug administration. The renal failure was characterized by a urine concentrating defect in kidney function as expressed by the marked drop in the urine/plasma-creatinine-ratio; a result also shown by others (Safirstein *et al.*, 1981; Chopra *et al.*, 1982). Cisplatin caused a marked increase in the serum creatinine concentration and a reduction in creatinine clearance. Chronic aminophylline treatment significantly attenuated cisplatin-induced acute renal failure. This beneficial effect was independent of the sodium intake. Although the urine volume of these animals was higher than in the rats that had received cisplatin alone, aminophylline significantly improved creatinine clearance and ameliorated the urine concentrating defect. Aminophylline-induced diuresis is a very unlikely explanation for its protective effect since a single dose of aminophylline shortly before cisplatin administration had no effect on the drug-induced nephrotoxicity, as has been shown for diuretics like acetazolamide and furosemide (Ward *et al.*, 1977, Heidemann *et al.*, 1983).

Enprophylline is a xanthine derivate lacking adenosine receptor antagonism in comparison to aminophylline (Persson *et al.*, 1982; Lunnell *et al.*, 1982; 1983). Hermes & Osswald (1985) investigated the effect of enprophylline on renal haemodynamics. These authors found no effect of the drug on GFR, single nephron GFR and sodium excretion. They therefore came to the conclusion that enprophylline has no adenosine antagonizing action in the rat kidney. We can show that enprofylline, in contrast to aminophylline, has no protective effect on cisplatin nephrotoxicity. This result can be regarded as further evidence that adenosine may be involved in the haemodynamic changes of cisplatin-induced renal failure.

Theophylline or derivates have been shown to have a beneficial effect on the outcome of various models of acute renal failure. Bidani & Churchill (1983) could reduce the severity and duration of glycerol-induced acute renal failure in rats. This work was expanded by Bowmer *et al.* (1986) who ameliorated a glycerol-induced acute renal failure by the more specific adenosine receptor antagonist 8-phenyltheophylline, an agent that, in addition, is thought to have no inhibitory effects on phosphodiesterase. Lin *et al.* (1987) showed that theophylline increased both GFR and renal blood flow during the initiation phase of post-ischaemic acute renal failure in rats. More recently the same authors (Lin *et al.*, 1988), in addition to their previous work, investigated the effect of theophylline during the maintenance phase of post-ischaemic acute renal failure in rats. They found that five days after a 30 min ischaemia, both renal blood flow and GFR could be increased by theophylline. Aminophylline has also been shown to inhibit the renal vasoconstriction due to acute amphotericin B infusions in the dog (Gerkens *et al.*, 1983) and in the rat (Heidemann *et al.*, 1983). In conclusion, all this experimental evidence is consistent with the hypothesis that adenosine is a mediator of the renal haemodynamic changes in acute renal failure. Adeosine is a potent vasoconstrictor in the kidneys, while all other vascular beds tested so far exhibit vasodilatation (Osswald, 1983). The haemodynamic effects of adenosine on the kidney are dependent on the salt status of the organism. NaCl-loaded animals do not respond with a decrease in renal blood flow and GFR, whereas sodium-depleted animals show enhanced vasoconstriction (Osswald *et al.*, 1978). Adenosine is present in the kidney (Osswald *et al.*, 1977; Miller *et al.*, 1978) and is most probably formed by degradation of ATP (Osswald, 1983). Adenosine concentrations increase several fold during renal ischaemia after ATP tissue concentrations have fallen (Osswald *et al.*, 1977). It is therefore possible that during acute tubular necrosis a decrease in cellular phosphorylation potential could in turn increase adenosine generation.

Independent of the underlying mechanism, it is possible that the protective effect of aminophylline on cisplatin nephrotoxicity may have clinical relevance. Empirical observations and animal studies have shown that the incidence of nephrotoxicity can be significantly reduced by the application of rather large infusion volumes. It is possible that certain patient groups are withheld from beneficial cisplatin therapy because they have contraindications for high i.v. volumes. Aminophylline could possibly enable these patients to receive cisplatin.

In conclusion, aminophylline given during the maintenance phase of cisplatin-induced acute renal failure, improves kidney function. Aminophylline has no effect when given only prophylactically during the initiation phase. The beneficial effect of aminophylline is independent of the salt intake. Since aminophylline exerts its renal effect most probably by competitive antagonism at adenosine receptors, the present results suggest that adenosine is involved in cisplatin-induced nephrotoxicity.

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Vasoactive actions of local anaesthetics on human isolated umbilical veins and arteries

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1 An *in vitro* study, using helical preparations of human umbilical arteries and veins obtained from healthy women at term pregnancy, was designed to determine: (a) whether three local anaesthetics commonly utilized in obstetric anaesthesia (bupivacaine, 2-chloroprocaine, and lignocaine) can induce contraction or relaxation of resting umbilical vessels; (b) whether these agents can induce contraction or relaxation of umbilical vessels which have been previously induced to contract by a known activator, potassium chloride (KCl); and (c) the relative potency of these agents as compared to KCl.

2 The results indicate that: (a) these local anaesthetics are vasoactive on human umbilical vascular smooth muscle; (b) bupivacaine induces contraction in over 90% of the resting vessels examined, while 2-chloroprocaine consistently causes relaxation and lignocaine causes a small degree of contraction in 40% of vessels examined; (c) bupivacaine causes further contraction (or potentiation) of KCl-contracted muscle in 50% of the vessels studied, while 2-chloroprocaine and lignocaine both induce relaxation of these contracted vessels.

Introduction

Regional anaesthesia using local anaesthetics is widely used in obstetrics for labour and delivery. Foetal effects of maternal local anaesthetic administration have been under intense scrutiny for the past few years. Local anaesthetic toxicity has been reported in parturients who received regional anaesthesia and, in addition, placental transfer of these agents has been confirmed by the presence of measurable quantities in umbilical cord blood (Ralston & Shnider, 1978). Foetal and neonatal well-being has been evaluated from foetal heart rate tracings, neonatal Apgar scores, and neurobehavioural scores; and results have been compared in the presence or absence of regional anaesthesia (Ralston & Shnider, 1978). However, the direct action of local anaesthetics on the vascular smooth muscle of the umbilical cord has not been extensively studied, although this tissue is an essential component in the regulation of the umbilical-placental circulation.

Umbilical blood flow studies in man are difficult to perform and therefore largely speculative (Walsh *et al.*, 1974). Umbilical blood flow in the physiological state is known to be pressure-dependent and relies on the resistance in the umbilical/placental unit (Walsh *et al.*, 1974). Microscopic studies of umbilical cords have demonstrated that innervation of umbilical vessels is lacking except for the intrafoetal segment (Hülsemann, 1971; Roach, 1976). In view of this, the reactivity of the umbilical vessels to vasoactive agents is of great importance, since umbilical resistance, and therefore, blood flow may be affected. Preliminary studies showed that human umbilical vessels can be used successfully in the study of vasoactive substances. Sympathomimetic amines were shown to induce responses in isolated umbilical arterial and venous strips (Somlyo *et al.*, 1965). Using umbilical vessels from human cords, varying contractile responses to 10 different vasoactive agents were demonstrated (Altura *et al.*, 1972).

Local anaesthetics are known to have vasoactive properties on a variety of vascular tissues (Covino, 1986). It is of interest to study what effect can be demonstrated on umbilical vasculature, since it is plausible that adverse physiological effects of local

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anaesthetics, e.g., foetal bradycardia following paracervical block, or other clinical effects caused by maternal local anaesthetic administration, result from their direct effect on the umbilical vessels. This question was raised in a previous article which described the effects of local anaesthetics on the umbilical artery (Tuvemo & Willdeck-Lund, 1982). However, this previous study did not include the umbilical vein, which is solely responsible for delivery of oxygenated blood to the foetus, and did not include 2-chloroprocaine, a local anaesthetic which is widely used in obstetrics.

The present study, utilizing vascular smooth muscle obtained from human umbilical arteries and veins was designed to determine: (1) whether three commonly used local anaesthetics (bupivacaine, 2-chloroprocaine and lignocaine) can induce contraction or relaxation of resting umbilical vessels; (2) whether these agents can induce contraction or relaxation of umbilical vessels which have been previously induced to contract by a known activator, KCl; and (3) the relative potency of these agents compared to the latter activator.

Methods

A total of 36 umbilical cords from normal pregnancies and healthy parturients (vaginal deliveries or Caesarean sections) were utilized for this study. Approximately 10 to 25 cm segments were cut from the cords, double-clamped, and placed immediately in cold Krebs-Ringer solution buffered with sodium bicarbonate. The solution was prepared daily from a refrigerated stock solution. The composition of the working Krebs-Ringer solution, made up with deionised water, was (in mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4 \cdot 7H_2O$ 1.2, $NaHCO_3$ 25.0, dextrose 10, pH 7.8–8.0. The chemicals used to make up the Krebs-Ringer bicarbonate solutions, as well as the KCl were all American Chemical Society certified reagent grade. The local anaesthetics were all of a grade conventionally used for regional anaesthesia in obstetric patients: bupivacaine hydrochloride (Marcaine hydrochloride 0.25%), 2-chloroprocaine hydrochloride (Nesacaine CE 2% with sodium bisulphite 0.7 mg ml^{-1}), and lignocaine hydrochloride (Xylocaine 1%).

The arteries and veins were carefully dissected out of the cords using microdissecting instruments, while continuously bathing the cords and vessels in cold Krebs-Ringer bicarbonate buffer. The arteries and veins were cut helically and then trimmed to make vascular strips of the following dimensions: 1.5 mm × 20 mm for arteries and 2.5 mm × 25 mm for veins, similar to that described previously (Altura *et al.*, 1972). They were each suspended in a Harvard Universal Jacketed Isolated Organ Bath and kept at 37°C by a Harvard Thermocirculator. The bath apparatus consisted of a cylindrical 20 ml bath which allowed free solution inflow and outflow and bubble-through aeration. The baths were oxygenated continuously with a 95% O_2-5% CO_2 mixture which held the pH at 7.3–7.5. The Krebs-Ringer solution used for washing the preparations between experiments was warmed to 37°C by passing through a coil warmed by the thermocirculator. One end of the muscle preparation was tied to the base of the bath while the opposite end was tied to a moveable Grass FT .03 force-displacement strain gauge connected to a 6 channel RD Beckman Instruments Dynograph. Tension on the muscle was adjusted by moving the strain gauge and was measured by pre-calibrated movement of the dynograph pen.

One artery and one vein from the same cord were utilized for each experiment. They were initially allowed 2–3 h to equilibrate in the bath under a resting tension of 2 g and were frequently washed with Krebs-Ringer bicarbonate solution (Altura *et al.*, 1972). The tissues were then tested with potassium chloride (KCl), a known contractile activator (Altura *et al.*, 1972), to confirm the integrity of the preparation and periodically throughout the experiment, as a control, to estimate vessel sensitivity and consistency. This was done by adding KCl to the bath medium and measuring the contractile force developed. The umbilical arteries and veins were exposed to each of three agents: bupivacaine, 2-chloroprocaine and lignocaine. Each agent was added to the bath medium in a cumulative manner, starting with a low concentration followed by 11 sequential additions. Ten minutes elapsed between additions to allow time for plateaus of vessel reactivity. Concentrations of the local anaesthetics and KCl were chosen such that total volumes added to the 20 ml baths never exceeded 1.0 ml. Periods of 30 min (for KCl) to 1 h (for local anaesthetics) were used to remove a drug from the bath medium by frequent washes with Krebs-Ringer solution.

Cumulative concentration-response curves were obtained according to a method previously described, where additions are made to the muscle bath to yield increasing cumulative concentrations (Van Rossum, 1963). The first phase of the experiment studied the action of the local anaesthetics on resting arteries and veins. For bupivacaine, concentrations ranged from 8.6 μM to a maximum of 430 μM. For 2-chloroprocaine, the concentrations were 0.065 mM to 3.3 mM; for lignocaine, they were 0.043 mM to 2.1 mM. In addition, cumulative concentration-response curves were obtained for bupivacaine, 2-chloroprocaine and lignocaine on arteries and veins previously contracted with sub-

maximal concentrations of KCl, using the same method and concentrations described above. In each latter case, a control was done to confirm that the chosen KCl concentration produced a sustained contraction that was stable for the length of time it took to perform the local anaesthetic experiment.

For drugs which produced contraction on resting vascular muscle, results were expressed and plotted as negative log of molar contraction vs. percentage of maximal KCl-induced contraction. Where relaxation occurred, the negative log of the concentration was plotted against % relaxation of the resting 2 g tension which was applied to all tissues. For experiments on KCl-contracted muscle, negative log of concentration was plotted against % change (contraction or relaxation) of the induced contraction. The KCl concentration which reliably produced a maximal contraction in all vessels was 80 mM; submaximal concentrations used varied from 20 mM to 60 mM. The means and standard errors of the means (s.e.mean) were calculated for all induced responses. Within groups statistical significance was established by one factor analysis of variance. Unpaired one-tailed t test was used for comparisons between groups, and between arteries and veins, where appropriate. Statistical significance was taken as $P < 0.05$. Relative potencies of the local anaesthetics were examined using pD_2 values and maximum tension. The pD_2 value was obtained by calculating the negative logarithm of the EC_{50}, or the effective concentration which produced 50% of maximal response (Ariëns, 1964).

Drugs

Marcaine hydrochloride 0.25% (Winthrop-Breon Laboratories, N.Y., New York, U.S.A.); Nesacaine CE 2% with sodium bisulphite 0.7 mg ml^{-1} (Astra Pharmaceutical Products, Westborough, Massachusetts, U.S.A.); Xylocaine 1% (Astra). No preservatives were present in the local anaesthetic solutions. The pH was adjusted by the manufacturer using hydrochloric acid or sodium hydroxide.

Results

Behaviour of human umbilical arterial and venous strips in vitro

As found previously, human umbilical vessel preparations showed a wide degree of variability and exhibited some spontaneous activity (Somlyo *et al.*, 1965; Altura *et al.*, 1972). For example, following the equilibration period, preparations differed from one another with respect to baseline resting tension stability, maximal isometric tension developed in response to activator and consistency over the several hours during which the experiment was run. Occasionally a vessel would spontaneously exhibit a maximal or supramaximal contraction (as compared to a KCl-induced contraction) from which recovery to baseline was complete. Inducing a maximal sustained contraction with KCl would often reduce the occurrence of these spontaneous contractions and tend to 'stabilize' the vessel. Maximal or submaximal contractions were also seen on occasion in response to washing the preparations. As described previously (Altura *et al.*, 1972), maximal activator-induced responses were often less during the first few hours of an experiment than those seen during subsequent stimulations.

KCl-induced contraction

KCl induced contraction in all vessels examined. It was chosen as the control activator because of the magnitude of the induced response, its reproducibility between preparations in terms of concentration required to produce maximal contraction, and the ease with which the muscle preparations would recover to baseline upon washing KCl out of the bath medium. Response to KCl stimulation displayed two components described previously as 'phasic' and 'tonic' (Bolton, 1979). At low concentrations, the muscle response showed an initial peak of tension which then declined back to baseline ('phasic component'). As the concentration of KCl increased, the phasic response was followed by a sustained level of tension ('tonic component') which was sometimes less than phasic tension. The tonic contraction increased in magnitude and stability as the KCl concentration increased, until, at high KCl concentrations, a sustained maximal contraction resulted (Figure 1). In general, preparations varied widely with respect to the shape of the KCl contraction and the lowest KCl concentration required to sustain a contraction.

Response of resting umbilical arterial and venous smooth muscle to local anaesthetics

Bupivacaine induced contraction in over 90% of vessels exposed to this drug. One factor analysis of variance within groups revealed $P < 0.0001$ for veins, and $P < 0.0002$ for arteries. The pattern of contraction differed from preparation to preparation. In most cases, the contraction displayed an initial phasic upstroke followed by a tonic contraction which was lower than the peak response. This tonic phase often consisted of repeated, or 'rhythmical

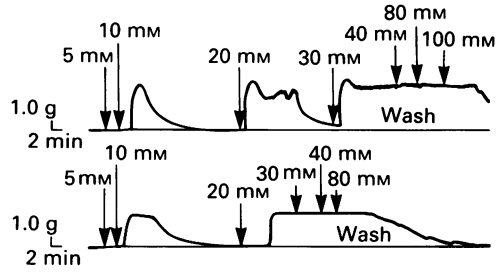


Figure 1 Responses of umbilical vessels following addition of KCl. Upper tracing shows umbilical vein; lower tracing shows umbilical artery. Arrows indicate cumulative additions of KCl. Initial upstroke of contraction is not sustained until higher cumulative KCl concentrations are attained.

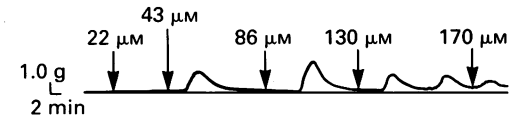


Figure 2 Responses of umbilical vein to bupivacaine. Arrows indicate cumulative additions of bupivacaine. Two phases of contractile response can be defined: initial rapid upstroke of contraction followed by sustained level of contraction.

contractions' (Bolton, 1979) between which relaxation was incomplete, thus resulting in an increase in baseline tension. In other cases, bupivacaine caused sustained increases in baseline tension without the presence of rhythmical contractions. Developed isometric tension was measured from the lowest point of tension which resulted during the ten minute interval after each succeeding concentration was added (Figure 2). The concentration-response curve showed maximal venous response at a bupivacaine concentration of 220 μM (Figure 3a). At this concentration, the contraction varied from 13%–41% (mean 22.4 ± 2.6) of maximal KCl contraction depending on the particular preparation. The lowest concentration that elicited a response in some venous preparations was 22.0 μM. For arterial muscle, the maximal response was obtained at 350 μM (Figure 3b) and contractions at this concen-

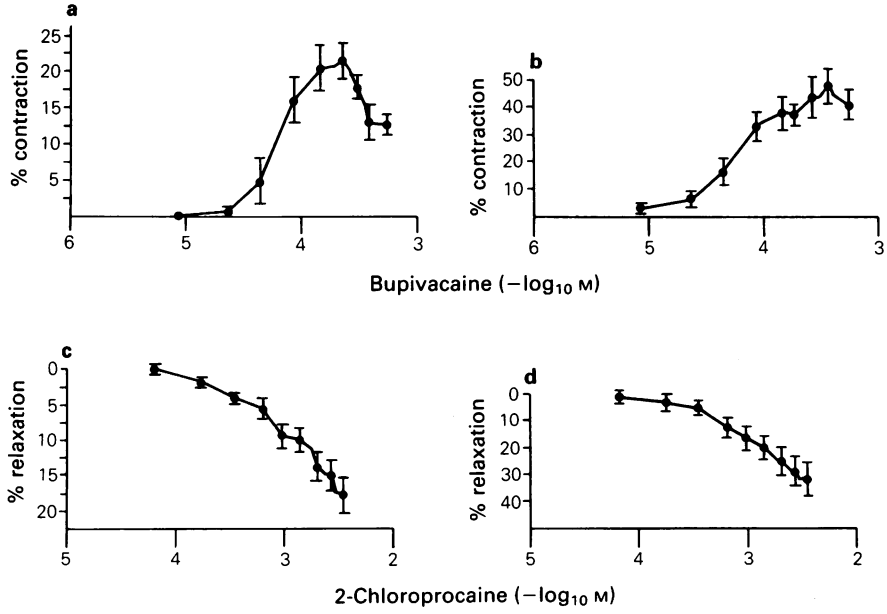


Figure 3 Cumulative – log concentration-isometric response curves. (a) Bupivacaine-induced contraction of resting umbilical venous smooth muscle. (b) Bupivacaine-induced contraction of resting umbilical arterial smooth muscle. Points indicate mean % of KCl maximal response. (c) 2-Chloroprocaine-induced relaxation of resting umbilical venous smooth muscle. (d) 2-Chloroprocaine-induced relaxation of resting umbilical arterial smooth muscle. Points indicate mean % relaxation of resting 2.0 g tension. In (a–d) vertical lines show s.e.mean; in (a) $n = 9$, (b) $n = 6$, (c) $n = 7$ and (d) $n = 6$ (n = number of vascular strips).

tration were from 28%–66% (mean 47 ± 6.3) of maximal KCl-induced contraction. Some arterial preparations contracted at the lowest concentration level used, 8.6 μM.

In contrast to bupivacaine, 2-chloroprocaine caused relaxation of all venous and arterial preparations in a concentration-dependent manner. At the median 2-chloroprocaine concentration used in these experiments (i.e., 1.6 mM), venous muscle relaxed 11.5 ± 1.7% and arterial muscle relaxed 24.1 ± 4.7% (Figure 3c and d) as compared to the resting 2 g tension applied to all tissues. Responses were seen at the lowest concentration used, 0.065 mM, in both arteries and veins. Statistical significance was obtained using analysis of variance, with $P < 0.0001$ in veins and $P < 0.0006$ in arteries.

For lignocaine, the results obtained were less consistent, making concentration-response curves difficult to construct: 40% of the vessels examined developed increases in baseline tension; 33% had relaxation of resting 2 g tension; and 27% showed no response. In the vessels which responded, both contraction and relaxation were concentration-dependent. Maximal contractile responses were 3.9% and 9.0%, respectively, for veins and arteries; maximal relaxation was 4.5% for veins and 13.5% for arteries.

Response of KCl-contracted vascular muscle to local anaesthetics

When umbilical vessels were induced to contract with submaximal concentrations of KCl, bupivacaine demonstrated an ability to increase both the magnitude of the sustained contraction and the frequency of rhythmical contractions (Figure 4). Sustained contraction increase was observed in approximately 50% of the veins and arteries at a concentration which was lower than that which elicted maximal contraction on resting muscle (Figure 5). However, analysis of variance failed to reveal statistically significant sustained contraction increase ($P < 0.06$ for veins and $P < 0.37$ for arteries). At higher concentrations, relaxation of the developed tension was observed in those vessels which had initially demonstrated potentiation of the

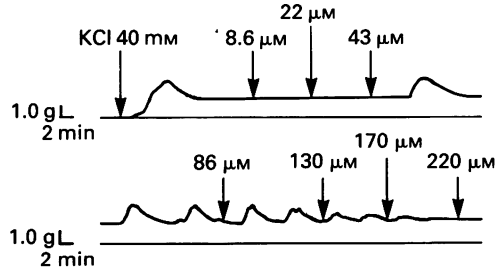


Figure 4 Response of umbilical artery to bupivacaine following KCl-induced submaximal contraction. Lower panel tracing represents continuation of upper panel tracing. When umbilical vessels are induced to contract with submaximal concentrations of KCl, bupivacaine demonstrates an ability to increase the magnitude of the sustained contraction as well as the frequency of repeated contractions. Following KCl, arrows indicate cumulative additions of bupivacaine.

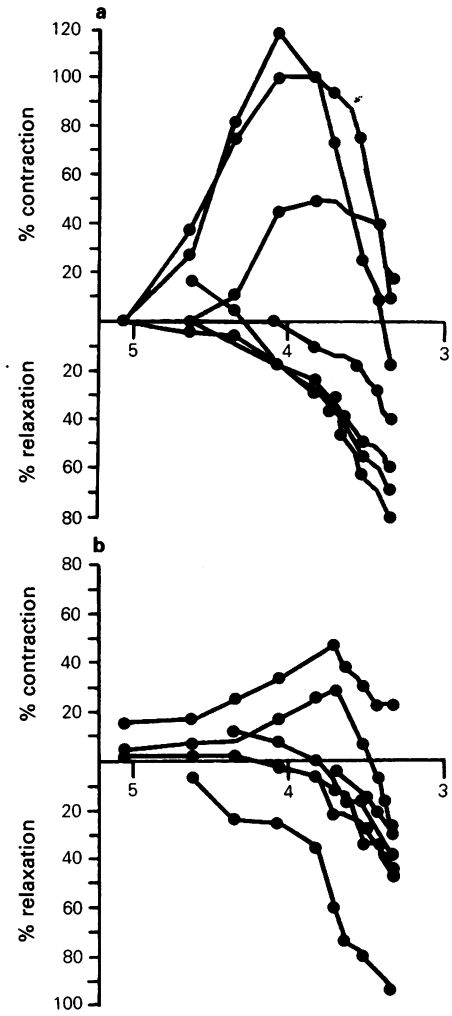


Figure 5 Effect of bupivacaine on KCl-contracted umbilical vessel smooth muscle (cumulative −log concentration-isometric response curves). Abscissa scales represent −log molar concentration of bupivacaine. Each tracing represents individual umbilical cord preparation. (a) Venous smooth muscle. (b) Arterial smooth muscle.

KCl-induced submaximal contraction. The KCl-contracted vessels which did not show an increased level of contraction with bupivacaine responded with concentration-dependent relaxation of the sustained submaximal contraction. Of those vessels that showed only relaxation, analysis of variance for the veins revealed significant relaxation at $P < 0.0001$. However, no statistically significant relaxation of the arteries occurred ($P < 0.16$).

Both 2-chloroprocaine and lignocaine caused relaxation of KCl-contracted arteries and veins in a concentration-dependent manner (Figure 6). For both 2-chloroprocaine and lignocaine, significance was reached at $P < 0.0001$ for veins and arteries using within groups analysis of variance. At the median 2-chloroprocaine concentration used, i.e. 1.6 mM, 71.2 ± 3.9% relaxation of induced contraction occurred in veins and 57.8 ± 10.5% occurred in arteries (Figure 7a and b). For lignocaine, the median concentration, 1.1 mM caused 56.3 ± 12.2% relaxation in veins and 51.3 ± 10.6% relaxation in arteries (Figure 7c and d).

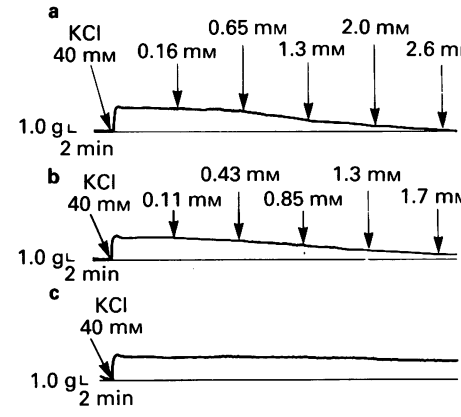


Figure 6 Response of the umbilical vein to local anaesthetics following KCl-induced submaximal contraction. Both 2-chloroprocaine (a) and lignocaine (b) caused a concentration-dependent relaxation of the KCl-induced contraction. (c) Control KCl-induced submaximal contraction, in which maximal spontaneous tension reduction at 100 min is 20%.

Relative potencies of local anaesthetic agents on human umbilical arterial and venous preparations

An analysis of the local anaesthetics relative potency based on pD_2 values and maximum tension (Table 1) showed that, at resting tension, bupivacaine, which is clinically the most potent agent, also had the highest pD_2, demonstrating high affinity for all vessels, and induced the strongest contractile responses on both

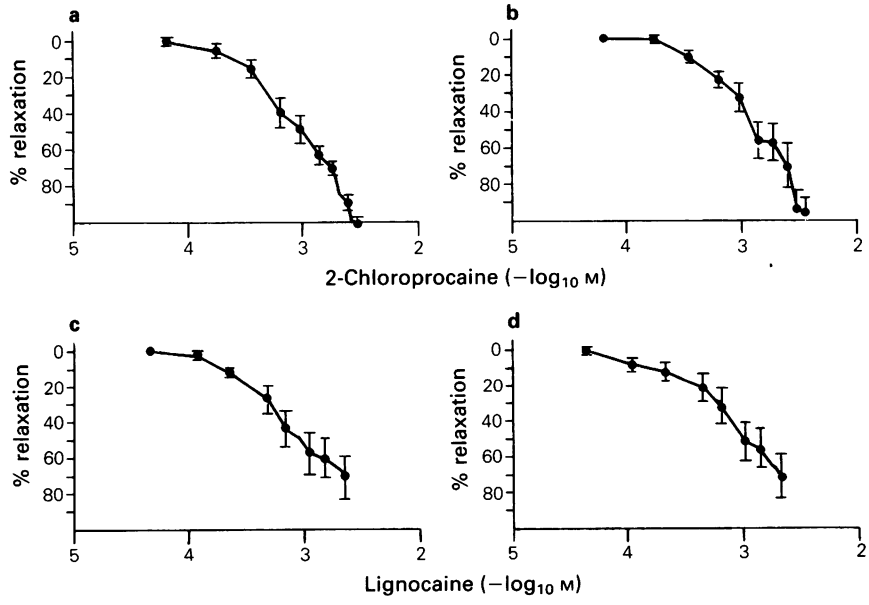


Figure 7 Relaxation of KCl-contracted umbilical vessel smooth muscle (cumulative −log concentration-isometric response curve) induced by the local anaesthetics. (a) Effect of 2-chloroprocaine on venous smooth muscle. (b) Effect of 2-chloroprocaine on arterial smooth muscle. (c) Effect of lignocaine on venous smooth muscle. (d) Effect of lignocaine on arterial smooth muscle. Points indicate mean % relaxation and vertical lines show s.e.mean. (a and d) $n = 6$, (b and c), $n = 7$ (n = number of vascular strips).

Table 1 Comparative potency of local anaesthetics on isolated umbilical vascular smooth muscle*

Agent	*Preparation*	*Vessel*	n	*pD_2*§	*Maximum tension*¶
Bupivacaine	Resting	Vein	9	4.1 ± 0.06	+744 ± 128
		Artery	6	4.3 ± 0.11	+1150 ± 210
	KCl-contracted	Vein	3	4.4 ± 0.09	+633 ± 117
			4	3.7 ± 0.09	−1408 ± 360
		Artery	3	4.4 ± 0.13	+243 ± 74
			3	3.7 ± 0.10	−1010 ± 242
2-Chloroprocaine	Resting	Vein	7	3.0 ± 0.06	−346 ± 51
		Artery	6	3.0 ± 0.07	−635 ± 121
	KCl-contracted	Vein	7	3.0 ± 0.06	−2733 ± 395
		Artery	7	2.9 ± 0.17	−1893 ± 300
Lignocaine	Resting	Vein	3	3.3 ± 0.23	+160 ± 21
			2	2.9 ± 0.1	−90 ± 10
			3		0†
		Artery	3	3.4 ± 0.03	+267 ± 17
			3	3.3 ± 0.19	−270 ± 38
			1		0†
	KCl-contracted	Vein	6	3.2 ± 0.05	−1588 ± 415
		Artery	6	3.2 ± 0.07	−1463 ± 234

* See text for statistical analysis.
§ pD_2 is the mean $-\log$ of EC_{50} (the mean molar concentration required to produce 50% of the maximal response).
¶ Maximum tension is the maximal attainable response (mean ± s.e.mean) in mg; (+) contraction, (−) relaxation.
† No response (see text).

veins and arteries. One-tailed test t failed to show a significant difference between the pD_2 values of veins compared to arteries ($P < 0.08$). 2-Chloroprocaine, with a less potent local anaesthetic action, had considerably less potency, i.e., much lower pD_2, and displayed only relaxation. Again, pD_2 values did not differ between veins and arteries ($P < 0.36$). Lignocaine, which in some vessels showed contraction and in others relaxation, occupied an intermediate position, with no significant difference between veins and arteries ($P < 0.35$). In general, for all three agents, arteries showed more sensitivity than veins to direct action of local anaesthetics at resting tension, although statistical significance was not reached. On resting vessels, comparisons between individual agents were made utilizing unpaired one-tailed t test and pD_2 values. Statistical significance at $P < 0.001$ was obtained for contractile response from lignocaine as compared to bupivacaine on both veins and arteries. Significant difference in relaxation from lignocaine as compared to 2-chloroprocaine was obtained for arteries ($P < 0.04$) but not for veins ($P < 0.23$).

When the vessels were first challenged with KCl, bupivacaine showed a tendency to contract further only some of the vessels, with a higher affinity and maximum tension for the veins than the arteries, although, as noted before, statistical significance was not reached for this contractile response. With this exception, all vessels exhibited relaxation of KCl-induced contraction when the local anaesthetics were applied. An examination of this relaxant effect to the three local anaesthetics showed the following order of potency: 2-chloroprocaine > lignocaine > bupivacaine for pD_2 values, and, as expected, 2-chloroprocaine > lignocaine > bupivacaine when maximum tension was considered. In this experimental condition, one tailed t test did not show significant differences between the veins and arteries for any of the agents ($P < 0.43$ for bupivacaine, $P < 0.38$ for 2-chloroprocaine, and $P < 0.35$ for lignocaine). On KCl-contracted vessels, analysis of variance was used to compare venous and arterial relaxation to all three local anaesthetic agents. Between-group variance was significantly different from within group variance, with $P < 0.0001$ for veins and $P < 0.02$ for arteries.

Discussion

To our knowledge, there have been few, and incomplete, previous experiments done to study the effect of local anaesthetics on the umbilical vasculature. Animal studies have concentrated on local anaesthetic action on vascular smooth muscle obtained

from sources other than the umbilical cord. It has been suggested that local anaesthetics may induce non-specific inhibition of vascular tone, as several of these agents were shown to cause relaxation of drug and hormone-induced contraction (Altura & Altura, 1974a). Some studies have demonstrated a contractile effect of procaine and lignocaine on animal vascular smooth muscle (Altura & Altura, 1974a) and of lignocaine on human uterine arteries (Cibils, 1976). One study, utilizing human umbilical arteries, found that prilocaine and bupivacaine produced contraction consistently (Tuvemo & Willdeck-Lund, 1982).

Since umbilical vessels lack innervation, circulating vasoactive substances, such as local anaesthetics, may interfere with the regulation of foetal blood flow by their direct action on vascular smooth muscle. This study demonstrates that local anaesthetics are vasoactive on umbilical vessel smooth muscle preparations. Contraction or relaxation of isolated vascular strips as measured in this experiment may relate to the *in vivo* physiological action of umbilical vessels in regulating blood flow to the foetus.

The concentrations of local anaesthetics used in this study were slightly higher than maternal plasma concentrations measured following administration of local anaesthetics for labour or Caesarean section (Ralston & Shnider, 1978). Rapid *in vivo* hydrolysis of 2-chloroprocaine reduces absorbed concentrations to nanogram levels. High maternal protein binding is responsible for reduced placental transfer of bupivacaine. However, inadvertent intravascular injection of local anaesthetics leads to plasma concentrations well in excess of those that were measured under controlled circumstances (Moore *et al.*, 1980). Furthermore, the intact microcirculation is 10,000 to 100,000 times more sensitive to drugs than are some isolated large vessels (Altura & Altura, 1974b). Therefore, although the drug concentration to which the *in vitro* isolated smooth muscle is exposed appears large; in fact, *in vivo*, the microcirculation will respond to many times lower concentrations than will the rest of the vasculature.

In the concentrations used for our studies, the only effect of 2-chloroprocaine on umbilical vasculature was relaxation. This was seen in both resting and contracted vessels and was consistently obtained. One could, therefore, postulate that even in the event of high maternal concentrations of 2-chloroprocaine, umbilical blood flow would not be reduced based on a direct action of this agent on the umbilical vasculature.

No consistent or marked response of resting umbilical vessels to lignocaine was seen in our study, although relaxation of KCl-contracted muscle was reproducible. Earlier accounts of depressed neurobehavioural scores in neonates whose mothers had received epidural anaesthesia with lignocaine have been refuted (Abboud *et al.*, 1983b; Kileff *et al.*, 1984). The aetiology of foetal bradycardia in paracervical block with lignocaine is still unknown, and it is possible that there is some relationship between this complication and the vasoactive properties of lignocaine. The results of our study did not demonstrate conclusively that lignocaine causes adverse foetal effects by a direct action on umbilical cord vascular smooth muscle.

Bupivacaine consistently produced contractions in resting umbilical arteries and veins. The magnitude of maximal sustained contraction ranged from 13%–41% in venous muscle and from 28%–66% in arterial muscle as compared to maximal KCl-induced contraction. In addition, umbilical vascular smooth muscle in many cases displayed contractile peaks which were up to four times higher than the level of sustained tension (Figure 2). Bupivacaine also displayed an ability to increase the tension of a KCl-induced contraction.

These findings are of particular interest in view of reports implicating bupivacaine in adverse maternal and foetal effects. Although favourable foetal outcome as assessed by Apgar scores, acid-base status, and neurobehavioural scores following regional anaesthesia with bupivacaine has been obtained (Scanlon *et al.*, 1976; Abboud *et al.*, 1983a; Kileff *et al.*, 1984), questions have been raised concerning its safety in uncomplicated regional anaesthesia. Data have shown a significantly increased incidence of foetal bradycardia following epidural anaesthesia with bupivacaine as compared to epidural anaesthesia with lignocaine or 2-chloroprocaine; and following paracervical block with bupivacaine (Teramo, 1969; Abboud *et al.*, 1982). There are accounts of bupivacaine-induced maternal central nervous system and cardiovascular toxicity, with the latter accounting for cases of refractory cardiovascular collapse. In these cases, inadvertent intravascular injection is the presumed aetiological factor. Poor foetal outcome has been attributed to adverse maternal cardiovascular effects, although foetal asphyxia has been noted in cases where bupivacaine induced maternal seizures in the absence of cardiovascular depression (Albright, 1985).

Our study suggests that intravascular injection of intended epidural bupivacaine may lead to plasma concentrations sufficiently high to cause umbilical vessel contraction with adverse foetal effects resulting via this mechanism. Although plasma bupivacaine concentrations are not reported in cases of severe maternal toxicity, there have been published case reports which include plasma bupivacaine concentrations following presumed inadvertent intravascular injection in surgical patients (Moore *et al.*,

1980). Experimental and human volunteer studies using concentrations which are considered to be in excess of bupivacaine toxic concentrations are similar to *in vitro* concentrations used in our study; e.g., contractile responses in some umbilical arteries at 8.6 μM (2.5 $\mu g\,ml^{-1}$) and in some veins at 22.0 μM (6.25 $\mu g\,ml^{-1}$) (Mather *et al.*, 1971; Denson *et al.*, 1984; Kotelko *et al.*, 1984; Sage *et al.*, 1985; Kasten, 1986).

Caution must be exercised when one extrapolates findings of an *in vitro* study to an *in vivo* situation. However, the data presented here suggest that bupivacaine may have clinically significant adverse effects based on its ability to contract umbilical vascular smooth muscle. 2-Cloroprocaine showed only smooth muscle relaxation, and the effect of lignocaine was not predictable, with only a small degree of contraction or relaxation seen in some preparations. In view of these findings, it seems prudent to explore further this avenue of research. The importance of the umbilical vasculature to foetal well-being suggests that agents used in pregnancy should be more closely examined with respect to their structure-activity relationships on the human umbilical-placental vasculature.

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Dissipation mechanisms for 5-hydroxytryptamine in the coronary circulation of the isolated perfused heart of the rat

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1 The contribution of uptake into vascular endothelial cells, of neuronal uptake and of extraneuronal uptake in the dissipation of 5-hydroxytryptamine (5-HT) perfused through the coronary circulation of the rat heart was examined.

2 Hearts from reserpine-pretreated rats were isolated and perfused *in vitro* with 5-HT, in the absence or presence of inhibitors, and rates of appearance of the deaminated metabolite, 5-hydroxyindoleacetic acid (5-HIAA), in the venous effluent were measured using an h.p.l.c. assay.

3 The steady-state rates of 5-HIAA appearance in the venous effluent in hearts perfused with 1 μM 5-HT (422 ± 8.48 pmol g^{-1} min^{-1}, $n = 12$) were reduced by pretreatment of the rats with 6-hydroxydopamine (22% inhibition), and by inclusion in the perfusion solution of 30 μM cocaine (28% inhibition), 100 μM 3-O-methylisoprenaline (64% inhibition), 100 μM corticosterone (58% inhibition), or 30 μM cocaine and 100 μM 3-O-methylisoprenaline (87% inhibition).

4 The extraneuronal deamination of 5-HT in the heart was saturable ($K_m = 101$ μM, $V_{max} = 31.2$ nmol g^{-1} min^{-1}). The neuronal deamination of 5-HT was saturated by about 50 fold lower concentrations of 5-HT than was extraneuronal deamination, but K_m and V_{max} values could not be determined.

5 In the coronary circulation of the rat heart, 5-HT was dissipated by the uptake processes for catecholamines, extraneuronal uptake (predominantly) and neuronal uptake, and subsequent metabolism by monoamine oxidase. There was no evidence that a cocaine-sensitive uptake of 5-HT into vascular endothelial cells made any significant contribution to 5-HT dissipation in the heart.

Introduction

The physiological role of 5-hydroxytryptamine (5-HT) is still not clear (Vanhoutte & Cohen, 1983), but the effects on local vascular function of 5-HT released from platelets have been implicated in various pathological conditions. These conditions include the coronary vasospasm of Prinzmetal's (variant) angina (Angus *et al.*, 1982; Bove & Dewey, 1983); cerebral vasospasm after subarachnoid haemorrhage (Allen *et al.*, 1974); digital vasospasm of Raynaud's phenomenon (Halpern *et al.*, 1960; Stranden *et al.*, 1982); and migraine (Raskin, 1981). Also, vasoconstrictor responses of blood vessels to 5-HT have been shown to be increased by atherosclerosis (Henry & Yokoyama, 1980; Heistad *et al.*, 1984). If 5-HT is involved in these conditions, then the presence of dissipation mechanisms for 5-HT in blood vessels may be important in limiting its effect. It is well documented that 5-HT can be avidly taken up by platelets (Pletscher, 1968; Sneddon, 1969), and can be taken up and then metabolized by monoamine oxidase (MAO) in endothelial cells (Junod, 1972; Strum & Junod, 1972; Shepro *et al.*, 1975; Junod & Ody, 1977). There is also evidence for uptake and storage of 5-HT in noradrenergic neurones (Thoa *et al.*, 1969; Verbeuren *et al.*, 1983; McCulloch *et al.*, 1984; Paiva *et al.*, 1984) and for extraneuronal uptake and metabolism of 5-HT in vascular smooth muscle (Buchan *et al.*, 1974; Paiva *et al.*, 1984; Fukuda *et al.*, 1986a,b).

In the present study, the dissipation of 5-HT in the coronary circulation has been examined, in view of the suggestions that 5-HT may be involved in the coronary vasospasm of Prinzmetal's angina (*vide supra*) and in the cyclic variations in blood flow

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which occur in stenosed coronary arteries (Ashton *et al.*, 1986). The aim of the study was to examine the contributions of uptake into vascular endothelial cells, of neuronal uptake and of extraneuronal uptake in the dissipation of 5-HT perfused through the coronary circulation of the rat heart *in vitro*.

Some of the results of this work were presented to the August 1985 meeting of the Australian Physiological and Pharmacological Society (Williams *et al.*, 1985), the December 1985 meeting of the British Pharmacological Society (Bryan *et al.*, 1986), and the December 1985 meeting of the Australasian Society of Clinical and Experimental Pharmacologists (Bryan *et al.*, 1987).

Methods

Male albino Wistar rats (170–245 g) were injected intraperitoneally with 1 mg kg^{-1} reserpine 18–24 h before the experiments (shown in preliminary experiments to reduce endogenous noradrenaline and 5-HT in the heart to negligible levels), and intraperitoneally with 5000 u kg^{-1} heparin 20 min before the animals were killed by cervical dislocation and bleeding. Some rats were injected intravenously with 50 mg kg^{-1} 6-hydroxydopamine (6-OHDA) 24 h before the experiments to achieve chemical sympathectomy. This dose of 6-OHDA abolished the presence of noradrenergic neurones in the heart as determined by fluorescence histochemistry (Bryan & O'Donnell, unpublished observations). In the experiments with 6-OHDA-pretreated rats, other rats were injected intravenously with 1 ml kg^{-1} of the vehicle used for the 6-OHDA (0.53 mM ascorbic acid in normal saline, *vide infra*). The pretreatment of rats with the vehicle only did not affect the rates of 5-hydroxyindoleacetic acid (5-HIAA) appearance in the venous effluent, compared with untreated rats. Hence, the results from rats with and without the vehicle pretreatment were pooled. In some experiments, rats were injected intraperitoneally with 75 mg kg^{-1} pargyline, 18 h and 2 h before the experiment, to inhibit MAO.

The hearts from the rats were isolated and perfused using a Desaga PLG peristaltic pump by the Langendorff technique with Tyrode solution at a constant rate of about 10 ml g^{-1} min^{-1} at 36.5°C. The Tyrode solution contained (in mM): Na^+ 149.2, K^+ 2.7, Ca^{2+} 1.3, Mg^{2+} 1.05, Cl^- 144.3, $H_2PO_4^-$ 0.4, HCO_3^- 11.9, glucose 5.0 and Na_2EDTA 0.04, and was aerated with 95% O_2 and 5% CO_2. The perfusion pressure was monitored throughout the experiments and ranged from 25–30 mmHg. The hearts were initially perfused for 15 min with 5-HT-free Tyrode solution, and then with Tyrode solution containing 5-HT for 80.5 or 90.5 min. The perfusion pressure was not affected by the 5-HT in the perfusion solution. 5-HT concentrations of 1 μM and greater were used throughout the study, so that, even when inhibitors were present, 5-HIAA in the venous effluent could be determined quantitatively using a high performance liquid chromatography (h.p.l.c.) assay (*vide infra*). In experiments with inhibitors (see Results), the inhibitor was included in the Tyrode solution from the 6th min of the initial perfusion and throughout the perfusion with 5-HT. Samples of venous effluent were collected at appropriate intervals during the perfusion with 5-HT (see Results). At the end of each experiment, the heart was removed from the cannula, blotted, weighed and placed in 0.31 M trichloroacetic acid containing 2.7 mM Na_2 EDTA at 4–6°C. After at least 2 h, the hearts were homogenized and then centrifuged at 10,000 *g* for 10 min. Also, samples of the perfusion solution were collected immediately before and after each experiment (arterial solutions).

Samples (2 ml) of venous effluent and of the arterial solutions (after dilution with Tyrode solution for 5-HT concentrations greater than 30 μM) were taken. Ice-cold 5 M acetic acid (200 μl) and 0.27 M Na_2 EDTA (20 μl) were added immediately after collection of the samples. The samples were stored on ice and frozen until the h.p.l.c. assay of 5-HT and 5-HIAA was carried out. Samples of the supernatant from the heart homogenate were also frozen for subsequent h.p.l.c. assay.

H.p.l.c. assay of 5-HT and 5-HIAA

The h.p.l.c. apparatus consisted of a Waters Associates Model 510 pump and solvent delivery system, a Waters U6K universal injector, a Waters CN reversed-phase guard column and a Novapak C_{18} reversed-phase steel column (5 μm, 3.9 mm internal diameter × 15 cm). The electrochemical detection system comprised a Waters Model 410 electrochemical detector and a glassy carbon electrode, connected to a chart recorder. The optimal electrode potential was found to be +0.65 V, relative to a Ag/AgCl reference electrode. The mobile phase contained Na_2HPO_4 70 mM, sodium heptane sulphonate 5 mM, Na_2EDTA 0.1 mM and acetonitrile 6.5%, and was adjusted to a pH of 3.6 with 8.7 M phosphoric acid. The flow rate was adjusted to 1 ml min^{-1} during assay of the samples and 0.1 ml min^{-1} when the h.p.l.c. was not in use for sample analysis. The mobile phase was recirculated through the system, was replaced at weekly intervals, and was degassed daily under vacuum in a sonicator.

Samples (20–100 μl) of venous effluent, arterial solution and supernatant of the heart homogenate were injected directly onto the h.p.l.c without any sample clean-up. The arterial samples were injected

to assay the 5-HT concentration in the perfusion solution and to check that no peaks other than 5-HT appeared in the chromatograms. The retention times for 5-HIAA and 5-HT were 5.7 min and 9.7 min, respectively. All samples were analysed on the day of the experiment. Standards containing appropriate concentrations of 5-HIAA and 5-HT were prepared daily, in Tyrode solution containing inhibitors when required, and were treated as described above for experimental samples. The amounts of 5-HIAA and 5-HT in the samples were measured by comparison of the peak heights of the experimental and standard solutions.

The only inhibitor drug that was detected by the electrochemical detector was 3-O-methylisoprenaline (OMI), which had a retention time on the h.p.l.c. column of 14 min. Hence, in experiments with OMI, the h.p.l.c. assay time was increased, but the OMI did not interfere with the 5-HIAA or 5-HT peaks. Corticosterone presented some problems in that it appeared to bind to the h.p.l.c. column and increase the background noise of the detector, so the column was washed with dimethylsulphoxide (DMSO) to remove the corticosterone after each experiment. Cocaine did not interfere with the h.p.l.c. assay at all.

Drugs and solutions

The drugs used were: cocaine hydrochloride (Drug Houses of Australia, Sydney, N.S.W., Australia); corticosterone (Sigma Chemical Company, St. Louis, MO, U.S.A.); heparin sodium (as ampoules of 5000 u ml^{-1}; Weddel Pharmaceuticals Ltd, London, England); 6-hydroxydopamine (6-OHDA) hydrobromide (Sigma); 5-hydroxyindoleacetic acid (5-HIAA; Sigma); 5-hydroxytryptamine (5-HT) creatinine sulphate complex (Sigma); 3-O-methylisoprenaline (OMI) hydrochloride (Boehringer, Ingelheim, F.R.G.); pargyline hydrochloride (Sigma); reserpine (as Serpasil ampoules, 1 mg ml^{-1}; Ciba-Geigy Australia Ltd, Sydney, N.S.W. Australia).

Stock solutions of 5-HT, 5-HIAA and OMI (10 mM) were prepared in 10 mM HCl. Corticosterone (300 mM) was dissolved in DMSO and diluted with Tyrode solution, such that the final concentration of DMSO in the perfusion solution was 4.6 mM. Pargyline hydrochloride (92.5 mg ml^{-1}) was dissolved in normal saline (154 mM NaCl), and 6-OHDA hydrobromide (74 mg ml^{-1}) was dissolved in normal saline containing 0.53 mM ascorbic acid. Other drugs and all dilutions were prepared in Tyrode solution.

Calculation of results

Steady-state rates of 5-HIAA appearance were calculated as the mean of the rates of 5-HIAA appearance in venous effluent samples collected in the 66th, 71st, 76th and 80th min of perfusion with 1 μM 5-HT, or in samples collected in the 80th, 84th, 87th and 90th min of perfusion with concentrations of 5-HT greater than 1 μM.

Rate constants for efflux of 5-HIAA from the hearts were calculated as the steady-state rate of 5-HIAA appearance divided by the 5-HIAA content of the heart at the end of the experiment. The half-time for efflux of 5-HIAA was then calculated as 0.693 divided by the corresponding rate constant for efflux of 5-HIAA.

Rate constants for MAO activity k_{MAO} were calculated as the steady-state rate of 5-HIAA appearance divided by the intracellular concentration of 5-HT (s_i) in the heart at the end of the experiment (Grohmann, 1987). Values of s_i were calculated as the concentration of 5-HT in the perfusion solution multiplied by the tissue/medium ratio of 5-HT in the heart after correction for distribution of 5-HT in the extracellular space (0.365 ml g^{-1}, Bryan *et al.*, 1983).

Rate constants for efflux and for MAO are expressed as geometric means with 95% confidence limits. All other results are expressed as arithmetic means $\pm$ s.e. The significance of differences between values was assessed by Student's *t* test.

In the kinetic analysis of the deamination of 5-HT, the steady-state rates of neuronal or extraneuronal deamination (v) at various 5-HT concentrations (s) were calculated as differences between mean steady-state rates of deamination in the absence and presence of inhibitors and expressed with the s.e. of the differences. Values of s/v versus s for extraneuronal deamination were analysed by linear least squares regression analysis (Snedecor & Cochran, 1980), and K_m and V_{max} values were obtained by a method for least squares estimation of non-linear parameters (Marquardt, 1963).

Results

(A) *Metabolism of 5-HT in hearts perfused with 1 μM 5-HT*

When rat hearts were perfused with 1 μM 5-HT, venous effluent samples contained detectable amounts of 5-HIAA from the 10th min of perfusion, but not at the 2nd min of perfusion (Figure 1). The limit of sensitivity of the h.p.l.c. assay of 5-HIAA corresponded to a rate of appearance in the venous effluent of approximately 20 pmol g^{-1} min^{-1}. 5-HIAA appeared to be the only metabolite of 5-HT that was formed in the heart, in that no other peaks were present in the chromatograph from the assay of either venous effluent samples or supernatant from the homogenate of the heart (Figure 2). The rates of 5-HIAA appearance in the venous effluent were

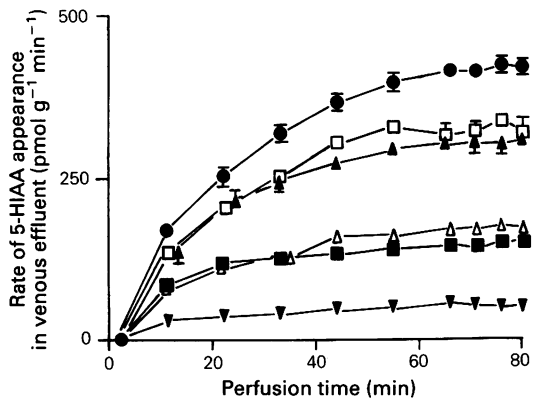


Figure 1 Rates of 5-hydroxyindoleacetic acid (5-HIAA) appearance in the venous effluent (pmol g^{-1} min^{-1}) plotted against perfusion time (min) for rat hearts perfused with 1 μM 5-hydroxytryptamine (5-HT) in the absence (●, $n = 11$) or in the presence of inhibitors: 30 μM cocaine (▲, $n = 7$), 100 μM corticosterone (△, $n = 4$), 100 μM 3-O-methylisoprenaline (OMI, ■, $n = 4$) or 30 μM cocaine plus 100 μM OMI (▼, $n = 3$), or for hearts from rats that had been pretreated with 50 mg kg^{-1} 6-hydroxydopamine (□, $n = 4$). Results are means of rates of 5-HIAA appearance from n hearts; vertical lines show s.e.

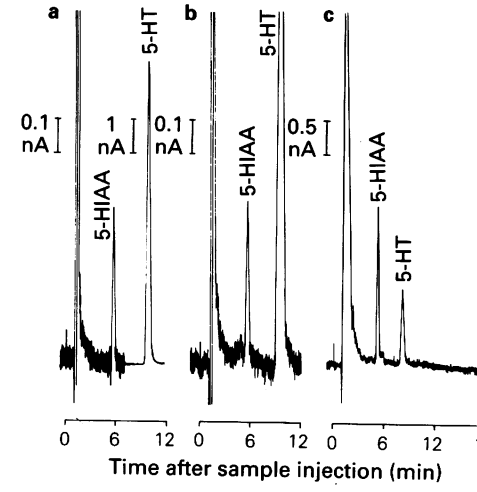


Figure 2 Chromatograms of (a) a standard mixture of 1 pmol 5-hydroxyindoleacetic acid (5-HIAA) and 20 pmol 5-hydroxytryptamine (5-HT); (b) a 25 μl sample of venous effluent, collected over the 76th min of perfusion, from a heart prefused with 1 μM 5-HT; and (c) a 20 μl sample of supernatant from the homogenate of the heart in (b) at the end of the experiment.

reduced by more than 95% (i.e. to levels that were not detectable) when the rats were pretreated with pargyline.

The rate of 5-HIAA appearance in the venous effluent reached steady state after perfusion with 1 μM 5-HT for 65 min (Figure 1). Hence, steady-state rates of 5-HIAA appearance were determined from four samples taken between the 66th and 80th min of perfusion with 5-HT (Table 1).

Table 1 Effects of inhibitors and of 6-hydroxydopamine (6-OHDA) pretreatment on the steady-state rate of 5-hydroxyindoleacetic acid (5-HIAA) appearance in the venous effluent during perfusion of rat hearts with 1 μM 5-hydroxytryptamine (5-HT)

Inhibitor	*Steady-state rate of 5-HIAA appearance in venous effluent*[a] (pmol g^{-1} min^{-1})	n	% *inhibition*[b]
No inhibitor	422 ± 8.48	12	—
6-OHDA pretreatment	328 ± 13.5*	4	22%
Cocaine 30 μM	305 ± 11.2*	8	28%
OMI 100 μM	150 ± 4.66*	4	64%
Corticosterone 100 μM	176 ± 4.61*	4	58%
Cocaine 30 μM + OMI 100 μM	54 ± 7.37*	3	87%

In all experiments, the rats were pretreated with reserpine. Inhibitors were present in the perfusion solution from the 6th min of the 15 min preliminary perfusion and throughout the 80.5 min perfusion with 5-HT (see Methods for details). Data are mean results from n hearts.

[a] Steady-state rates of 5-HIAA appearance were calculated as the mean of rates determined at the 66th, 71st, 76th and 80th min of perfusion.

[b] % inhibition was calculated as the difference between the steady-state rate of 5-HIAA appearance in the absence and presence of an inhibitor, expressed as a percentage of the rate with no inhibitor.

OMI = 3-O-methylisoprenaline.

* Significantly different from rate with no inhibitor, $P < 0.001$.

(B) *Effects of cocaine, 3-O-methylisoprenaline, corticosterone and pretreatment with 6-OHDA on metabolism of 5-HT*

In hearts perfused with 1 μM 5-HT, chemical sympathectomy, by pretreatment of rats with 6-OHDA, reduced the rates of 5-HIAA appearance in the venous effluent throughout the 80 min perfusion period (Figure 1). Cocaine (30 μM), which inhibits both neuronal uptake (Iversen, 1963) and uptake into vascular endothelial cells (Junod, 1972), also reduced the rate of 5-HIAA appearance in the venous effluent (Figure 1). The steady-state rate of 5-HIAA appearance (Table 1) in the presence of cocaine was not significantly different from that in hearts from 6-OHDA pretreated rats ($t = 1.23$, d.f. 10, $P > 0.05$).

The extraneuronal uptake inhibitors, OMI (100 μM) and corticosterone (100 μM), markedly reduced the rates of 5-HIAA appearance in the venous effluent throughout the 80 min perfusion period (Figure 1). Comparison of the steady-state rates of 5-HIAA appearance (Table 1) showed that the extraneuronal uptake inhibitors caused about 60% inhibition, and the inhibition by OMI was slightly, but significantly, greater than that by corticosterone ($t = 3.97$, d.f. 6, $0.01 > P > 0.001$). This difference between the effects of OMI and corticosterone was not due to an increase in efflux of 5-HIAA from the heart in the presence of corticosterone, since the rate constant for efflux of 5-HIAA from the heart was significantly lower in the presence of corticosterone (0.0476 min^{-1}, 95% confidence limits: 0.0399 and 0.0569 min^{-1}, $n = 4$) than in the presence of OMI (0.0775 min^{-1}, 95% confidence limits: 0.0696 and 0.0862 min^{-1}, $n = 4$).

Uptake into noradrenergic neurones, uptake into endothelial cells and extraneuronal uptake were inhibited by the inclusion of cocaine (30 μM) and OMI (100 μM) in the perfusion solution. This very markedly reduced the rate of 5-HIAA appearance (Figure 1), although there was still significant 5-HIAA appearance in the venous effluent at steady state (Table 1). The effect (Table 1) of the combined inhibitors (87% inhibition) was approximately equivalent to the additive effects of each inhibitor added separately to the perfusion solution (total of 92% inhibition).

(C) *Concentration-dependence of extraneuronal and neuronal deamination of 5-HT*

Hearts were perfused with 5-HT (1, 10, 30, 100 or 300 μM) in the absence or presence of 100 μM OMI, and steady-state rates of 5-HIAA appearance in the venous effluent were determined (see Methods). Steady-state rates of extraneuronal deamination of 5-HT were calculated as differences between the steady-state rates of 5-HIAA appearance in the absence and presence of OMI, and were corrected for incomplete inhibition of extraneuronal uptake by 100 μM OMI, as described by Bryan *et al.* (1984). The extraneuronal deamination of 5-HT in the hearts appeared to be saturable (Figure 3a), and obeyed Michaelis-Menten kinetics, in that a plot of s/v versus s was linear ($r = 0.993$, Figure 3b). The K_m value was 101 μM and V_{max} was 31.2 nmol g^{-1} min^{-1}.

In another series of experiments, hearts were perfused with 5-HT (1,3,10 μM) in the absence or presence of 30 μM cocaine. Steady-state rates of neuronal deamination of 5-HT were calculated as the differences between the steady-state rates of 5-HIAA appearance in the absence and presence of cocaine. The neuronal deamination of 5-HT in the hearts appeared to be saturable (Figure 3c), but it was saturated by about 50 fold lower concentrations of 5-HT than was extraneuronal deamination (Figure 3a). It was not possible to carry out any further analysis of these data for neuronal deamination because of the large variability at the higher concentrations of 5-HT (Figure 3c). This variability was caused by the small contribution of neuronal deamination to the total deamination of 5-HT at these 5-HT concentrations. For example, when the concentration of 5-HT was 10 μM, only 10% of the total deamination was inhibited by cocaine.

(D) *Activity of neuronal and extraneuronal monoamine oxidase*

The rate constant, k_{MAO}, characterizing the activity of MAO *in situ* in the heart, was calculated both for neuronal MAO (from data in section B with 1 μM 5-HT in the presence of 100 μM OMI) and for extraneuronal MAO (from data in section B with 1 μM 5-HT in the presence of 30 μM cocaine). The values of k_{MAO} were 0.188 min^{-1} (95% confidence limits: 0.108 and 0.325 min^{-1}, $n = 4$) for neuronal MAO and 0.222 min^{-1} (95% confidence limits: 0.117 and 0.420 min^{-1}, $n = 8$) for extraneuronal MAO. Hence, the activities of MAO for metabolism of 5-HT in neuronal and extraneuronal sites did not differ significantly. It should be noted that k_{MAO} for neuronal MAO may have been slightly underestimated, since a small degree of saturation of neuronal uptake of 5-HT would be evident at 1 μM 5-HT (see section C).

Discussion

Previous studies have shown that many cell types have an uptake process for 5-HT. After uptake, the 5-HT may be stored, e.g. in granules of platelets

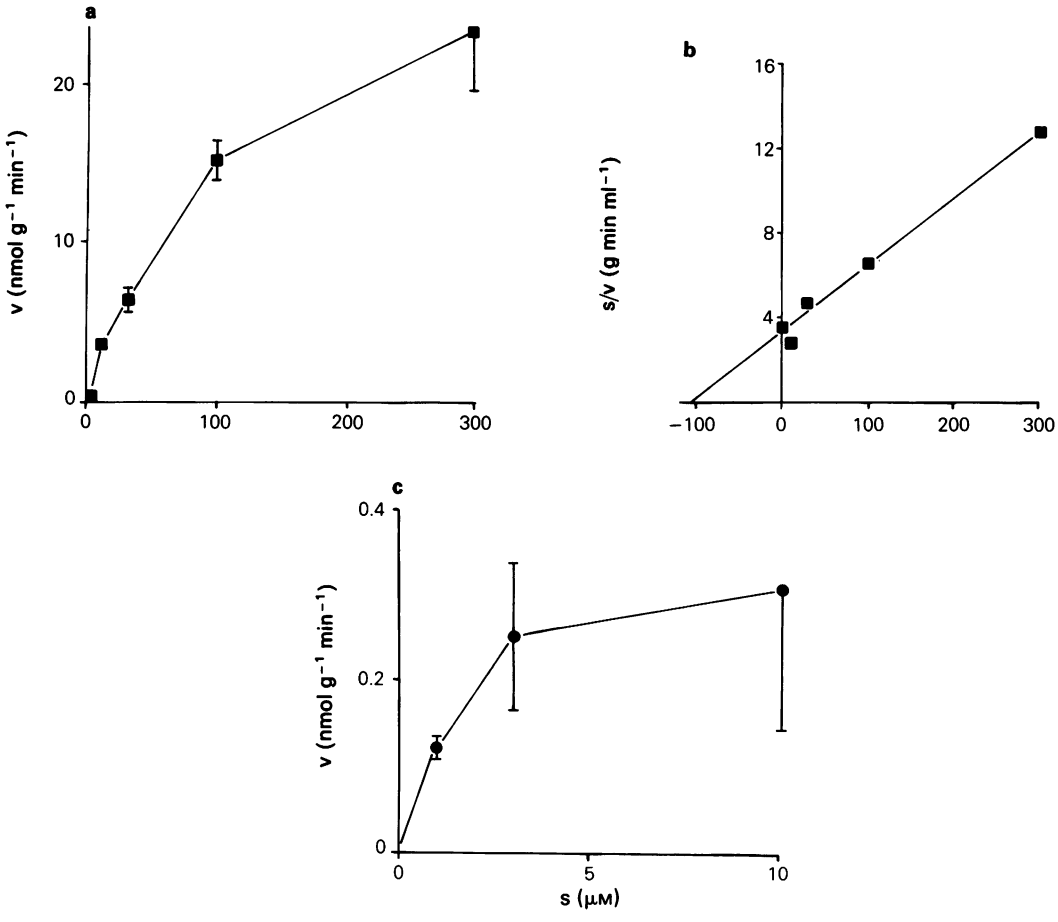


Figure 3 Kinetic analysis of the extraneuronal (a and b) and neuronal (c) deamination of 5-hydroxytryptamine (5-HT) in the rat perfused heart. (a) and (c) Mean steady-state rates (v, in nmol g^{-1} min^{-1}) of (a) extraneuronal deamination or (c) neuronal deamination, calculated as described in Results, are plotted against 5-HT concentration (s, in μM) from data for 4 to 12 hearts; vertical lines show s.e. Note: The scales on the abscissal and ordinate axes are different for (a) and (c). (b) s/v is plotted against s from the data in (a) for extraneuronal deamination. The regression line shown was obtained by linear least squares regression analysis (Snedecor & Cochran, 1980).

(Stahl & Meltzer, 1978; Da Prada & Picotti, 1979) or in vesicles of either 5-hydroxytryptaminergic neurones (Aghajanian & Bloom, 1967) or noradrenergic neurones (Thoa *et al.*, 1969; Verbeuren *et al.*, 1983; McCulloch *et al.*, 1984; Paiva *et al.*, 1984). Alternatively, the 5-HT may be metabolized by MAO, e.g. in endothelial cells (Junod, 1972; Junod & Ody, 1977) or in smooth muscle cells (Paiva *et al.*, 1984). The present study has shown that, when 5-HT was perfused through the coronary circulation of the rat heart *in vitro*, 5-HT is dissipated by both extraneuronal uptake (inhibited by OMI or corticosterone) and by neuronal uptake into noradrenergic neurones (prevented by chemical sympathectomy with 6-OHDA). No evidence was obtained for uptake into vascular endothelial cells in the heart. It should be noted that the rats were pretreated with reserpine, to prevent uptake into storage vesicles in the noradrenergic neurones, so that any 5-HT that entered these neurones would be exposed to intraneuronal MAO and detected as 5-HIAA after metabolism. When the hearts were perfused with 1 μM 5-HT, 13% of the metabolism of 5-HT was not inhibited by cocaine or OMI, and this probably represented a small amount of passive diffusion of 5-HT into MAO-containing cells in the heart. It has been shown previously that, at a concentration of 1 μM, about 17% of the accumulation of isoprenaline in the rat heart occurred by passive diffusion (Bönisch, 1978).

Endothelial cells in the pulmonary circulation of perfused lung preparations have been shown to take up and metabolize 5-HT (Junod, 1972; Strum & Junod, 1972; Iwasawa *et al.*, 1973). In the systemic circulation, endothelial cells isolated from the aorta (Shepro *et al.*, 1975; Junod & Ody, 1977) and from capillaries of adipose tissue (Robinson-White *et al.*, 1981) have also been shown to take up and metabolize 5-HT. In the coronary circulation of the perfused heart of the rat, indirect evidence suggested that endothelial cells did not contribute to the dissipation of 5-HT. This evidence was that there was no significant difference between (i) the rates of metabolism of 5-HT in hearts from rats pretreated with 6-OHDA (to prevent neuronal uptake by destroying the noradrenergic neurones) and (ii) the rates in hearts perfused with solution containing cocaine, an inhibitor of both neuronal uptake (Iversen, 1963) and of the uptake of 5-HT into endothelial cells in perfused lungs (Junod, 1972). Thus, in contrast with the endothelial cells of perfused lungs, where there is avid cocaine-sensitive uptake and metabolism of 5-HT, there did not appear to be cocaine-sensitive uptake of 5-HT into vascular endothelial cells of the perfused heart of the rat. Cocaine was used in the experiments on rat hearts, instead of imipramine, an inhibitor that has been used to characterize uptake in both pulmonary endothelial cells (Junod, 1972) and endothelial cells from systemic blood vessels (Shepro *et al.*, 1975; Junod & Ody, 1977; Robinson-White *et al.*, 1981). This was because imipramine, in the concentrations required to inhibit neuronal uptake and uptake into endothelial cells (5 μM), caused heart block in the rat hearts, as has been found previously by Callingham (1967).

The neuronal uptake and subsequent metabolism of 5-HT in the hearts of reserpine-pretreated rats is compatible with reports of 5-HT uptake into noradrenergic neurones in rat atria (McCulloch *et al.*, 1984), in blood vessels including dog saphenous vein (Verbeuren *et al.*, 1983; Paiva *et al.*, 1984) and dog cerebral arteries (Verbeuren *et al.*, 1983), and in guinea-pig vas deferens (Thoa *et al.*, 1969). It was not possible to determine values for the K_m and V_{max} of 5-HT for neuronal uptake in the heart (see Results, section C), but it was clear that neuronal uptake of 5-HT was saturated by 5-HT concentrations about 50 fold lower than those required to saturate extraneuronal uptake ($K_m = 101\,\mu$M). Hence, the K_m of 5-HT for neuronal uptake in the rat heart appears to be of the same order of magnitude as that for 5-HT uptake into endothelial cells of perfused lungs *in vitro* (rat, 5.9 μM; Junod, 1972) and *in vivo* (rabbit, 1.1 μM; Catravas & Gillis, 1983), and in cultured endothelial cells of the bovine pulmonary artery (1 μM; Lee & Fanburg, 1986).

There are data on the extraneuronal uptake of 5-HT in various blood vessels, including those in the rat heart (Grohmann & Trendelenburg, 1984; present study), rat arteries (Fukuda *et al.*, 1986a,b), rabbit ear artery (Buchan *et al.*, 1974) and dog saphenous vein (Paiva *et al.*, 1984). In most of these studies, inhibition of 5-HT uptake by drugs that inhibit extraneuronal uptake of catecholamines has been used as evidence that the same uptake process transported both 5-HT and catecholamines. In an attempt to obtain direct evidence as to whether there was competition between 5-HT and isoprenaline for the same extraneuronal uptake sites, the effect of isoprenaline on 5-HT metabolism in the rat heart was examined. Unfortunately, isoprenaline interfered with the assay of 5-HIAA by h.p.l.c. However, the alternative experiment, showing that 5-HT inhibited extraneuronal uptake of isoprenaline (Grohmann & Trendelenburg, 1984), has demonstrated that these two amines enter extraneuronal sites in the rat heart by the same uptake process.

In the rat heart, the K_m and V_{max} values obtained for the extraneuronal deamination of 5-HT (101 μM and 31.2 nmol g^{-1} min^{-1}, respectively) were very close to the corresponding values for extraneuronal uptake of 5-HT (181 μM and 42.8 nmol g^{-1} min^{-1}, respectively; Grohmann & Trendelenburg, 1984). The similarity of these values suggested that the rate-determining step in the deamination of 5-HT in the heart was the uptake of 5-HT, and not MAO. The similarity also suggested that saturation of deamination of 5-HT reflected saturation of uptake of the amine, and not saturation of extraneuronal MAO. This relationship between kinetic constants has previously been shown for the extraneuronal uptake and deamination of noradrenaline in the rat heart (Fiebig & Trendelenburg, 1978) and also for the neuronal uptake and deamination of noradrenaline (Trendelenburg *et al.*, 1987).

The K_m of 5-HT for extraneuronal uptake in the rat heart is of the same order of magnitude as that of (−)-adrenaline and (−)-noradrenaline, but greater than that of (−)-isoprenaline (Grohmann & Trendelenburg, 1984). The only other reported K_m value of 5-HT for extraneuronal uptake is 391 μM in the dog saphenous vein (Paiva *et al.*, 1984). The higher K_m of 5-HT for extraneuronal uptake in the dog saphenous vein compared with that in the rat heart may reflect a tissue or species difference, or may be due to the tendency for corresponding K_m values determined in incubated tissues to be higher than those determined in perfused organs (Grohmann & Tredelenburg, 1984).

In the rat heart perfused with 1 μM 5-HT, extraneuronal deamination (64% of the total) exceeded neuronal deamination (22–28% of the total). Differences in uptake, and not differences in MAO activity, appeared to account for this, in that the activities of

neuronal and extraneuronal MAO in the rat heart were very similar (as shown by the calculated values of k_{MAO}; see Results, section D). The relative amounts of neuronal and extraneuronal dissipation of 5-HT in different blood vessels, e.g. rat aorta, mesenteric artery and tail artery, has been shown to differ according to the density of noradrenergic innervation (Fukuda *et al.*, 1986b). In the dog saphenous vein, extraneuronal dissipation of 5-HT appeared to represent at least 30% of the total uptake and metabolism of 5-HT (Verbeuren *et al.*, 1983; Paiva *et al.*, 1984).

Since dissipation of 5-HT in the coronary circulation of the rat heart occurred via uptake processes for catecholamines, it is of interest to compare the results for 5-HT with those obtained previously for catecholamines. Steady-state rates of extraneuronal deamination (at non-saturating amine concentrations) were greater than steady-state rates of neuronal deamination for 5-HT (present sutdy) and (−)-adrenaline (Grohmann, 1987), but the reverse was found for (−)-noradrenaline and dopamine (Grohmann, 1987). Hence, 5-HT and the catecholamine, (−)-adrenaline, appeared to be dissipated in the rat heart by the same processes, with the exception that extraneuronal adrenaline is metabolized by catechol-*O*-methyltransferase, as well as by MAO (Grohmann, 1987). Other comparisons that could be made between catecholamines (Grohmann, 1987) and 5-HT in the rat heart were the activities of neuronal and extraneuronal MAO. The rank order of k_{MAO} values for neuronal deamination was (−)-noradrenaline > dopamine > 5-HT > (−)-adrenaline, but that for extraneuronal deamination was dopamine > 5-HT > (−)-noradrenaline > (−)-adrenaline. Hence, the relative activities of MAO for these amines differed in neuronal and extraneuronal sites, particularly for (−)-noradrenaline.

In summary, the study has shown that, in the coronary circulation of the rat heart, 5-HT was dissipated by the uptake processes for catecholamines, extraneuronal uptake and neuronal uptake. The contribution of extraneuronal uptake was greater than that of neuronal uptake, even at the low concentrations of 5-HT that have been measured free in human plasma under normal conditions (0.03–0.07 μM; Genefke *et al.*, 1968; Frattini *et al.*, 1979). It is not possible to predict the local 5-HT concentrations that would occur if 5-HT were released in pathological conditions, e.g. Prinzmental's angina, but the present results suggested that the dissipation of 5-HT in the coronary circulation would occur via extraneuronal uptake and neuronal uptake. There was no evidence that cocaine-sensitive uptake and metabolism of 5-HT in vascular endothelial cells made any significant contribution to 5-HT dissipation in the heart. Hence, to ensure that the pathological effects of any released 5-HT on vascular function in the coronary circulation would be terminated as quickly as possible, it would be important that drugs that inhibit the neuronal or extraneuronal uptake processes should not be present in the circulation.

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Effects of oxodipine on ^{45}Ca movements and contractile responses in vascular smooth muscle

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1 The inhibitory effects of oxodipine, a new dihydropyridine, were compared with those of nifedipine on contractile responses in rat isolated aortic strips and on spontaneous mechanical activity in portal vein segments.

2 In rat isolated aorta oxodipine ($IC_{50} = 7.8 \pm 1.8 \times 10^{-9}$ M) and nifedipine ($IC_{50} = 8.5 \pm 2.5 \times 10^{-9}$ M) dose-dependently inhibited the contractile responses induced by high K (80 mM), whereas responses to noradrenaline (NA, 10^{-6} M) were only slightly affected ($IC_{50} > 10^{-7}$ M). These inhibitory actions were observed with both drugs added either before or after the induced contractions.

3 Contractile responses induced by addition of Ca to 0 Ca high-K solution were also dose-dependently inhibited by oxodipine ($IC_{50} = 4.5 \pm 2.5 \times 10^{-9}$ M). However, oxodipine up to 10^{-6} M did not modify the contractile responses obtained in strips incubated in 0 Ca when Ca was added in the presence of NA.

4 Oxodipine and nifedipine also inhibited the development of spontaneous mechanical activity in portal vein segments.

5 Oxodipine inhibited ^{45}Ca uptake stimulated by high K ($I_{50} = 8.7 \pm 2.5 \times 10^{-9}$ M) or by NA ($I_{50} > 10^{-7}$ M). However, it did not modify ^{45}Ca uptake and ^{45}Ca efflux in resting strips or ^{45}Ca efflux stimulated by NA.

6 These results indicate that the effects of oxodipine on vascular smooth muscle may be due to the blockade of Ca entry through potential- and receptor-operated channels; it was at least 10 times more selective for potential-operated channels. Oxodipine did not modify Ca entry through passive leak channels and NA-induced intracellular Ca release.

Introduction

It is now well recognized that calcium entry blockers (CEBs) are effective in the prophylaxis and treatment of coronary artery disease, arterial vasospasm and hypertension (Godfraind *et al.*, 1984; Opie, 1984; Epstein *et al.*, 1985) which indicates that vascular smooth muscle is one of the tissues in which these compounds show their characteristic effects. These therapeutic effects of CEBs in vascular smooth muscle fibres have been attributed to inhibition of Ca entry through voltage- and receptor-operated channels (Bolton, 1979; Cauvin *et al.*, 1983; Fleckenstein, 1983; Godfraind *et al.*, 1986). In aortic strips dihydropyridine CEBs exhibit a potent antagonism against depolarization-induced contractile responses (Fleckenstein, 1983; Cauvin *et al.*, 1983), whereas their antagonistic effect against noradrenaline-induced contractions occurs only at high concentrations of these compounds (Schumann *et al.*, 1975; Golenhofen & Hermstein, 1975; Bolton, 1979). Oxodipine [4-(2,3-methylen-dioxyphenyl)-1,4-dihydro-2,6-dimethyl,3-carboxyethyl-5-carboxymethyl-piridine] is a new dihydropyridine derivative (Tejerina *et al.*, 1987) which in contrast to nifedipine is not photolabile. The present study was undertaken to examine the effects of oxodipine on rat isolated aortic strips and portal vein segments and to compare its effects with those of nifedipine, the best known dihydropyridine.

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Methods

Experimental procedure

Sprague-Dawley rats of either sex (200–250 g) were killed by a blow on the head. The thoracic aorta and

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the portal vein were dissected and placed immediately in a modified Krebs-Henseleit solution (KS) of the following composition (mM): NaCl 118, KCl 4.7, $NaHCO_3$ 25, $MgCl_2$ 1.2, $CaCl_2$ 1.8 and glucose 11. The Ca-free KS was prepared by adding 2 mM EGTA instead of 1.8 mM $CaCl_2$. After excess fat and connective tissue were removed, the aortae were cut into helical strips of about 1.0 mm width and 20 mm in length. Aortic strips and vein segments were mounted vertically under 2 g and 1 g of tension, respectively, in organ baths containing 10 ml KS. The tissue bath was maintained at 34°C and bubbled with 95% O_2–5% CO_2 gas mixture. Contractile responses were recorded isometrically by a force-displacement transducer (Grass FT03) connected to a Grass polygraph. Each preparation was allowed to equilibrate for at least 2 h before initiation of experimental procedures and during this period the incubation media were changed every 15 min.

After equilibration the following experiments were performed: (1) In some experiments aortic strips were exposed to single submaximal concentrations of KCl (80 mM) and noradrenaline (NA, 10^{-6} M). An initial 25 min control contraction was obtained in each experiment with the appropriate stimulating agent and strips were then rested for a minimum of 45 min. Control contractile responses for each agonist were obtained at the beginning of the experiment until two successive responses were almost identical in height. This was followed by exposure to oxodipine or nifedipine for a 10 min period in both cases before the addition of KCl or NA and during the 25 min re-exposure to the stimulating agents. Only one agonist was used in each experiment. The results of these experiments are expressed as a percentage of the maximal control agonist-induced contractile responses. (2) To determine whether oxodipine and nifedipine could relax an existing contraction, aortic strips were contracted by single submaximal concentrations of NA or KCl. When the contractile response to either agonist was maximum, oxodipine or nifedipine was added in progressively increasing cumulative concentrations (10^{-7} M–5×10^{-4} M). Strips were allowed to reach a new steady-state tension before each successive concentration of the relaxant drug. The results were expressed as percentage of the maximal control agonist-induced responses. (3) In additional experiments dose-response curves to $CaCl_2$ (1–5 mM) were obtained in aortic strips incubated in Ca-free KS containing 2 mM EGTA for 2 h and then in Ca-free high-K (80 mM) solution for 10 min before adding the drugs (Barrigón *et al.*, 1984). The results were expressed as a percentage of the maximal contractile response induced by 5 mM $CaCl_2$. (4) To study the effects of oxodipine on a membrane store of Ca mobilized by NA the experimental protocol employed was similar to that previously described by Ebeigbe & Aloamaka (1985). Strips were stimulated by 10^{-5} M NA. When the contractile response was stable, strips were rinsed with Ca-free KS for 10 min ('Ca-washing') followed by 5 min exposure to 1.6 mM Ca KS ('Ca-loading') and finally, exposure to Ca-washing KS for 1 min, followed by 10^{-5} M NA stimulation. The above protocol was repeated in the presence of oxodipine, added 5 min into the 'Ca-washing' period or 2 min at the end of the 'Ca-loading' period. Control tissues incubated with ethanol (i.e. solvent for oxodipine and nifedipine) were always run in parallel in each of these experimental designs, the change in responsiveness of the control strips during the whole time course of these experiments being 6.9 ± 4.6%.

Measurements of ^{45}Ca movements

Techniques employed to estimate ^{45}Ca movements in aortic strips were as previously described by Godfraind (1983) and Barrigón *et al.* (1984). To determine ^{45}Ca uptake, aortic strips were bisected and in each experiment half of the aorta served as control and the other half as the experimental strip. Both halves were equilibrated for 1 h under 1 g of tension in Tris Buffer solution (TS) of the following composition (mM): NaCl 160, KCl 4.6, $CaCl_2$ 1.8, $MgCl_2$ 1.2, glucose 11 and tris-hydroxymethyl-amino-methane 6.0. This solution was adjusted to pH 7.4 with 4 N HCl and gassed with 100% O_2. After equilibration strips were further incubated for 5 min in TS containing ^{45}Ca (specific activity 0.4 μCi m^{-1}; The Radiochemical Centre, Amersham) either in the presence or absence of oxodipine and then for another 5 min in the same solution with or without the addition of 80 mM KCl or 10^{-6} M NA. At this time strips were washed in 50 mM $LaCl_3$-Ca-free TS for 5 min to remove extracellular bound Ca. Afterwards strips were removed, blotted and weighted. The strips were then placed in scintillation vials and 0.5 ml of Soluene-100 (Packard) added and the strips digested overnight at 50°C. Radioactivity was assayed in a liquid scintillation counter (Intertechnique Model SL-3000) as previously described (Barrigón *et al.*, 1983; 1984).

To study ^{45}Ca efflux in non-stimulated aortic strips, preparations were labelled with ^{45}Ca TS (sp. act. 2 μCi ml^{-1}) for 2 h and then placed in successive tubes containing 2 ml of Ca-free TS every min for the 30 min duration of the washout. In some vials oxodipine was added to the Ca-free TS. The radioactivity lost into the tubes and present at the end of the experiment was measured as described by Barrigón *et al.* (1984). The data obtained were plotted as desaturation curves which illustrate the decline of

tissue ^{45}Ca with time. ^{45}Ca efflux stimulated by NA (10^{-5} M) was studied following the technique previously described by Godfraind (1983). Aortic strips were preincubated for 2 h in ^{45}Ca containing KS ($2\,\mu Ci\,ml^{-1}$), oxodipine being added or not during the last 20 min. The strips were then transferred to non-radioactive KS also with or without oxodipine and 5 min later to the same solution containing or not containing NA (10^{-6} M). Afterwards strips were treated as for ^{45}Ca uptake experiments.

Drugs

The following drugs were used: oxodipine (IQB, S.A.), nifedipine (Bayer) and noradrenaline bitartrate (Sigma). Oxodipine and nifedipine were initially dissolved in 99% ethanol and further dilutions were made in KS, but control experiments demonstrated that the highest ethanol level used at 10^{-6} M have no effect on vascular smooth muscle contraction. Values were expressed as the mean ± s.e.mean and statistical analysis was performed by use of Student's *t* test. The differences between control and experimental values were considered significant when $P < 0.05$. Dose-response slopes were analysed to give the concentration of oxodipine or nifedipine producing a 50% inhibition of the maximal contractile response (IC_{50}) or of the maximal NA and high-K dependent ^{45}Ca uptake (I_{50}) using a linear regression analysis (method of least squares).

Results

Effects on resting and spontaneous mechanical activity

The effects of oxodipine and nifedipine were studied on changes in base-line tension and spontaneous mechanical activity in aortic strips and portal veins, respectively. Both drugs had no effect on resting tension but inhibited in a dose-dependent manner the rate and amplitude of spontaneous myogenic activity in portal veins. The IC_{50} values for oxodipine and nifedipine inhibition of the amplitude of spontaneous activity were $2.5 \pm 1.2 \times 10^{-8}$ M and $7.0 \pm 0.5 \times 10^{-8}$ M, respectively, this difference being statistically significant ($P < 0.05$).

Effect on contractions induced by KCl and NA

The inhibitory effect of oxodipine and nifedipine on contractile responses induced by KCl (80 mM) and NA (10^{-6} M) were quantified and compared in isolated aortic strips. Both agonists produced equipotent submaximal contractile responses, 642.8 ± 36.0 mg and 842.0 ± 88.9 mg, respectively ($n = 8$; $P > 0.05$), whereas maximal contractile responses were obtained with 100 mM KCl (700.2 ± 28.5 mg, $n = 8$) and 10^{-5} M NA (940.8 ± 32.9 mg, $n = 8$). The effects of oxodipine, 10^{-9} M–10^{-7} M, on the time courses of the KCl- and NA-induced contractions are shown in Figure 1. Oxodipine produced a dose-dependent inhibition of the contractile response induced by both stimulating agents. However, whereas 10^{-7} M oxodipine almost suppressed the contractile response induced by KCl, this concentration left unaltered about 50% of the response induced by NA. The IC_{50} values for oxodipine and

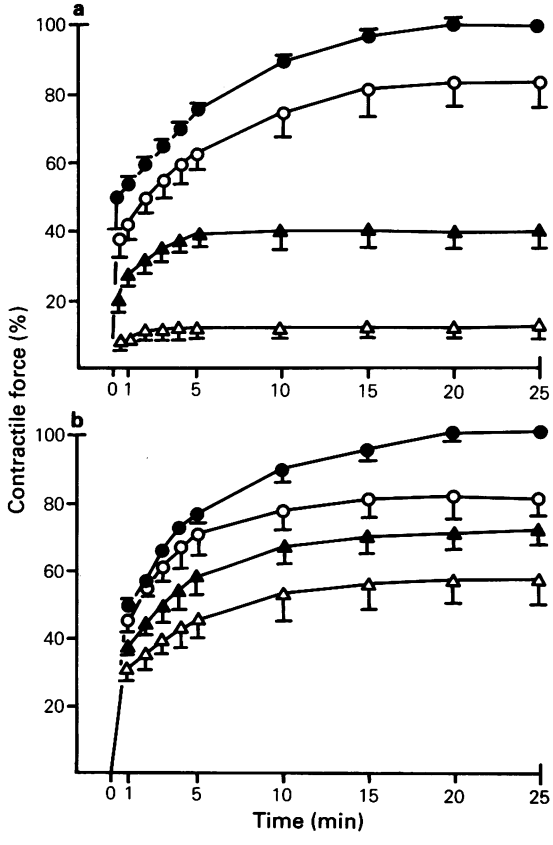


Figure 1 Time course of the contractions induced by (a) high K (80 mM) and (b) noradrenaline (10^{-5} M) in rat aortae pretreated or not with oxodipine. Each point represents the mean of 8 experiments; vertical lines indicate s.e.mean. (●) Controls; (○) after oxodipine, 10^{-9} M, (▲) 10^{-8} M and (△) 10^{-7} M.

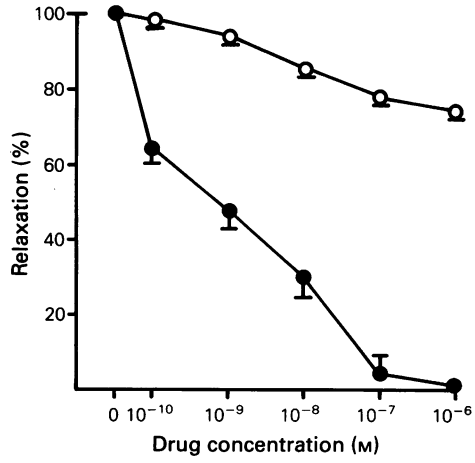


Figure 2 Relaxation of high-K- (●, 80 mM) and noradrenaline-induced (○, 10^{-5} M) contractions by oxodipine. Ordinate scale: relaxation (% of control values). Each point represents the mean of 8 experiments; vertical lines indicate s.e.mean.

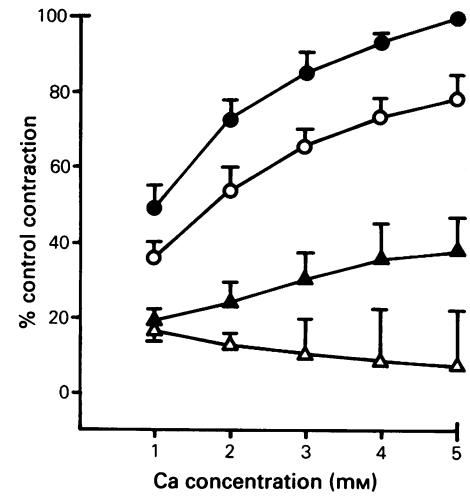


Figure 3 Effects of oxodipine on the restoration of the isometric contractions of aortic strips by addition of calcium (1–5 mM) to Ca-free high-K (80 mM) medium. Ordinate scale: percentage of the maximum control contractions obtained with the highest concentration of calcium in each experiment. Each point represents the mean of 6 experiments; vertical lines indicate s.e.mean. (●) Controls; (○) after oxodipine 10^{-10} M, (▲) 10^{-9} M and (△) 10^{-8} M.

nifedipine-induced depression of high-K-induced responses were $7.8 \pm 1.8 \times 10^{-9}$ M and $8.5 \pm 2.5 \times 10^{-9}$ M ($P > 0.05$), respectively, which indicates that both drugs are almost equipotent on the contractile responses induced by 80 mM KCl.

Figure 2 shows the relaxant effect of cumulatively increasing concentrations of oxodipine on aortic strips previously contracted with either 80 mM KCl or 10^{-6} M NA. Cumulative increases in the concentration of oxodipine and nifedipine (10^{-10} M–10^{-6} M) to strips previously contracted with KCl resulted in a dose-dependent relaxation with an IC_{50} of $3.0 \pm 2.1 \times 10^{-9}$ M and $7.5 \pm 2.1 \times 10^{-9}$ M, respectively ($P > 0.05$). Conversely, at the highest concentration tested (10^{-6} M) oxodipine and nifedipine relaxed strips previously contracted with NA by only $15.3 \pm 3.2\%$ and $26.1 \pm 1.8\%$ of its maximum, respectively.

Effects on Ca-induced contractions

The inhibitory effects of oxodipine on the $[Ca]_o$-dependent contractile responses are shown in Figure 3. Following incubation for 2 h in a Ca-free high-K (80 mM) KS, the addition of Ca to the bathing media (1–5 mM) caused a contraction of the aortic strips in a dose-dependent manner. The maximum contractile response induced by 5 mM $CaCl_2$ was similar to that induced by 10^{-6} M NA (1001.2 ± 83.4 mg as compared to 842.0 ± 88.9 mg, $n = 8$, $P > 0.05$). Oxodipine, 10^{-10} M–10^{-8} M, not only produced a progressive decrease in the amplitude of Ca-induced contractions of K-depolarized aortic strips but also caused a progressive shift of Ca concentration-response curves downward and to the right. Regression analysis of the inhibition yielded an IC_{50} of $4.5 \pm 2.5 \times 10^{-9}$ M.

In another group of experiments the effects of oxodipine were studied on the contractile responses induced by NA in strips which were incubated for 10 min in Ca-free KS, then in 'Ca-loading' KS for 5 min and finally in Ca-free KS for 1 min. At this time stimulation with NA (10^{-5} M) resulted in a phasic contraction of an amplitude of 729.0 ± 72.9 mg ($n = 6$), presumed to reflect Ca mobilization from a membrane-bound store (Karaki *et al.*, 1979). In 6 experiments oxodipine, 10^{-6} M, had no effect on the magnitude of the NA-induced phasic contraction when applied before 'Ca-loading' (619.7 ± 71.5 mg, $P > 0.05$) or during 'Ca-loading' (625.0 ± 50.2 mg, $P > 0.05$).

Effects on ^{45}Ca uptake and ^{45}Ca efflux

The effects of oxodipine, 10^{-8} M–10^{-6} M, were studied on the La^{3+}-resistant ^{45}Ca uptake in resting,

Table 1 Effect of oxodipine on lanthanum-resistant ^{45}Ca uptake in resting aortae and in aortae stimulated with 80 mM KCl or 10^{-6} M noradrenaline

Drug Concentration (M)	^{45}Ca *uptake* (μmol kg^{-1} *wet wt.*) *Unstimulated (controls)*	*Stimulated* KCl	*Stimulated* Noradrenaline
0	86.0 ± 6.8	317.0 ± 11.0	215.2 ± 10.2
10^{-10}		299.1 ± 12.3	203.1 ± 14.3
10^{-9}		281.2 ± 12.8*	190.6 ± 10.7
10^{-8}	85.2 ± 5.8	203.5 ± 10.0***	170.6 ± 12.5**
10^{7}	88.6 ± 6.9	110.4 ± 10.5***	148.5 ± 10.5***

Results shown are means ± s.e.mean, $n = 9$
*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

non-stimulated, as well as in aortic strips stimulated by 80 mM KCl and 10^{-5} M NA. Oxodipine, 10^{-7} M, had no significant effects on ^{45}Ca uptake in resting, non-stimulated aortic strips (86.0 ± 6.8 as compared to 88.6 ± 6.9 umol kg^{-1} aorta; $n = 6$, $P > 0.05$), but produced (Table 1) a significant dose-dependent inhibition of ^{45}Ca uptake stimulated by KCl (80 mM) and NA (10^{-6} M). In order to analyse the dose-dependency of the inhibitory effects of oxodipine on high-K and NA-induced ^{45}Ca uptake the procedure of Meisheri *et al.* (1981) was used. ^{45}Ca uptake was measured in unstimulated strips and from these, averaged values for the increase in Ca uptake over control by the stimulating agent, in the absence as well as the presence of oxodipine were calculated. % inhibitions of ^{45}Ca uptake by oxodipine were then obtained to produce Figure 4. Oxodipine, 10^{-10} M–10^{-7} M dose-dependently inhibited ^{45}Ca uptake stimulated by 80 mM KCl (Figure 4a). Concentration-inhibition curves for contraction and ^{45}Ca uptake were almost superimposed and thus IC_{50} and I_{50} values were similar (7.8 ± 1.8 × 10^{-9} M and 8.7 ± 2.5 × 10^{-9} M, respectively). Oxodipine also decreased ^{45}Ca uptake in strips stimulated by 10^{-5} M NA. As is shown in Figure 4b, concentration-inhibition curves for contraction and ^{45}Ca uptake were also superimposed (IC_{50} and $I_{50} \geqslant 10^{-7}$ M, respectively). These results seem to indicate that even when oxodipine-induced inhibition of high K- and NA-induced contractions can be related to the blockade of Ca entry induced by both agonists, ^{45}Ca uptake stimulated by NA was more resistant to oxodipine inhibition than high K-stimulated ^{45}Ca uptake.

The effects of oxodipine were also studied on ^{45}Ca efflux in non-stimulated as well as in aortic strips stimulated by 10^{-5} M NA. In non-stimulated strips and following exposure for 2 h to ^{45}Ca-labelled TS the strips were rinsed with Ca-free-TS for the first 18 min of the 30 min washout and with Ca-free-TS containing 10^{-6} M oxodipine for the final 12 min. The desaturation curves show (Figure 5) that oxodipine had no effect on the percentage of ^{45}Ca remaining in the aorta.

Furthermore, even at the highest concentration tested, 10^{-6} M, oxodipine had no effect on the tissue content of ^{45}Ca after 2 h incubation in ^{45}Ca-containing solution (0.975 ± 0.11 mmol kg^{-1} wet wt.) as compared to the values obtained in control tissues (0.980 ± 0.09 mmol kg^{-1} wet wt.; $n = 8$, $P > 0.05$). When 10^{-5} M NA was added for 2 min, a significant decrease ($P < 0.05$) of similar magnitude in residual ^{45}Ca content was observed both in control (0.685 ± 0.06 mmol kg^{-1} wet wt.) and oxodipine-pretreated strips (0.711 ± 0.08 mmol kg^{-1} wet wt.; $n = 8$, $P > 0.05$). These results indicate that oxodipine does not modify NA-stimulated ^{45}Ca efflux.

Discussion

The results of this paper demonstrate that oxodipine, a new dihydropyridine derivative, exhibits on rat isolated aortic strips and portal vein segments effects similar to those previously described with nifedipine (Godfraind, 1983) and other dihydropyridines (Godfraind, 1981; Cauvin *et al.*, 1983; Fleckenstein, 1983). Thus, the mechanism through which oxodipine relaxes vascular smooth muscle fibres seems to be related to its ability to inhibit the influx of extracellular Ca through potential- (POCs) and receptor-operated channels (ROCs) of the cell membrane.

Different experimental results demonstrated that oxodipine inhibited Ca entry through POCs: (1) oxodipine inhibited in a dose-dependent manner the contractile responses induced by K depolarization or by increases of extracellular Ca in K-depolarized aortic strips. These responses are entirely dependent

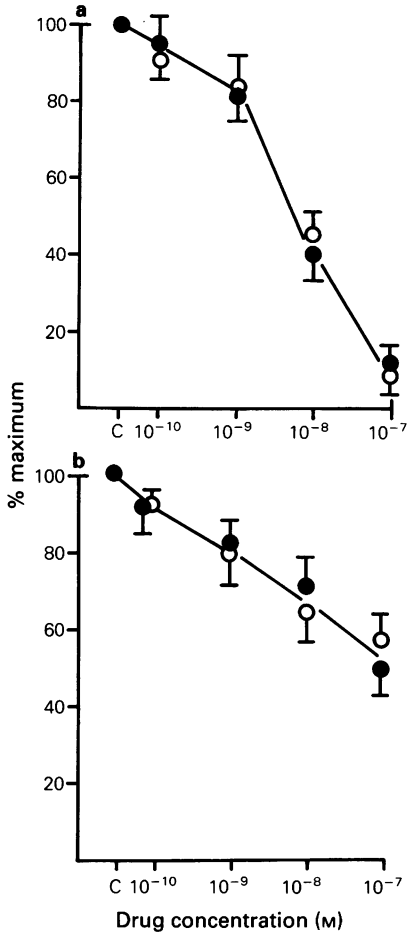


Figure 4 Inhibitory effect of oxodipine on (a) high K (80 mM) and (b) noradrenaline-induced (10^{-6} M) ^{45}Ca uptake (○) and contractile force (●) in rat isolated aortae. Ordinate scales: oxodipine-sensitive component as % of the maximum. Each point represents the mean of at least 6 experiments; vertical lines indicate s.e.mean.

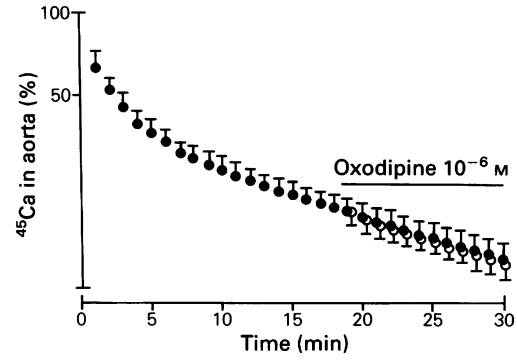


Figure 5 Effect of oxodipine, 10^{-6} M, on ^{45}Ca efflux from rat isolated aortic strips. Muscles were incubated for 2 h in ^{45}Ca before washout in Ca-free solution. Each point represents the mean of 6 experiments. (●) Controls. (○) Oxodipine-treated strips (17 to 30 min).

upon the influx of extracellular Ca through POCs (Hudgins & Weiss, 1968; Godfraind & Kaba, 1969) and have been used to provide a simple means of studying drugs with possible Ca entry blocking properties (Haeusler, 1972; Godfraind, 1981). (2) Oxodipine was more potent than nifedipine at inhibiting the spontaneous myogenic activity in rat portal veins. This myogenic activity is also dependent on extracellular Ca (Sigurdson *et al.*, 1975) and has been related to Ca entry through POCs (Golenhofen & Lammel, 1972). (3) At the same range of concentrations at which it inhibited K-induced contractions, oxodipine also inhibited dose-dependently the ^{45}Ca uptake stimulated by high K without altering resting ^{45}Ca uptake. Moreover, concentration-inhibition curves for K-induced contractions and ^{45}Ca uptake were almost superimposed. This indicates that an inhibition of Ca entry through POCs was responsible for the inhibitory effect of oxodipine on contractile responses induced by high K and Ca in aortic strips, as well as for the suppression of the myogenic activity in rat portal veins. However, as has been previously described with other CEBs (Cauvin *et al.*, 1983; Godfraind, 1983; Godfraind *et al.*, 1986), even at high concentrations oxodipine had no affect on ^{45}Ca uptake in resting aortic strips. This suggests that Ca entry through passive membrane leak channels is insensitive to oxodipine.

Furthermore, most of the CEBs are relatively more potent at inhibiting K-induced contractions than NA-induced contractions (Godfraind, 1981; Cauvin *et al.*, 1983; Kazda *et al.*, 1983) and this is also true for oxodipine. In fact, oxodipine inhibited NA-induced contractions at concentrations about three orders of magnitude higher than those required to inhibit Ca-induced contractions in K-depolarized aortic strips. NA-induced contractions can be resolved into two components, an initial phasic component which is due to the release of Ca from intracellular stores (Hudgins & Weiss, 1968; Godfraind & Kaba, 1969) and a tonic component which is associated with an increase in Ca influx through ROCs (Hudgins & Weiss, 1968; Deth & Van Breemen, 1974). In the present experiments the inhibitory effect of oxodipine on total contractile

responses to NA paralleled its attenuation of the tonic component. In fact, oxodipine-sensitive ^{45}Ca influx and contractions showed a similar concentration-response relationship, suggesting that oxodipine-induced inhibition of NA contractions can be related to the blockade of Ca entry through ROCs. However, if at the high concentrations used NA depolarized the membrane potential of vascular smooth muscle fibres, the inhibitory effect of oxodipine on NA-induced contractions could be attributed to inhibition of Ca entry through both POCs and ROCs. Furthermore, when these results are compared with those obtained in aortic strips stimulated by high K, it is evident that oxodipine was at least two orders of magnitude more selective at inhibiting Ca entry through POCs than through ROCs. These results confirm recent data in rat cultured aortic smooth muscle cells (Ruegg *et al.*, 1988) demonstrating that ^{45}Ca uptake stimulated by high K was blocked by CEBs (various DHPs, verapamil, diltazem), their potency reflecting the potencies in inhibiting K-induced contractions. However, ^{45}Ca uptake stimulated by angiotensin II and vasopressin was not blocked by these concentrations of CEBs. Therefore, the present results support the concept that POCs are more sensitive to CEBs than the ROCs (Cauvin *et al.*, 1983; Godfraind *et al.*, 1986).

However, the phasic component of the NA-induced contractions was unaffected suggesting that the release of Ca from intracellular stores is insensitive to oxodipine. To obtain further information on the effect of oxodipine on the NA-sensitive Ca store, the effects of oxodipine on NA-induced phasic contractions were studied. The phasic contractile response induced by NA in Ca-free media is similar to those found in rat (Ebeigbe & Alaomaka, 1985) and rabbit aorta (Karaki *et al.*, 1979) and has been attributed to mobilization of Ca from a membrane-bound store. In the present study oxodipine did not affect the amplitude of the NA-induced phasic contraction in Ca-free medium whether applied before or during 'Ca-loading'. This result suggests that oxodipine had no effect on the release of Ca from a membrane-bound store induced by NA and/or that the Ca store responsible for the phasic contraction is filled with extracellular Ca via an oxodipine-insensitive pathway. A similar explanation was proposed by Karaki *et al.* (1979) to explain why verapamil and D600 in concentrations of 10^{-6} M did not affect this phasic response. Furthermore, at the maximum concentration tested which inhibited by almost 70% the NA-dependent ^{45}Ca uptake, oxodipine had no effect on ^{45}Ca efflux in resting, non-stimulated, as well as in aortic strips stimulated by NA. These results strongly suggest that at the concentrations at which it inhibits Ca entry, oxodipine fails to inhibit NA-induced intracellular Ca release. Similar results have been found in the presence of PY 108-068 (Hof *et al.*, 1982) and nifedipine (Godfraind, 1983; Kanmura *et al.*, 1983).

In conclusion, oxodipine is a new dihydropyridine derivative which like nifedipine inhibits Ca entry through voltage- and receptor-operated channels. However, oxodipine failed to inhibit Ca entry through passive leak channels and NA-induced intracellular Ca release.

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Tissue noradrenaline and the polyol pathway in experimentally diabetic rats

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1 The effects of a six week period of streptozotocin-induced diabetes on tissue catecholamines and on *in vivo* noradrenaline turnover were assessed in rats.

2 Noradrenaline concentrations measured in heart ventricle, terminal ileum, vas deferens, spleen and adrenal tissue from the diabetic rats were all found to be elevated compared to those found in control rat tissues. The adrenaline contents of the adrenal glands were also raised in these animals.

3 Noradrenaline turnover in heart ventricle, terminal ileum and vas deferens was estimated from the decline in tissue content of the amine following inhibition of its synthesis with α-methyl-*p*-tyrosine. Turnover was found to be increased in all three tissues.

4 The involvement of the polyol pathway in the above changes was investigated by examining the effects of continuous treatment with an aldose reductase inhibitor, Statil (ICI 128436) or dietary myo-inositol supplementation. Either treatment was found to prevent or reduce the increases in tissue noradrenaline and in its turnover. Myo-inositol treatment also partially prevented the rise in adrenal adrenaline.

5 It is concluded that the elevation of tissue catecholamines and of noradrenaline turnover by diabetes was related to myo-inositol depletion secondary to excessive sorbitol synthesis. Possible mechanisms for the observed increase in noradrenaline turnover could involve Na^+, K^+-ATPase depression.

Introduction

Abnormalities of sympathetic nervous function have been reported to occur in diabetic patients (Low *et al.*, 1975). Sympathetic activity in experimentally diabetic rats as assessed by noradrenaline (NA) turnover has been found to be decreased (Yoshida *et al.*, 1987) or increased (Ganguly *et al.*, 1987). The mechanism(s) which underlie these and other diabetes-induced nervous abnormalities remain uncertain. Hypoxia (Low *et al.*, 1986), protein glycosylation (Brownlee *et al.*, 1984) and excessive activity of the aldose reductase pathway (Gabbay, 1975; Kador *et al.*, 1985) have each been proposed.

With the development of effective inhibitors of aldose reductase the possible involvement of the last mentioned has recently received considerable attention (Kador *et al.*, 1985). Aldose reductase inhibition has been shown to prevent and, in some cases, reverse a number of the changes that are observed in nerves of diabetic animals e.g. reductions in nerve conduction velocity and in the speed of some types of axonal transport (Mayer & Tomlinson, 1983; Mayer *et al.*, 1984). Yoshida *et al.* (1987) reported that the reduced NA turnover which they observed was prevented by aldose reductase inhibition as well as by insulin therapy.

Myo-inositol (MI) concentration in nervous tissue is depressed in short term experimental diabetes, an effect which may be prevented by either aldose reductase inhibition or dietary MI supplementation. This MI depression may mediate some of the above mentioned diabetic-induced changes in nervous function e.g. the reduced nerve conduction velocity and the depression of some types of axonal transport processes (Mayer & Tomlinson, 1983; Mayer *et al.*, 1984).

The present study examines the effects of short term experimental diabetes on tissue catecholamine concentrations and their turnover. The effects of

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either aldose reductase inhibition with Statil (ICI 128436) or dietary MI supplementation on any diabetes induced changes were also investigated.

Methods

Experimental animals

Diabetes was induced in male Wistar rats (200–300 g) by a single injection of 2 ml kg^{-1} of a freshly prepared solution of streptozotocin (20 mg ml^{-1} in pH 4.5 citrate buffer) via a tail vein. Controls received buffer alone. Three days after injection, glucose concentrations were assessed in a drop of blood from a tail vein (obtained by puncturing the vein with a sterile needle) using test strips (BM-test-BG, Boehringer Mannheim). Only rats showing values at or above 22 mM were assigned to diabetic groups. MI-treated rats received 1 g l^{-1} MI in their drinking water from day 3 until the time of death (this resulted in a dose of approximately 1 g kg^{-1} day^{-1}).

We have previously found this treatment to prevent sciatic nerve MI depletion (Kofo-Abayomi & Lucas, 1988). Statil (ICI 128,436; 3-(4-bromo-2-fluorobenzyl)-4oxo-3H-phthalazine-1-yl acetic acid) was administered orally in 5% Tween 80 (10 mg Statil ml^{-1}) at a dose of 25 mg kg^{-1} day^{-1}. All animals not receiving Statil were given equivalent oral doses of 5% Tween 80.

All animals were given free access to food and water.

Tissue catecholamines and blood glucose treatment

Tissue catecholamines were determined by h.p.l.c. with electrochemical detection. The equipment consisted of a pump (Gilson model 302 with manometric module, model 802), an injection valve (Rheodyne model 7125), a reversed phase column (20 cm × 4 mm i.d. packed with ODS 5 μm hypersil particles), and electrochemical detector (BAS model LC 3A) set at 0.65V and a recorder (Gilson model N1). The mobile phase contained NaH_2PO_4 (0.1 M), NaCl (2 mM), EDTA (0.1 mM) and 10% methanol with octyl-sulphonate (1.2 mM) as the ion-pairing agent.

The solution was degassed under vacuum before use. The flow was 1 ml min^{-1}.

Six weeks after the tail vein injection the rats were weighed, anaesthetized with pentobarbitone (60 mg kg^{-1}, i.p.) and tissues removed and placed in liquid nitrogen. They were subsequently stored at −20°C until use (usually within 1 month, a period which preliminary studies showed, did not result in significant loss of catecholamines under these conditions). At assay, tissues were homogenized in ice-cold 0.1 M perchloric acid containing EDTA (2.5 mM) and sodium metabisulphite (10 mM) and an amount of epinine approximately equivalent to the expected tissue catecholamine content was added to each tube as an internal standard. The homogenates were then centrifuged at 35,000 *g* for 20 min at 4°C, the supernatants transferred to fresh tubes and the pH raised to 8.6 by the addition of 1 M Tris buffer; 50 mg of acid washed alumina was then added to each tube after which they were shaken vigorously for 20 min in a mechanical shaker. The supernatants were removed and the alumina washed twice with 1 ml distilled deionized water. The catecholamines were finally eluted from the alumina with 0.2 ml 0.1 M perchloric acid. Aliquots (10 μl) of this solution were injected onto the column. *In vivo* noradrenaline turnover in heart ventricles, terminal ileum and vas deferens was assessed by measuring the rate of decrease in its concentration in these tissues following the administration of the synthesis inhibitor α-methyl-*p*-tyrosine (AMPT; Spector *et al.*, 1965). The methyl ester of AMPT was dissolved in isotonic saline at a concentration of 60 mg ml^{-1} and 2 ml kg^{-1} of this solution was injected i.p. either 4 or 8 h before the animals were killed. The 8 h groups were reinjected 4 h before they were killed in order to maintain tissue AMPT levels.

NA turnover is expressed as turnover rate constant (h^{-1}). This is the negative regression coefficient with associated standard deviations calculated from the decline of log tissue NA concentration with time (h).

Differences in tissue mass between groups could affect the interpretation of changes in concentrations of NA. Wet tissue weights were, therefore, measured in five additional control, six six-week diabetic rats and in five similarly diabetic rats which received Statil as above.

Glucose concentrations were measured in 0.1 ml samples of blood taken at the time of death by the use of a colourimetric copper reduction method (Asatoor & King, 1954).

Statistics

Differences between the four or three groups in tissue NA concentrations, adrenal adrenaline or in turnover rate constants were assessed initially by one way analysis of variance. Where P values of less than 0.05 were obtained, individual means or slopes were compared by the Duncan's method for multiple comparisons; where group sizes differed the harmo-

Table 1 Blood glucose concentrations and growth in control and diabetic rats (SDR) and in diabetic rats treated with myo-inositol (MI) or Statil

	Controls (22)	*SDR* (24)	*SDR + MI* (19)	*SDR + Statil* (20)
Blood glucose (mM)	5.4 ± 2.3	28.9 ± 9.8***	31.8 ± 9.6***	32.8 ± 7.6***
Growth (g)	72.8 ± 17.4	−19.8 ± 37.2***	−24.0 ± 35.7***	−15.6 ± 37.1***

Results are of means ± s.d. with numbers per group in parentheses. The significances of differences from the control values are indicated by: *** $P < 0.001$. No significant differences between the diabetic groups were observed.

nic mean of n was used in the latter test (Bruning & Kintz, 1968). Again P values of <0.05 were taken to indicate significance.

Results

Diabetes was confirmed in all diabetic groups by their raised blood glucose concentrations and their reduced growth rates (Table 1). These measurements were not significantly changed by treatment with either MI or Statil.

Tissue NA levels were higher in the untreated diabetic group compared to their controls (Table 2). The mean increases were: heart + 95%, spleen + 59%, terminal ileum + 179%, vas deferens + 247% and adrenals + 142%. In each tissue, with the exception of the spleens of the MI-treated group, in which NA was not measured, treatment with MI or Statil appeared to prevent these increases completely, NA levels being similar to control values and significantly lower than those of the untreated diabetic group. Adrenal adrenaline was also increased in the untreated diabetic group by an average of 108% compared to control values. This elevation was significantly smaller in the MI but not in the Statil-treated group.

NA turnover *in vivo* was increased in the heart ventricles and terminal ilea from the untreated diabetic group compared to values obtained from control rat tissues ($P < 0.001$ and $P < 0.01$ respectively, Table 3). The mean rate constant for NA turnover in the vas deferens was also greater in the untreated diabetic group but the difference was not significant ($P > 0.05$). NA turnover in the heart ventricles and in the ileum of the Statil-treated animals was similar to control values and significantly ($P < 0.001$ and $P < 0.01$ respectively) less than in the untreated diabetic rats.

MI-treatment also appeared to prevent the increased turnover in the terminal ileum in the diabetic animals. A reduced NA turnover rate was observed in the vas deferens of the MI-treated group compared to the untreated diabetic group. The effect of MI on NA turnover in heart ventricles was not assessed. Heart ventricles from the additional diabetic rats weighed approximately 20% less than those from the control animals ($P < 0.01$). In contrast, ilea from these animals were approximately twice the weight of those from controls (Table 3). Statil treatment appeared to have no effect on these changes. No differences between groups were found

Table 2 Noradrenaline turnover (rate constants, h^{-1}) in control and diabetic rats (SDR) and in diabetic rats treated with myo-inositol (MI) or Statil

	Controls (22)	*SDR* (24)	*SDR + MI* (19)	*SDR + Statil* (20)
Heart ventricle	0.059 ± 0.070***	0.123 ± 0.059	ND	0.034 ± 0.210***
Terminal ileum	0.091 ± 0.150**	0.157 ± 0.216	0.064 ± 0.092*	0.088 ± 0.094**
Vas deferens	0.158 ± 0.136	0.223 ± 0.157	0.080 ± 0.083**	0.187 ± 0.112

Results are the negative regression coefficients ± s.d. for the decline of $\log_e$ tissue NA concentrations with time (h). The number of results used to calculate these values are indicated in parentheses. The significances of differences from the untreated diabetic group data: * $P < 0.5$, ** $P < 0.01$ and *** $P < 0.001$.

Table 3. Tissue weights in control and diabetic (SDR) rats and in diabetic rats treated with Statil

	Controls (5)	*SDR* (6)	*SDR + Statil* (5)
Heart ventricle (mg)	1000 ± 115	801 ± 116**	788 ± 71**
Spleen (mg)	944 ± 132	759 ± 205	761 ± 172
Ileum (g)	7.43 ± 0.79	14.3 ± 2.8***	16.60 ± 1.15***
Vas deferens (mg)	69.1 ± 4.9	67.0 ± 8.3	68.5 ± 7.7
Adrenals (2) (mg)	63.8 ± 4.5	72.7 ± 7.2	71.1 ± 9.8

Results were obtained in additional animals and are mean ± s.d. The significance of differences from control values are indicated by ** $P < 0.01$ and *** $P < 0.001$.

in the weights of the spleen, vas deferens or adrenal glands.

Discussion

An increase in NA concentrations in various tissues in short-term experimental diabetes has been observed previously in rats (Ganguly *et al.*, 1987; Yoshida *et al.*, 1987) and mice (Garris, 1987). The present results are in agreement with these reports, but, in addition show, that the diabetes-induced elevation of tissue NA may be related to MI depletion since it was prevented either by MI supplementation or by aldose reductase inhibition. Both have been shown to prevent MI depletion in nerve (Mayer & Tomlinson, 1983).

Changes in tissue weights were observed in the additional control, diabetic and Statil-treated diabetic rats. If the reduction in heart ventricle mass by diabetes was not accompanied by any change in the number of sympathetic neurones then the increase in noradrenaline content per neurone in untreated diabetic rats was approximately 56% rather than the 95% indicated by the tissue NA concentration data of Figure 1. The effects of MI and Statil on NA content per neurone would be correspondingly greater since Statil, and presumably MI, did not prevent the hypoplasia. In contrast, the hyperplasia observed in the ileum means that increases in NA per neurone, again providing the latter remained constant in number, was much greater than that indicated in Figure 1 for the untreated diabetic group. The prevention of the elevation of neuronal NA by MI or Statil would, however be less complete since Statil, and, presumably, MI did not prevent the hyperplasia. The finding of increased catecholamine turnover in tissues of the untreated diabetic animals is in agreement with the study in cardiac muscle reported by Ganguly *et al.* (1987) but not with the

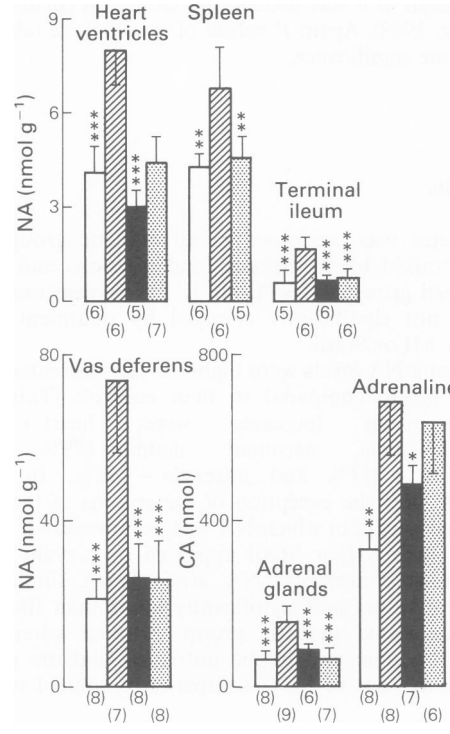


Figure 1 Tissue catecholamine levels in control and diabetic (SDR) rats and in diabetic rats treated with myo-inositol (MI) or Statil. The histograms represent the means and the vertical lines the s.d.'s. Open columns: controls; hatched columns: diabetic rats; solid columns: diabetic rats treated with MI; stippled columns: diabetic rats treated with Statil. Values for the vas deferens, heart ventricles, spleen and terminal ileum are nmol noradrenaline per g of wet tissue while those for the adrenal glands are total noradrenaline or adrenaline content (CA) of both glands. The numbers in each group are given in parentheses and the significance of differences from the untreated diabetic group values are indicated by * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

report by Yoshida *et al.* (1987) who found reduced turnover in heart, brown fat and pancreas. The reason for the disparity between the results of the latter study and those of this work are not clear. The model and duration of diabetes appeared similar. In both studies turnover was assessed from the decline in tissue catecholamines after synthesis inhibition with AMPT.

There are at least two possible mechanisms which could explain an increase in tissue NA turnover in the untreated diabetic group. Na^+, K^+-ATPase activity has been reported to be depressed in diabetic nerve, this depression being reversible by either MI or aldose reductase inhibition (Green *et al.*, 1983). Acute inhibition of Na^+, K^+-ATPase has been found to increase both acetylcholine and NA turnover (Vizi *et al.*, 1982; Magyar *et al.*, 1986).

Thus, neuronal MI depletion in the untreated diabetic rats may, by depressing Na^+, K^+-ATPase, cause the observed increase in NA turnover. Alternatively, hypothyroidism might be involved as Tahiliani & McNeil (1985) have reported depressed plasma levels of both T_3 and T_4 in 6-week streptozotocin-diabetic rats and hypothyroidism has been found to increase cardiac sympathetic activity whether measured by [^{3}H]-NA turnover or, as here, by tissue NA decline following AMPT (Landsberg & Axelrod, 1968). Na^+, K^+-ATPase activity has been reported to be depressed by hypothyroidism and increased by T_3 administration (Norgaard *et al.*, 1983). The untreated diabetic rats in the present study may, therefore, have had reduced neuronal Na^+, K^+-ATPase activity due to hypothyroidism which resulted in the observed increase in NA turnover. Such a hypothesis does not, however, explain its prevention by MI or Statil unless such treatment prevented the hypothyroidism. The elevation of tissue NA in the absence of AMPT is unlikely to be due to hypothyroidism since Landsberg & Axelrod (1968) found no NA elevation in their hypothyroid rats and Ganguly *et al.* (1987) found no effect of T_3 administration on the elevated tissue NA which they observed in diabetic rats. The latter paper reported a decrease in the proportion of [^{3}H]-NA recovered from a cardiac tissue fraction containing the storage vesicles. The possibility of such a storage defect for endogenous NA and, if it occurs, whether it is prevented by aldose reductase inhibition requires investigation.

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What confers specificity on glycine for its receptor site?

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1 The structural requirements for activation of the glycine receptor were studied in isolated ventromedial hypothalamic neurones of rats by use of a 'concentration-clamp' technique under single-electrode voltage-clamp conditions.

2 α-Amino acids (L-α-alanine, and D-α-alanine, and L-serine), and glycine-methylester, glycine-ethylester and β-amino acids (β-alanine and taurine) produced a transient inward Cl^- current, which was similar to that induced by glycine.

3 The responses to individual α- and β-amino acids were selectively antagonized by strychnine, but were not affected by bicuculline, picrotoxin or the taurine antagonist, TAG (6-aminomethyl-3-methyl-4H,1,2,4-benzothiadiazine-1,1-dioxide hydrochloride), suggesting that α- and β-amino acids activate the same glycine receptor.

4 β-Amino acids were slightly more potent than the α-amino acids in causing cross-desensitization of the glycine response.

5 From the results of the structure-activity analysis of the optical isomers of α-alanine, serine and cysteine, a tentative structure of the glycine receptor is proposed.

Introduction

Glycine plays an important role as an inhibitory neurotransmitter in the central nervous systems of various animals (Curtis *et al.*, 1968; Krnjević, 1974; Aprison *et al.*, 1976; Pycock & Kerwin, 1981). In spite of its simple chemical structure, many kinds of glycine binding sites have been reported in vertebrate tissues: (a) a conventional glycine receptor which operates through a Cl^- channel and which is sensitive to strychnine (Curtis *et al.*, 1968; Graham *et al.*, 1985), (b) a strychnine-insensitive glycine binding site (De Feudis, 1977; Bristow *et al.*, 1986), one of which may allosterically regulate the N-methyl-D-aspartate (NMDA) receptor (Johnson & Ascher, 1987), and (c) a glycine transport carrier (Johnson & Iversen, 1971; Wilkin *et al.*, 1981). In general, for compounds with complex chemical structures and flexibility in the molecule, there exists the possibility of activity at different recognition sites, due to their conformational variation. It is, for example, generally accepted that GABA can exist in two distinct conformations: an extended or a folded form. GABA is believed to interact with its receptor in an extended conformation, while binding to the transport carrier protein requires the folded form (Krogsgaard-Larsen *et al.*, 1975; Johnston *et al.*, 1976). Similarly, the ability of acetylcholine to exist in different conformational states, accounts for its ability to activate either nicotinic or muscarinic receptors. However, the interactions of glycine with its receptor sites are unlikely to be explained in this way, since glycine seems to exist only in one conformation. It is thus important to know what confers specificity upon the interaction between glycine and its receptor-ion channel complex in mammalian CNS neurones. Although previous electrophysiological studies have provided considerable information about the specificity of the glycine receptor, quantitative data have been lacking, because conventional methods such as bath perfusion, ionophoretic and pressure applications of glycine, were accompanied by significant desensitization before glycine equilibrated with the receptor.

Recently, we have developed a new type of concentration-jump technique termed 'concentration-clamp' which combines internal perfusion and very rapid drug application. This technique makes it possible to apply drugs within a few milliseconds (Akaike *et al.*, 1986). This high application speed greatly decreased or abolished the contamination of the activation phases with desensitization. In the present study, we have applied this 'concentration-clamp' technique to isolated ventromedial hypothalamic neurones of the rat (Kaneda *et al.*, 1988)

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and examined the effects of α- and β-amino acids and glycine derivatives on the glycine receptor. From this structure-activity analysis of glycine receptor interaction, the tentative structure of the glycine receptor is proposed.

Methods

Preparation

Experiments were performed on ventromedial hypothalamic (VMH) neurones isolated from 1 to 3 week-old Wistar rats by use of a previously described dissociation procedure (Kaneda *et al.*, 1988).

Solutions

The ionic compositions of the external and internal solutions were (in mM): (A) incubation solution for the enzyme treatment and isolation of cells: NaCl 160, KCl 5, $CaCl_2$ 2, glucose 10, N-2-hydroxyethylpiperidine-N'-2-ethanesulphonic acid (HEPES) 10; (B) experimental external solution: NaCl 150, KCl 5, $CaCl_2$ 2.6, $MgCl_2$ 1.1, glucose 10 and HEPES 10. The pH was adjusted to 7.4 by adding tris (hydroxymethyl)aminomethane (Tris-base). The internal perfusion solution (mM): KF 50, K-aspartate 50, Tris-Cl 30, $MgCl_2$ 1, ATP-Na_2 1 and HEPES 10; the pH was adjusted to 7.2 with Tris-base.

Internal perfusion by suction-pipette

Freshly dissociated VMH neurones were taken up by suction into a tapered capillary with a fire-polished tip having a diameter of approximately 500 μm and transferred into a culture dish (3 cm in diameter). Single neurones were clearly visible under an inverted microscope with magnification of 320×. A suction-pipette technique was used for internal perfusion (Hattori *et al.*, 1984; Ishizuka *et al.*, 1984; Akaike *et al.*, 1986).

A Pyrex-glass tube with a 1.5 mm outer diameter was pulled to a shank length of 3 to 4 mm. The pipette tip was fire-polished to give an inner diameter of about 3–4 μm with fire-polishing equipment (Narishige, type MF-83). One of the dendrites was drawn into the suction-pipette with slight negative pressure (5 to 10 cmHg). This portion of the dendric membrane, was ruptured by applying square command pulses of +100 mV of 10 ms duration. Adequacy of internal perfusion with the present suction-pipette technique was evaluated by determining how close the reversal potential for the glycine-gated Cl^- current (E_{Gly}) was to the Cl^- equilibrium potential (E_{Cl}) of about −40 mV, calculated from the Nernst equation based on the Cl^- activities estimated by a F10121Cl Cl^- electrode connected to an ION85 Analyzer (Radiometer, A/S).

Rapid drug application using 'concentration-clamp' technique

The drug-application method used was described in detail in a previous paper (Akaike *et al.*, 1986). After setting a cell in the tip of the suction-pipette as described above, the tip was placed in a plastic tube through a circular hole with a diameter of 500 μm. The lower end of this tube can be placed in each of several dishes on a turntable which contains external solution and the required drugs. Onset and offset of quick perfusion of the solutions are operated by an electromagnetic valve, driven by 24 volts d.c. supplied by a stimulator (Nihon Koden, SEN-7103). The exchange of external solution took 2 to 4 ms for the isolated VMH neurones.

Electrical measurement

The membrane current was measured through a Ag-AgCl wire in the suction-pipette holder. The reference electrode was also a Ag-AgCl wire in a Ringer-agar plug. The resistance between the suction-pipette filled with the internal solution and the reference electrode was about 2 MΩ. Both electrodes were connected to a patch-clamp amplifier (List-Medical, EPC-7). The current and voltage were monitored on a digital storage oscilloscope (National, VP-5730A), simultaneously recorded on an ink recorder (Rikadenki, R-22) and stored on an FM data recorder (TEAC, MR-30).

Drugs

Drugs employed in the present experiments were as follows. Collagenase (Sigma, type I), trypsin (Difco, 1: 250); α-amino acids: glycine (Gly) (Nakarai), L-α-alanine (L-α-Ala), D-α-alanine (D-α-Ala), L-serine (L-Ser), D-serine (D-Ser), L-cysteine (L-Cys) (Tokyo Kasei); β-amino acids: β-alanine (β-Ala), taurine (Tau) (Tokyo Kasei); γ-aminobutyric acid (GABA) (Tokyo Kasei); other glycine derivatives: 2-amino-isobutyric acid (2-AIBA), glycine methylester (Gly-Met), glycine ethylester (Gly-Et), oxamic acid and N-acetyl glycine (N-Ac-Gly) (Tokyo Kasei). Specific antagonists: strychnine [Sigma], bicuculline methiodide [Sigma], picrotoxin [Sigma], and 1,2,4-benzothiadiazine-1,1-dioxide (TAG). TAG was kindly donated by Dr Okamoto. Drugs were dissolved in the test solutions just before use. All experiments were carried out at room temperature (22–25°C).

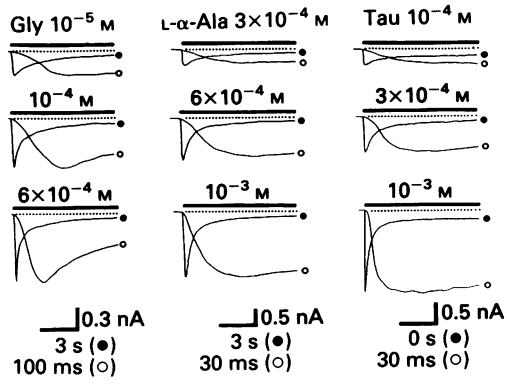


Figure 1 Glycine (Gly)-, L-α-alanine (L-α-Ala)- and Taurine (Tau)-induced Cl^- currents (I_{Cl}) recorded from rat isolated ventromedial hypothalamic (VMH) neurones. Holding potential (V_H) was −70 mV. Data were obtained from 3 different cells. Current traces were superimposed by different time scales.

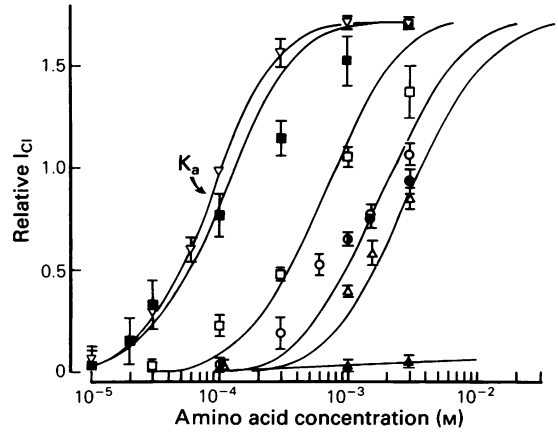


Figure 2 Dose-response curves for α- and β-amino acids at a V_H of −70 mV. Abscissa scale: concentration of amino acids. Ordinate scale: relative currents induced by various amino acids normalized to the peak current induced by 10^{-4} M glycine alone. Solid lines for all amino acids except glycine were fitted by eye. The theoretical curve for glycine was reconstructed from equation [1] using the maximal response (I_{max}) of 1.7, the concentration for half I_{max} (K_a) of 9×10^{-5} M and the Hill coefficient (n) of 1.8. Glycine (▽, $n = 8$); L-α-alanine (○, $n = 9$); D-α-alanine (●, $n = 6$); L-serine (△, $n = 5$); D-serine (▲, $n = 4$); taurine (□, $n = 6$); β-alanine (■, $n = 6$). Vertical bars give s.e.mean.

Results

Glycine, L-α-Ala, D-α-Ala, L-Ser, D-Ser, L-Cys, β-Ala, Tau, Gly-Met, Gly-Et, 2-AIBA, oxamic acid and N-Ac-Gly were applied to rat isolated VMH neurones by the 'concentration-clamp' technique. Gly, β-Ala, Tau, L-α-Ala and L-Ser produced inward Cl^- currents (I_{Cl}) which had similar response patterns in their activation and desensitization phases (Figure 1). Both Gly-Met and Gly-Et gave much weaker responses while 2-AIBA, L-Cys, D-Ser, oxamic acid and N-Ac-Gly did not elicit any response. In Figure 1, each response is shown at superimposed fast and slow sweep speeds in order to show the activation and desensitization processes clearly.

Responses to each amino acid were normalized to the peak I_{Cl} induced by 10^{-4} M glycine. The theoretical curve for the response to glycine was drawn according to equation [1] using the maximal response (I_{max}) of 1.7, the concentration for half I_{max} (K_a) of 9×10^{-5} M and the Hill coefficient (n) of 1.8:

$$I = I_{max} \frac{C^n}{C^n + K_a^n} \qquad [1]$$

where I is the observed drug-induced I_{Cl} and C is the drug concentration. β-Ala was almost equipotent with glycine as an agonist at concentrations up to 10^{-4} M, although it produced smaller responses than that of glycine at concentrations over 10^{-4} M. Other α- and β-amino acids such as Tau, L- and D-α-Ala and L-Ser were weaker agonists than β-Ala, as shown in the dose-response curves (Figure 2). The tested amino acids, except for β-Ala, did not achieve the same I_{max} as glycine, whereas the K_a for glycine was lower than for all other amino acids. Using the differences in potency and efficacy for each amino acid, the activity of each amino acid on the glycine receptor was evaluated in the order of glycine > β-Ala > Tau > L- and D-α-Ala > L-Ser ≫ D-Ser.

Inhibitory effects of strychnine, TAG, bicuculline and picrotoxin on the responses induced by amino acids were examined. Glycine, L-α-Ala and Tau were used at concentrations nearly equal to their K_a values of 10^{-4}, 10^{-3} and 10^{-3} M, respectively. Figure 3 shows typical examples of the effects of strychnine and bicuculline on I_{Cl} induced by these amino acids. Strychnine (10^{-8} M) inhibited the glycine-, L-α-Ala- and Tau-induced responses by about 30 to 50% without changing either the activation or desensitization kinetics. Both bicuculline and TAG failed to inhibit these responses at low concentrations and picrotoxin gave only a very slight inhibition (less than 5%) even at concentrations greater than 10^{-5} M.

Effects of antagonists on 10^{-4} M glycine and 10^{-3} M Tau-induced currents were evaluated in a dose-dependent manner. The threshold concentration for an effect of strychnine on glycine and Tau-induced I_{Cl} was 10^{-9} M, in both cases with an IC_{50} value of 2×10^{-8} M. Maximal inhibition for both

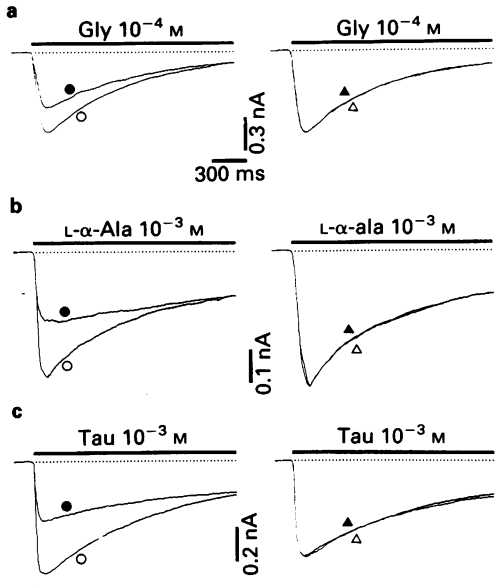


Figure 3 Effects of strychnine (10^{-8} M at ●) and bicuculline (10^{-5} M at ▲) on glycine (Gly)-, L-α-alanine-(L-α-Ala) and taurine (Tau)-induced I_{Cl}. Open symbols indicate controls. (a), (b) and (c) were obtained from 3 different neurones. V_H was −70 mV.

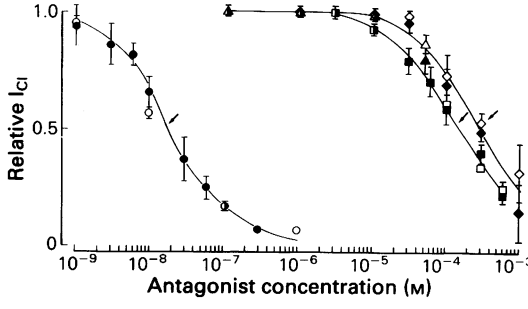


Figure 4 Inhibition of glycine (Gly)- and taurine (Tau)-induced I_{Cl} by various antagonists. Abscissa scale: concentration of antagonists. Ordinate scale: relative currents normalized to the peak current induced by 10^{-4} M glycine or 10^{-3} M taurine alone. For taurine 10^{-3} M: symbols indicate (○) + strychnine; (□) + picrotoxin (△) + bicuculline and (◇) + TAG(6-aminomethyl-3-methyl-4H, 1,2,4-benzothiadiazine-1, 1-dioxide hydrochloride). For glycine 10^{-4} M: symbols indicate (●) + strychnine; (■) + picrotoxin; (▲) + bicuculline and (◆) + TAG. Neurones were pretreated by antagonists before glycine or taurine plus one of the antagonists were applied. Arrows indicate IC_{50}. Each point is the average of 5 to 8 neurones and the vertical bars give s.e.mean.

responses was achieved at 10^{-6} M strychnine. Inhibitory effects of picrotoxin, bicuculline and TAG became evident only at a concentration of 10^{-5} M. The IC_{50} values obtained from the inhibition curves were 1.5×10^{-4} M for picrotoxin, 3×10^{-4} M for bicuculline and 3×10^{-4} M for TAG.

The cross-desensitization between α-, β- and γ-amino acid-induced responses was examined and the interactions of glycine, L-α-Ala, Tau and GABA are summarized in Figure 5. Figure 5a shows that pretreatment with GABA did not affect the successive glycine responses. The L-α-Ala-induced current was unaffected by pretreatment with GABA but was completely inhibited by pretreatment with glycine (Figure 5b). The Tau-induced current was not modified by pretreatment with GABA but completely inhibited by pretreatment with glycine (Figure 5c). Cross-desensitization with glycine was also observed with responses to other α- and β-amino acids such as D-α-Ala, L-Ser and β-Ala (not shown).

The effects of pretreatment with α-, β- and γ-amino acids on the glycine response were examined quantitatively. Figure 6a shows the experimental protocol. The results are summarized in Figure 6b. At concentrations up to 3×10^{-3} M, GABA pretreatment had no effect on the successive glycine responses. On the other hand, pretreatment with other α- and β-amino acids (L-α-Ala, D-α-Ala, L-Ser, Tau and β-Ala) suppressed the successive glycine responses in a dose-dependent manner. Pretreatment with D-Ser at concentrations above 10^{-3} M produced very slight suppression of the glycine response. The relative order of the IC_{50} values for each amino acid was identical to the relative order of their K_a values. The IC_{50} values were approximately 2×10^{-5} M for β-Ala, 10^{-4} M for Tau and 3×10^{-4} M for L- and D-α-Ala and L-Ser.

Cross-desensitization between glycine and various α-, β- or γ-amino acid-induced current is shown in Figure 7. There was no cross-desensitization between glycine and GABA-induced currents. On the other hand, α- and β-amino acid-induced currents had strong inhibitory effects on the succeeding glycine-induced current, indicating cross-desensitization.

Discussion

The responses induced by glycine, Tau, and other active α- and β-amino acids were preferentially inhibited by the specific glycine receptor antagonist, strychnine (Curtis *et al.*, 1968; Graham *et al.*, 1985). On the other hand, the selective $GABA_A$ receptor antagonist, bicuculline (Curtis *et al.*, 1971; Akaike *et al.*, 1985; Yakushiji *et al.*, 1987), the Cl^- channel blocker, picrotoxin (Constanti, 1978; Yakushiji *et al.*,

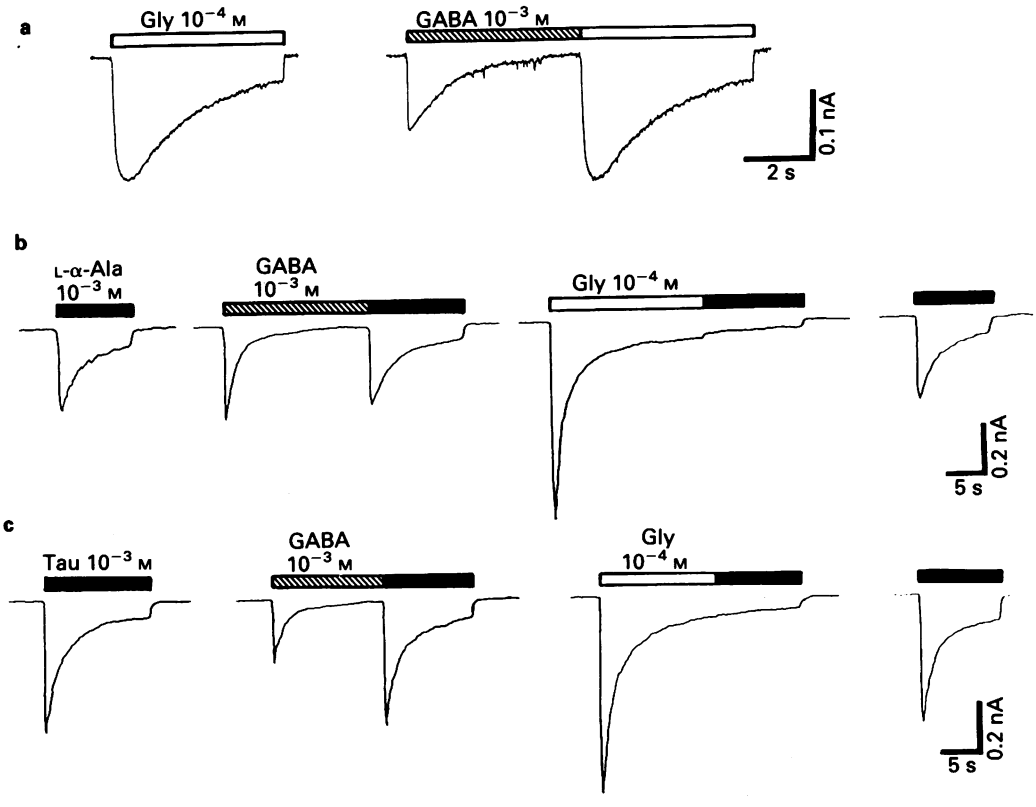


Figure 5 Interaction between responses induced by α-, β- and γ-amino acids at a V_H of −70 mV. (a) Effect of γ-aminobutyric acid (GABA) pretreatment on glycine (Gly)-induced current. (b) Effect of GABA or glycine pretreatment on (L-α-Ala)-induced current. (c) Effect of GABA or glycine pretreatment on taurine (Tau)-induced current. (a), (b) and (c) were obtained from different cells. Open bars: glycine treatment; hatched bars: GABA treatment; solid bars: L-α-Ala (b) or taurine (c) treatment.

1987) and the Tau receptor antagonist, TAG (Martin *et al.*, 1981; Yarbrough *et al.*, 1981; Okamoto *et al.*, 1983) failed to antagonize the α- and β-amino acid-induced I_{Cl}. These results indicate that the above effects induced by glycine, Tau and the active α- and β-amino acids are all mediated through the strychnine-sensitive glycine receptor.

On the basis of the values obtained for I_{max} and the efficacy of every amino acid (Figure 2), the relative order of activity was estimated as glycine > β-Ala > Tau > L- and D-α-Ala > L-Ser. Ionophoretic application of glycine and related amino acids depressed the firing of spinal dorsal horn interneurones, Renshaw cells and cortical neurones of cat (Curtis *et al.*, 1968). The potency-order was glycine > β-Ala > Tau > L-α-Ala > L-Ser. This similarity may also indicate that the glycine receptors in rat isolated VMH neurones are likely to be identical to the strychnine-sensitive glycine receptor in the previous reports.

In these experiments, pretreatment with GABA did not affect the successive glycine-induced response but pretreatment with glycine inhibited the successive α- and β-amino acid-induced responses. In VMH neurones the glycine-induced responses are mediated by Cl^- and there is no significant shift of E_{Cl} during glycine application (Akaike & Kaneda, unpublished observation). Furthermore, the GABA-induced response is also mediated by Cl^- (not shown). If the shift of E_{Cl} occurs because of pretreatment with GABA, successive glycine-induced response should be smaller than the glycine-induced response without pretreatment with GABA. But as shown in Figure 5, the pretreatment with GABA did not affect the successive glycine-induced response, indicating that there is no significant shift of E_{Cl} during GABA application. These results indicate that the observed inhibitory effects of α- and β-amino acid-induced currents by pretreatment with glycine are due to cross-desensitization between

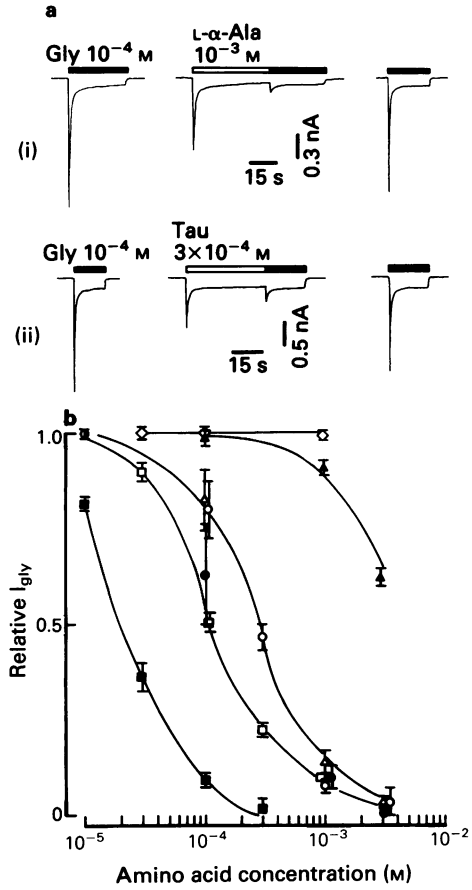


Figure 6 Effects of pretreatment with α-, β- and γ-amino acids on glycine-induced current. (a) Pretreatments with taurine (Tau) and L-α-alanine (L-α-Ala) decreased the glycine-induced current: (i) and (ii) were obtained from different cells. (b) Inhibitory effect of pretreatment with various amino acids on the glycine-induced response. Abscissa scale: concentration of amino acids during pretreatment. Ordinate scale: relative current of glycine (I_{Gly}) after pretreatment with various amino acids. Relative I_{Gly} was normalized to the control response induced by 10^{-4} M glycine alone: (○) L-α-alanine; (●) D-α-alanine; (△) L-serine; (▲) D-serine; (□) taurine; (◇) GABA and (■) β-alanine. Each point is the average of 4–7 neurones and bars indicate s.e.mean.

glycine and other amino acids. On the basis of the above results we evaluated the relationship between structure and activity of both α- and β-amino acids and several glycine derivatives on strychnine-sensitive glycine receptor. Figure 8 summarizes the structures of the various agonists used.

β-Amino acids (β-Ala and Tau) were more potent than the α-compounds in depressing the glycine response by cross-desensitization. β-Amino acids are

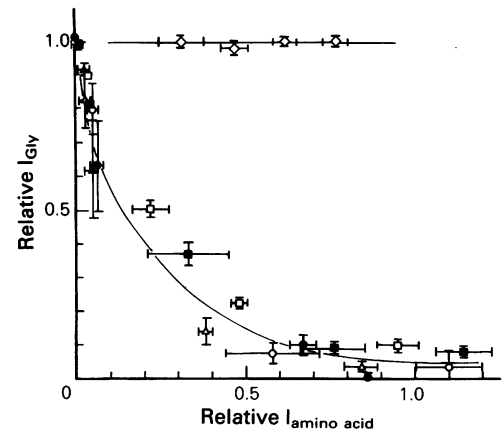


Figure 7 Cross-desensitization between glycine- and other amino acids. Abscissa scale: relative current induced by pretreatment with amino acids normalized to the control response of 10^{-4} M glycine. Ordinate scale: relative I_{Gly}, which followed the pretreatment with various amino acids, normalized to the control response of 10^{-4} M glycine. Data were quoted from Figure 6b; symbols as in Figure 6b.

regarded as being more potent agonists on the glycine receptor than α-amino acids except for glycine itself. As shown in Figure 8, β-amino acids are longer than the α-amino acids by a distance of one methylene group between the amino and the acidic group. It is therefore unlikely that β-amino acids act on the glycine receptor in an extended conformation. Construction of β-Ala and Tau molecules, using the C.P.K. model (space field model), can give a folded conformation with similar spatial positions for the amino group and the acidic group as those in the glycine molecule (not shown). Thus, we suggest that β-amino acids act in a folded conformation.

For the α-amino acids, including glycine, it is evident from the present study that D-Ser, L-Cys and 2-AIBA lack activity at the glycine receptor. The α-amino acids show structural variations in the functional group on the second position in the molecule. Both optical isomers of α-Ala, Ser and Cys have a methyl, methanol and methanethiol group on the second position, respectively. Glycine has no functional group on the second position, while 2-AIBA has two methyl groups (see Figure 8). Thus it is suggested that the functional group on the second position may decrease the affinity of the amino acid for the glycine receptor by steric hindrance around the second carbon in the molecule. 2-AIBA and L-Cys, which have two methyl groups and a methanethiol group, respectively, seemed to lack activity because of their large steric hindrance.

The L- and D-isomers of α-Ala were equipotent, while D-Ser was completely inactive, although L-Ser

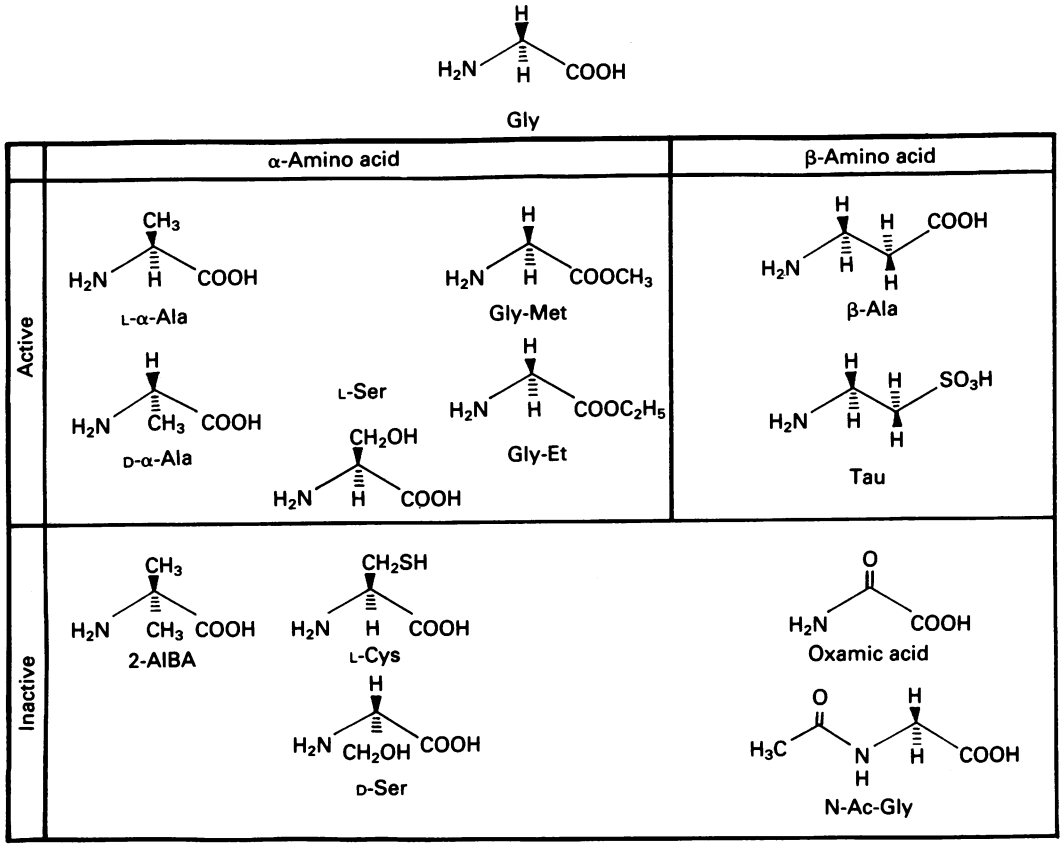


Figure 8 Chemical structures of the amino acids and their derivatives used in this study. Note the different functional group on the second carbon in different α-amino acids and the difference in molecular length between α-amino acids and β-amino acids.

was a weaker agonist. This means that the glycine receptor can discriminate between the L- and D-isomers of Ser. This discrimination involves other stereo-chemical factors, besides steric hindrance, because both optical isomers of Ser give an equivalent steric hindrance on the second C-position, analogous to L- and D-α-Ala. One of the strong modulating factors in intermolecular interactions is hydrogen bonding. Hydroxyl and thiol groups, in general, can form a hydrogen bond with carboxyl groups etc. The difference in potency between the L- and D-isomers of Ser might be attributed to hydrogen bond formation between the methanol group on the second position and the carboxyl group which probably exists in the glycine receptor protein (see Figure 8).

On the basis of the above discussion, we propose a tentative model for the specificity of the glycine receptor. The glycine receptor protein may be operated by three binding sites in its ligand-receptive zone. Two of them are thought to contribute to the usual receptor binding by glycine and other active amino acids, attracting their amino and carboxyl groups. This would relate to receptor activation. The third site would seem to limit the binding of α-amino acids to the glycine receptor, depending on the functional group on its second position. It provides the specificity for glycine as the strongest ligand for the glycine receptor among the active amino acids which are supposed to act directly on the glycine receptor, because glycine has no functional group in the second position. In the case of Ser, the third binding site may attract the methanol group by forming a hydrogen bond. This attraction and the steric hindrance around the second carbon would make L-Ser a weaker agonist than glycine and L- and D-α-Ala. And the great difference in activity between L- and D-Ser, D-Ser being inactive although the L-isomer is a weak agonist, may indicate an asymmetric localization of the third binding site in the glycine receptor protein. It may limit the binding of D-Ser to the conventional binding site more strongly than that of

L-Ser. For Cys there is the likelihood of hydrogen bond formation and steric hindrance by the methanethiol group on the second position. Both of these stereo-chemical factors are stronger in Cys than in Ser, making even the L-isomer of Cys inactive. Relatively strong binding activity of D-Ser for the glycine receptor was reported in rat brain slices (Kishimoto *et al.*, 1981). In the present study, D-Ser failed to activate the glycine receptor in the rat isolated CNS neurone. The relatively strong affinity of D-Ser for the glycine receptor may be related to the third binding site we propose here.

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Heterogeneity of nucleoside transport inhibitory sites in heart: a quantitative autoradiographical analysis

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1 The distribution of nucleoside transport inhibitory sites in rat and guinea-pig cardiac sections was investigated by use of [^{3}H]-nitrobenzylthioinosine ([^{3}H]-NBMPR) autoradiography. The distribution of these sites was heterogeneous in guinea-pig sections and homogeneous in rat sections.

2 The areas of high density of nucleoside transport inhibitory sites found in guinea-pig cardiac sections were compared to the distribution of an endothelial cell marker, von Willebrand Factor, determined by radioimmunocytochemistry. These two markers were co-localized suggesting that coronary endothelial cells from guinea-pig have a high density of NBMPR binding sites and thus may have a high nucleoside transport capacity.

3 Nucleoside transporter subtypes with differing affinity for NBMPR or dipyridamole were investigated by quantitative autoradiography. Sites in rat tissues had high affinity for NBMPR (K_D = 0.6 nM) but were of low sensitivity to dipyridamole (K_i = 3.1 μM). In guinea-pig sections, areas of high and low [^{3}H]-NBMPR binding site density were analyzed separately. In both areas, sites had high affinity for NBMPR (K_D = 1.4 nM, 4.5 nM, respectively) and dipyridamole (K_i = 108 nM, 245 nM, respectively).

4 While differences in density of nucleoside transport inhibitory sites are detectable between distinct regions of the heart, subtypes differing in affinity for NBMPR or dipyridamole were not evident. Therefore, more detailed structure activity studies are required to determine if subtypes of these sites exist within a single heart.

Introduction

Adenosine is an endogenous nucleoside that exerts important regulatory effects on cardiovascular function. At least two cell surface adenosine receptors have been recognised; stimulation of the A_1 adenosine receptor subtype results in negative inotropic, chronotropic and dromotropic effects in the heart while A_2 adenosine receptor activation causes vascular smooth muscle relaxation. The coronary vasculature is particularly sensitive to adenosine and it has been proposed that adenosine, released from cells during periods of hypoxia and high metabolic demand, could be an important link in regulating coronary blood flow and oxygen delivery to cardiac cells (Berne, 1980). In addition, adenosine may serve as an endogenous regulator of cardiac excitability and contractility (Dobson & Fenton, 1983). The cardiovascular effects of adenosine have been exploited therapeutically for the management of supraventricular tachycardia (DiMarco *et al.*, 1983) and for the production of controlled hypotension during anaesthesia (Sollevi *et al.*, 1984).

Adenosine is rapidly cleared from the vicinity of its cell surface receptors by rapid uptake systems consisting of membrane-located nucleoside transport mechanisms and subsequent intracellular metabolism. Facilitated diffusion nucleoside transport systems have been characterized in many cell types, including cardiac myocytes, and can be inhibited by pharmacologically diverse groups of drugs including coronary vasodilators, calcium channel blockers and benzodiazepines (Williams *et al.*, 1984; Hammond *et al.*, 1985; Ford *et al.*, 1985). Many of the so-called coronary vasodilator drugs, for example dipyridamole and dilazep, are believed to exert some of their therapeutic effects by interacting with transport inhibitory sites associated with the nucleoside transport mechanism. In this way, adenosine influx is inhibited and its levels close to adenosine receptor sites are sustained, leading to potentiation and prolongation of adenosine-mediated effects.

At present, it has not been determined where transport inhibitors bind on nucleoside transport complexes. Jarvis & Young (1982) proposed a model where inhibitors bound to the permeation site to produce inhibition of nucleoside transport; however, Koren *et al.* (1983) proposed an allosteric interaction

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between inhibitors binding to one site and producing inhibition of nucleoside transport at a separate site. In light of this controversy, we have adopted the term nucleoside transport inhibitory sites to refer to sites, occupancy of which inhibits nucleoside permeation.

The study of drug interactions with nucleoside transport mechanisms has been facilitated by the use of a high affinity probe, [^{3}H]-nitrobenzylthioinosine ([^{3}H]-NBMPR) (Cass *et al.*, 1974). NBMPR and congeners bind with high affinity and selectivity to membrane-located sites in many cell types including erythrocytes from various species and cardiac myocytes. Occupation of these sites inhibits nucleoside transport and inhibition constants of drugs for the inhibition of [^{3}H]-NBMPR binding are predictive of their transport inhibitory activity (Williams *et al.*, 1984).

Subtypes of facilitated diffusion nucleoside transport systems have recently been identified; systems of high or low sensitivity to NBMPR are present in several cloned and uncloned lines of cultured cells (Paterson *et al.*, 1987) and these systems may coexist in some cell types. Systems of high and low sensitivity to dipyridamole are detectable in guinea-pig and rat myocytes, respectively (Heaton & Clanachan, 1987; Clanachan *et al.*, 1987). However, it is not known if subtypes exist either in the same cell or in the different cellular elements within the heart. The discovery of regional differences in transporter distribution or regional subtypes differing in drug affinity would have important implications in the understanding of the mechanisms of adenosine efflux (production) and influx (inactivation). Selective inhibition of nucleoside transporter subtypes might allow the selective modulation of the regulatory actions of adenosine.

In this study we have determined NBMPR binding constants in different regions of guinea-pig and rat heart using quantitative autoradiography. This procedure also permitted the measurement of the affinity of dipyridamole for NBMPR sites in order to examine potential heterogeneity of transport inhibitory sites in these tissues.

Methods

Tissue preparation

Sprague-Dawley rats (male, 200–400 g) and Hartley guinea-pigs (male, 200–400 g) were killed by a blow to the head followed by exsanguination. The hearts were quickly removed and the ventricles were embedded in media (20 parts OCT Compound: 10 parts distilled water: 7 parts tragacanth gum), frozen in isopentane cooled with liquid nitrogen and stored at −70°C for up to two weeks. Transverse sections (10 μm) were cut (−22°C) with a cryostat (International Equipment Company model CTI), mounted onto gelatin-coated slides, desiccated overnight at 4°C and then used immediately or stored at −70°C for up to two weeks.

Biochemical analysis of [^{3}H]-NBMPR binding to tissue sections

The characteristics of [^{3}H]-NBMPR binding to tissue sections on microscope slides were determined for the purpose of subsequent autoradiographical studies. Two microscope slides with two (guinea-pig) or three (rat) sections per slide were used for each determination. The sections were preincubated for 5 min in ice-cold phosphate buffered saline (PBS composition (mM): NaCl 137, Na_2HPO_4 6, KCl 2.7, KH_2PO_4 1.5, $CaCl_2$ 0.9, $MgCl_2$ 0.5; pH 7.4), then incubated with graded concentrations of [^{3}H]-NBMPR (0.05–10.0 nM) for 30 min at 20°C. Fixation of the tissue sections resulted from the addition of formalin to the incubation medium (final conc. 0.4%) for 10 min. This method of fixation was found not to alter the total or non-specific binding. The tissue sections were washed twice for 5 min in ice-cold PBS then quickly rinsed three times in ice-cold distilled water. Non-specific binding of [^{3}H]-NBMPR was determined in alternate slides processed as above, but incubated with [^{3}H]-NBMPR in the presence of dipyridamole (30 μM for guinea-pig sections or 100 μM for rat sections) or dilazep (100 μM).

Dipyridamole competition studies for guinea-pig sections were performed in the presence of 2.8–3.2 nM [^{3}H]-NBMPR and graded concentrations of dipyridamole (1 nM–30 μM). For rat sections, [^{3}H]-NBMPR was used at concentrations of 0.7–0.9 nM, dipyridamole concentrations ranged from 0.1 to 100 μM, and the non-specific binding was determined with 100 μM dilazep. Sections were wiped from the slides with Whatman GF/B filters and the radioactivity was counted (Beckman LS6800). For every six slides used as above, one was scraped with a razor blade and the tissue sections were assayed for protein content by the method of Lowry *et al.* (1951) with bovine serum albumin as the standard. The data were analyzed to yield estimates of the dissociation constants (K_D; nM), maximal numbers of binding sites (B_{max}; fmol mg^{-1} protein) and dipyridamole inhibition constants (K_i; nM or μM).

Autoradiographical analysis of [^{3}H]-NBMPR binding to tissue sections

Equilibrium binding assays and dipyridamole competition assays were performed as above with guinea-pig and rat slide-mounted tissue sections.

Instead of wiping the sections from the slides, they were blown dry with cold air in a cold room (4°C) and desiccated overnight. In addition, competition assays with inosine, a transporter permeant, were performed with guinea-pig sections in the presence of 3.2 nM [^{3}H]-NBMPR and graded concentrations of inosine (3 μM–3 mM). Tissue sections were apposed to Ultrofilm ^{3}H in x-ray cassettes and stored in the dark at 4°C for three weeks. Included with each film were two sets of 6 standards described below. The films were developed with Microdol X Developer for 10 min, rinsed with distilled water, fixed with Kodak Fixer for 5 min, and washed in running tap water for 20 min, all at 20°C.

Preparation of standards Radioactive standards were required to enable quantitation of autoradiograms; cardiac tissue was used in order to resemble the experimental sections as closely as possible particularly in terms of quenching radioactivity. Ventricular tissue was minced into 10 mg pieces, homogenized in 20 volumes (w/v) of ice-cold 0.32 M sucrose (Polytron, setting 5 for 20–30 s), filtered through 250 μm mesh then centrifuged for 10 min at 20,000 *g*. Graded concentrations of a nonvolatile tritiated compound, [^{3}H]-inulin, were thoroughly mixed with the ventricular paste to make six standard ^{3}H concentrations (Unnerstall *et al.*, 1981). These were frozen and 10 μm sections were cut and mounted on microscope slides in a similar manner to the ventricular tissue sections. Samples from each standard were dried to constant weight, dissolved in NCS tissue solubilizer (300 μl), neutralized with glacial acetic acid (9 μl) and d.p.m. mg^{-1} dry weight was determined for each standard by liquid scintillation spectrometry.

Small samples of guinea-pig heart were dried to constant weight, solubilized in 1 N NaOH and protein concentrations were determined by the method of Lowry *et al.* (1951) to determine mg protein mg^{-1} dry weight of heart tissue.

Quantitation of autoradiograms

A computer image analysis system (Imageplus, Scientific MicroPrograms) was used to determine optical densities of different areas of the autoradiograms (high and low [^{3}H]-NBMPR binding site density components in guinea-pig sections and low density component in rat cardiac sections). Each experimental condition consisted of a minimum of 8 tissue sections. Autoradiograms were coded and analyzed in random order by an individual who was unaware of the experimental condition.

In order to convert the computer-derived optical density to the more meaningful unit of fmol mg^{-1} protein, a standard curve (ln d.p.m. mg^{-1} dry weight vs. ln optical density) was prepared for each sheet of Ultrofilm ^{3}H used, by the method of Unnerstall *et al.* (1982). Experimental values were converted to fmol mg^{-1} protein using the specific activity of the ligand and the value of mg dry weight mg^{-1} protein (determined as outlined above).

Von Willebrand Factor radioimmunocytochemistry

Von Willebrand Factor is synthesized by endothelial cells and is a reliable marker for these cells. Immunoreagents for von Willebrand Factor were used in conjunction with [^{3}H]-biotin to localize endothelial cells by autoradiography by use of a modification of the method described by Hunt & Mantyh (1984). Guinea-pig ventricular sections (10 μm) were preincubated for 5 min in ice-cold PBS. This was followed by 10 min in 0.4% formaldehyde in ice-cold PBS and two subsequent 5 min washes in ice-cold PBS. Sections were then incubated in presoak buffer (5% bovine serum albumin (BSA), 0.05% NaN_3, 0.3% Triton X100 in PBS) for 1 h at 20°C. Washes, at this and later steps, were performed with 1% ABS and 0.05% NaN_3 in PBS, 2 × 5 min at 20°C. Sections were then incubated with the primary antibody, rabbit polyclonal antibody to human von Willebrand Factor (1 : 1600 dilution in 1% BSA, 0.05% NaN_3, 0.3% Triton X100 in PBS), for 1 h at 20°C. Sections were washed, then incubated with the secondary antibody, biotinylated goat anti-rabbit IgG (1 : 400 dilution in the same buffer used for primary antibody), for 1 h at 20°C. Sections were washed again then incubated with [^{3}H]-biotin (15 μCi) and avidin (10 μg) (previously mixed for 20 min at 20°C in 1.5 ml 1% BSA, 0.05% NaN_3 in PBS and diluted to 30 ml) for 20 min at 20°C. Five washes of 10 min each were performed with the same wash buffer as previously. Sections were then blown dry, at 4°C, desiccated and apposed to Ultrofilm ^{3}H.

Specific binding to von Willebrand Factor was determined by the difference between sections incubated with and without the primary antibody: nonspecific binding of avidin-[^{3}H]-biotin complex was determined from sections not exposed to primary or secondary antibodies.

Materials

Compounds and reagents for these studies were obtained from the following sources: [G-^{3}H]-nitrobenzylthioinosine (sp. act. 37 Ci mmol^{-1}), from Moravek Biochemicals, CA, U.S.A. and repurified as necessary before use; dipyridamole, inosine and avidin, from Sigma Chemical Co., St. Louis, MO, U.S.A.; [^{3}H]-inulin (sp. act. 3 Ci mmol^{-1}), from Amersham Canada Ltd., Oakville, Ont.; α-[8,9-

^{3}H(N)]-biotin (sp. act. 33 Ci mmol^{-1}), from New England Nuclear, Boston, MA, U.S.A.; rabbit polyclonal anti-human von Willebrand Factor, biotinylated anti-rabbit IgG and horseradish peroxidase avidin D, from Dimension Laboratories Inc., Mississauga, Ont. Canada; Ultrofilm ^{3}H, from Fisher Scientific, Edmonton, Alta, Canada; OCT Compound from Canlab, Edmonton, Alta, Canada. Dilazep was donated by Hoffman-LaRoche.

Results

Biochemical analysis of [^{3}H]-NBMPR binding to tissue sections

Site-specific binding of [^{3}H]-NBMPR to slide-mounted sections of guinea-pig and rat ventricles was saturable. Scatchard plots were linear (Figure 1) and gave NBMPR binding constants, K_D (geometric mean and 95% confidence interval) and B_{max} ± s.e.mean, of 3.2 (1.4–7.1) nM and 931 ± 176 fmol mg^{-1} protein, respectively, for guinea-pig ($n = 4$) and 0.7 (0.5–1.0) nM and 419 ± 60 fmol mg^{-1} protein, respectively, for rat ($n = 3$). These values are comparable to those observed in ventricular myocytes (Clanachan *et al.*, 1987) and in crude ventricular membranes (Williams *et al.*, 1984) prepared from these species. To ensure that the binding in ventricular sections involved the same site that had been characterized previously in myocytes and membrane preparations, we evaluated the affinity of dipyridamole in competition experiments with [^{3}H]-NBMPR. The K_i values obtained were 70 nM for guinea-pig and 3 μM for rat and the Hill coefficients were 0.81 and 0.91 for guinea-pig and rat ventricular sections, respectively (Figure 1).

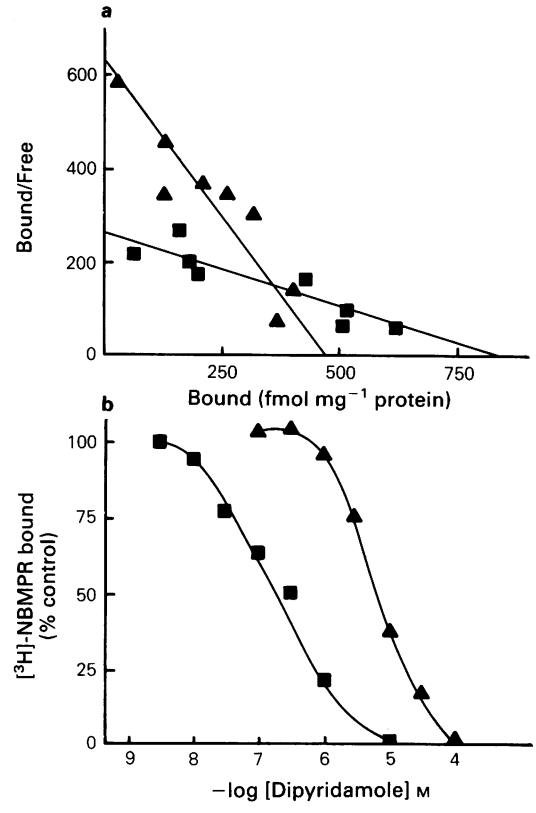


Figure 1 Analysis of [^{3}H]-NBMPR binding to guinea-pig (■) and rat (▲) cardiac sections. Sections were wiped from microscope slides and associated tritium was determined by scintillation spectrophotometry. (a) Scatchard analysis of specific binding. Abscissa scale: site-specific [^{3}H]-NBMPR bound (fmol mg^{-1} protein). Ordinate scale: site-specific bound [^{3}H]-NBMPR (fmol mg^{-1} protein)/free [^{3}H]-NBMPR (nM). Best fit regression lines were drawn through the points of single representative experiments. (b) Concentration-dependent inhibition by dipyridamole of specific [^{3}H]-NBMPR binding. Abscissa scale: $-\log_{10}$ [dipyridamole]M. Ordinate scale: [^{3}H]-NBMPR bound (% control in the absence of dipyridamole). Data points are from single representative experiments.

Autoradiographical localization

Autoradiography was performed to localize the total and non-specific [^{3}H]-NBMPR binding components in guinea-pig and rat ventricular sections. The difference between the total binding and non-specific binding autoradiograms represents site-specific binding. In guinea-pig tissue sections, the specific binding had a non-uniform distribution which consisted of high and low density components (Figure 2a,b). The high density binding component appeared to be associated with the coronary vasculature and endocardial lining. In contrast, a uniform distribution was evident in the total binding and non-specific binding autoradiograms for rat ventricular sections, indicating a homogeneous specific binding component (Figure 2e,f). Histological examination of rat sections used for autoradiography indicated that endothelial cells were present and had not been destroyed during the experiments.

Von Willebrand Factor radioimmunocytochemistry

Guinea-pig cardiac sections incubated with primary antibody to von Willebrand Factor followed by secondary antibody and then avidin-[^{3}H]-biotin

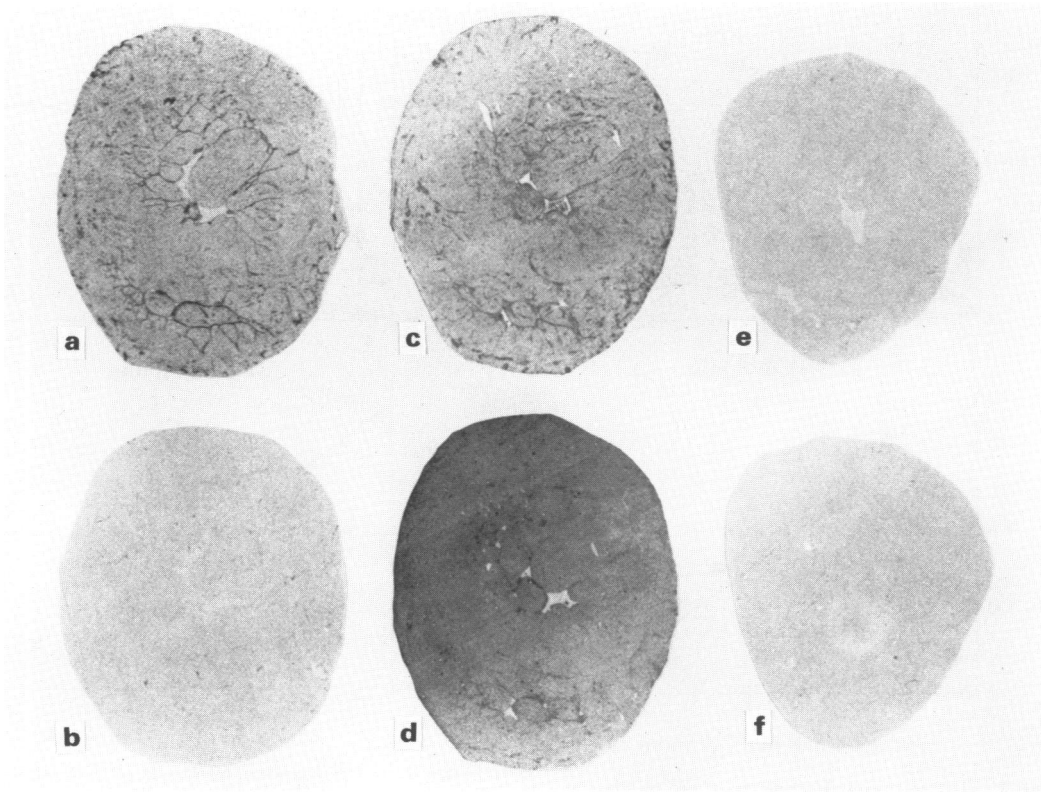


Figure 2 Autoradiographical localization of [^{3}H]-NBMPR binding sites in guinea-pig cardiac sections (a, b; 8 nM) compared to von Willebrand Factor (c, d) and [^{3}H]-NBMPR binding sites in rat cardiac sections (e, f; 2 nM). The difference between the total (a, e) and non-specific (b, in the presence of 30 μM dipyridamole and f, in the presence of 100 μM dilazep) represents the specific binding of [^{3}H]-NBMPR. Immunoreagents for von Willebrand Factor in conjunction with [^{3}H]-biotin were used to produce autoradiograms of endothelial cells (c) or control (d, in the absence of primary antibody). Non-specific binding of avidin-[^{3}H]-biotin is responsible for the high background (c, d). Rat sections that had been used for autoradiography were examined histologically and no evidence of destruction or removal of endothelial cells was found.

complex gave rise to autoradiograms such as that depicted in Figure 2c. Autoradiograms resulting from sections incubated in the secondary antibody and then in avidin-[^{3}H]-biotin complex are represented by Figure 2d. Autoradiograms were also produced from slides incubated only with avidin-[^{3}H]-biotin (data not shown) and a high non-specific association of this complex was apparent. Further experiments were performed with avidin-peroxidase to investigate this high non-specific association. Similar results (data not shown) were obtained with and without secondary antibody incubations prior to avidin-peroxidase incubations. Therefore the non-specific association of avidin to cardiac tissue caused the high non-specific binding of avidin-[^{3}H]-biotin complex (Figure 2d). The pattern produced by [^{3}H]-biotin specific to the endothelial cell-located von Willebrand Factor (Figure 2c) is similar to the high density [^{3}H]-NBMPR binding component in these tissue sections (Figure 2a).

Quantitative autoradiography of [^{3}H]-*NBMPR binding to tissue sections*

Computer image analysis of the autoradiograms from guinea-pig ventricular sections allowed the determination of NBMPR binding constants of the high and low binding site density components. K_D and B_{max} determinations were 1.4 nM and 1751 fmol mg^{-1} protein, respectively, for the high site density component and 4.5 nM and 990 fmol mg^{-1} protein, respectively, for the low site density component (Figure 3).

Similar analysis of rat verticular sections, that possessed a uniform binding site distribution, provided

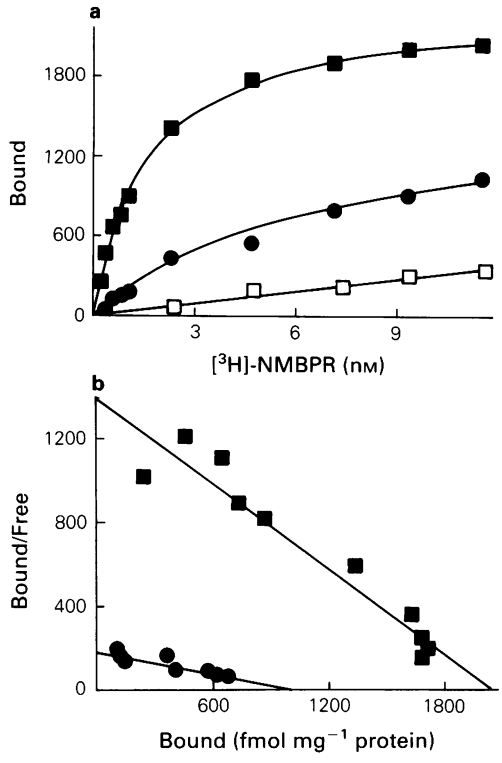


Figure 3 Concentration dependence of the binding of [^{3}H]-NBMPR to guinea-pig cardiac sections (a). Computer image analysis allowed the separation of the high density areas (■, total binding) associated with the vasculature, from the low density areas (●, total binding) representative of the cardiac myocytes. Non-specific binding (□) is linear with concentration. Abscissa scale: free [^{3}H]-NBMPR concentration (nM). Ordinate scale: bound [^{3}H]-NBMPR (fmol mg^{-1} protein). Points represent means of four sections from a single representative experiment. Non-specific line is a regression line through the points. Scatchard analysis (b) of specific binding was used to determine [^{3}H]-NBMPR binding constants for the two areas. Abscissa scale: site-specific bound [^{3}H]-NBMPR (fmol mg^{-1} protein). Ordinate scale: site-specific bound [^{3}H]-NBMPR (fmol mg^{-1} protein)/free [^{3}H]-NBMPR concentration (nM). Lines are best-fit regression lines through the points; data correspond to (a).

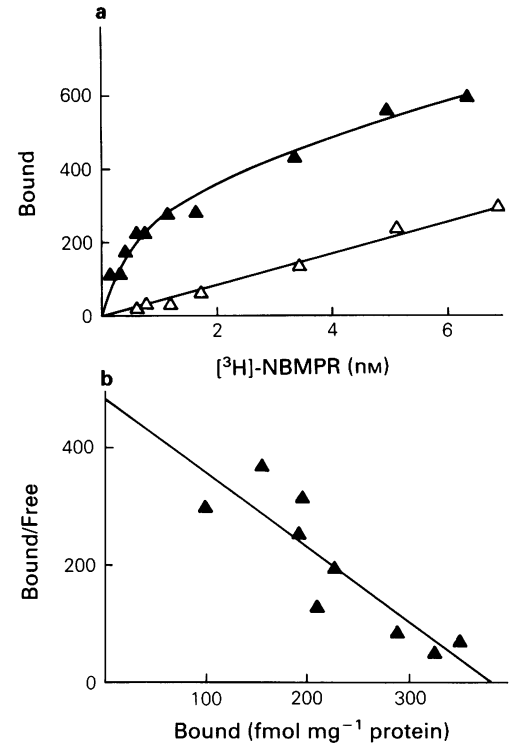


Figure 4 Concentration-dependence of the binding of [^{3}H]-NBMPR to rat cardiac sections (a). Computer image analysis was used to measure total (▲) and non-specific (△) binding. Abscissa scale: free [^{3}H]-NBMPR concentration (nM). Ordinate scale: bound [^{3}H]-NBMPR (fmol mg^{-1} protein). Points represent means of four sections from a single representative experiment. Non-specific line is a regression line through the points. Scatchard analysis of specific binding (b) was used to determine [^{3}H]-NBMPR binding constants. Abscissa scale: site-specific bound [^{3}H]-NBMPR (fmol mg^{-1} protein). Ordinate scale: site-specific bound [^{3}H]-NBMPR (fmol mg^{-1} protein)/free [^{3}H]-NBMPR concentration (nM). Line is best-fit regression line through the points; data correspond to (a). The scatter in the points around the line is due to the high affinity and low binding site density for [^{3}H]-NBMPR in rat sections.

NBMPR binding constants, K_D and B_{max}, of 0.6 nM and 526 fmol mg^{-1} protein, respectively (Figure 4).

Dipyridamole inhibited NBMPR binding with K_i estimates of 108 nM and 245 nM for the high and low density binding components, respectively, of guinea-pig ventricular sections and 3.1 μM for rat ventricular sections (Figure 5). Inosine inhibited NBMPR binding to the high and low density components of guinea-pig ventricular sections with K_i estimates of 179 μM and 238 μM, respectively (Figure 6).

Discussion

The distribution of NBMPR binding sites in cardiac sections was analyzed by autoradiography and a heterogeneous distribution in guinea-pig and homoge-

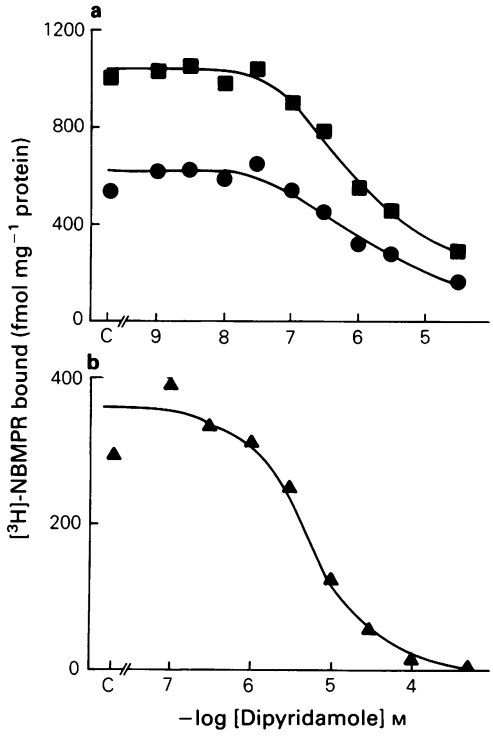


Figure 5 Concentration-dependent inhibition of total [^{3}H]-NBMPR binding by dipyridamole in guinea-pig (a; 3.7 nM [^{3}H]-NBMPR) or rat (b; 0.8 nM [^{3}H]-NBMPR) cardiac sections. Computer image analysis allowed the separation of the high (■) and low (●) [^{3}H]-NBMPR binding site density components found in guinea-pig sections. Abscissae: $-\log_{10}$[dipyridamole] M. Ordinates: Total [^{3}H]-NBMPR bound (fmol mg^{-1} protein). Points are means of four sections from single representative experiments.

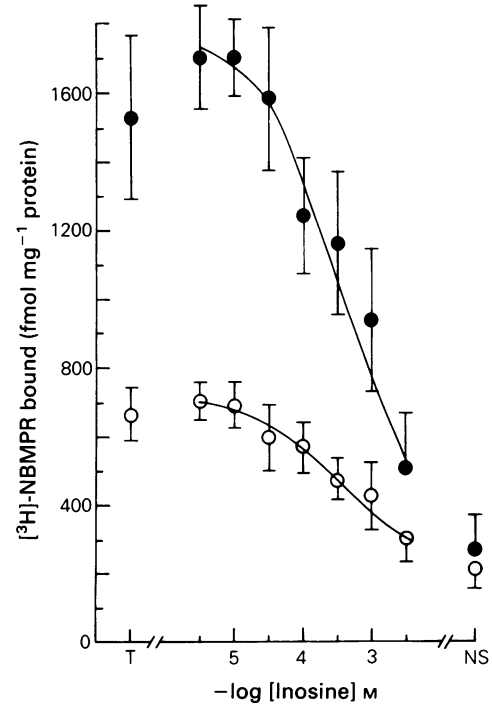


Figure 6 Concentration-dependent inhibition of total [^{3}H]-NBMPR (3.2 nM) binding by inosine in guinea-pig cardiac sections. Computer image analysis allowed the separation of the high (●) and low (○) [^{3}H]-NBMPR binding site density components. Abscissa scale: $-\log_{10}$[inosine] M. Ordinate scale: total [^{3}H]-NBMPR bound (fmol mg^{-1} protein). Points are means of four sections from single representative experiments.

neous distribution in rat sections was found. Comparison of the high density NBMPR binding site distribution with that for von Willebrand Factor indicated endothelial cells in guinea-pig heart have a high density of NBMPR sites.

Biochemical analysis of guinea-pig sections resulted in a K_D value (3.2 nM) for NBMPR binding that was similar to values obtained for the high (1.4 nM) and low (4.5 nM) binding site areas analyzed by quantitative autoradiography. The B_{max} value obtained for the biochemical analysis (931 fmol mg^{-1} protein) was equivalent to that obtained for the low binding site density areas (990 fmol mg^{-1} protein) and approximately half that obtained for the high binding site density areas (1751 fmol mg^{-1} protein) analyzed by quantitative autoradiography. Since endothelial cells account for only 3% of heart volume (Nees *et al.*, 1985), it is not unexpected that the B_{max} for the biochemical analysis approximates that for the low binding site density areas. Dipyridamole inhibition constants were similar when determined by biochemical analysis or quantitative autoradiography of the high and low binding site density areas. Both analytical techniques used 10 μm guinea-pig ventricular sections but while quantitative autoradiography separated the two binding areas, biochemical analysis provided values representative of all cell types averaged proportionately together.

Inosine inhibition constants were similar for the high (179 μM) and low (238 μM) binding site density areas, as determined by quantitative autoradiography. These K_i values for inhibition of NBMPR binding by inosine are typical of nucleoside transport systems of broad substrate specificity (Plagemann & Wohlhüeter, 1980).

Analysis of rat cardiac sections by the biochemical method and by quantitative autoradiography provided NBMPR binding constants that were similar

for the two methods: K_D values of 0.7 nM and 0.6 nM, respectively, and B_{max} values of 419 fmol mg^{-1} protein and 526 fmol mg^{-1} protein, respectively. Inhibition of NBMPR binding by dipyridamole also provided similar values for the two methods: K_i values of 3 μM by biochemical analysis and 3.1 μM by quantitative autoradiography. This provides further evidence that rat and guinea-pig cardiac tissue possess high affinity NBMPR sites that differ markedly in affinity for dipyridamole.

The species differences evident from this study are similar to those reported for cardiac membranes. Isolated cardiac myocytes from these species have also been investigated (Clanachan *et al.*, 1987) and the maximum NBMPR binding site density was two fold greater in guinea-pig myocytes than in rat myocytes. This corresponds to the difference in density between the low binding site density areas in guinea-pig cardiac sections and rat cardiac sections described in this study. This supports the interpretation that the low binding site density areas in guinea-pig sections are representative of cardiac myocytes.

A comparison of the distributions of von Willebrand Factor and the high density NBMPR binding component in consecutive sections of guinea-pig ventricles indicated co-localization of these markers. Von Willebrand Factor, a component of clotting Factor VIII required for platelet aggregation and attachment, is synthesized and released by endothelial cells and thus serves as a specific marker for endothelial cells (McComb *et al.*, 1982; Jaffe, 1984). Specifically, in heart tissue von Willebrand Factor has been localized in surface endocardial cells and coronary vascular endothelium (McComb, 1984). The co-localization of von Willebrand Factor and the high density NBMPR binding component found in this study indicates that guinea-pig ventricular endothelial cells have a high density of NBMPR binding sites and nucleoside transport sites.

Significant endothelial cell uptake of adenosine has been described in canine skeletal muscle (Gorman *et al.*, 1986), guinea-pig heart (Nees *et al.*, 1985; Sparks *et al.*, 1985), cultured guinea-pig coronary endothelium (Nees *et al.*, 1985) and cultured pig aortic (Pearson *et al.*, 1978; 1983) and pulmonary (Pearson & Gordon, 1985) endothelial cells. However, these studies did not distinguish between transport of adenosine into cells and subsequent metabolism. From our studies it would appear that guinea-pig coronary endothelial cells have a high capacity for nucleoside transport due to the high density NBMPR binding sites. However without kinetic data on membrane fluxes of nucleosides in these cells, their true capacity for nucleoside transport is not known.

Nucleoside transport systems differ among species and tissues; facilitated diffusion systems have usually been subclassified on the basis of inhibitor sensitivity. Rat tissues have been shown to be less sensitive to dipyridamole than tissues from other species, particularly guinea-pig, when adenosine potentiation (Hopkins & Goldie, 1971), inhibition of nucleoside transport or inhibition of NBMPR binding is measured (Hammond & Clanachan, 1985; Clanachan *et al.*, 1987). Additionally, some CNS membrane preparations with apparently uniform populations of NBMPR binding sites show biphasic dipyridamole inhibition of NBMPR binding (Hammond & Clanachan, 1984; 1985). This suggests two or more forms of the transporter which can be distinguished by dipyridamole but not by NBMPR. From this study it was apparent that the high and low density NBMPR binding components in guinea-pig heart were sensitive to dipyridamole.

While it has been clearly shown that NBMPR is a high affinity nucleoside transport inhibitor in rat and guinea-pig cardiac myocytes (Heaton & Clanachan, 1987; Clanachan *et al.*, 1987; Ford & Rovetto, 1987), it is possible that rat endothelial cells may have an NBMPR-insensitive transport component akin to transport systems of some cultured cells (Paterson *et al.*, 1987). For instance, rat erythrocytes possess both NBMPR-sensitive and -insensitive transport components (Jarvis, 1987) and these two systems may be co-localized on other cell types.

According to the adenosine hypothesis, adenosine is produced and released from cardiac cells during times of metabolic stress. It acts locally to produce many receptor-mediated effects that reduce excitability and contractility and increase oxygen supply (Berne, 1980; Dobson & Fenton, 1983). Subtypes of nucleoside transport systems among various regions of heart could have differing drug affinities thus allowing selective potentiation of some adenosine-mediated effects. Alternatively, regional differences in nucleoside transporter density could be important in controlling the rate of removal of adenosine from its sites of action. Nucleoside transport inhibitors would be predicted to exert greater potentiation of adenosine in areas of high transporter density. Therefore, regional differences in nucleoside transport systems, with respect to drug affinity or site density, could have important therapeutic implications.

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Inotropic and chronotropic effects of N^6-substituted derivatives of cyclic AMP as assessed in guinea-pig isolated right atria and papillary muscle

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1 The inotropic and chronotropic actions of N^6-substituted adenosine 3′:5′-cyclic monophosphate (cyclic AMP) derivatives (N^6-R cyclic AMPs) were studied in guinea-pig isolated right atrial preparations and in the papillary muscle preparations from guinea-pig right ventricle.

2 All the N^6-R cyclic AMPs except N^6-$C_{14}H_{29}$ produced positive inotropic effects in papillary muscle. The C_5H_{11}, C_6H_{13}, C_7H_{15}, C_8H_{17} and C_9H_{19} compounds were the most potent as inotropic agents, the potency being lower with compounds having longer or shorter N^6-side chains than these.

3 In right atria N^6-C_2H_5-C_7H_{15} cyclic AMPs produced negative chronotropic effects. However, after treatment of the preparations with 8-phenyltheophylline the negative chronotropic effects were either much attenuated or abolished, indicating the involvement of adenosine receptors.

4 All the N^6-R cyclic AMPs except N^6-$C_{14}H_{29}$ were more potent activators of bovine myocardial protein kinase than cyclic AMP. The partition coefficients between octanol and an aqueous phase of N^6-R cyclic AMPs became greater as the numbers of carbon atoms increased, and there appeared to be a relationship between partition coefficient and inotropic potency. It was concluded that membrane penetrativeness rather than potency as activators of protein kinase determined the potencies of N^6-R cyclic AMPs as positive inotropic agents.

5 Derivatives such as N^6-C_7H_{15} cyclic AMP, which have positive inotropic activity without any marked negative chronotropic effect, may be useful as cardiotonic agents in heart failure.

Introduction

Adenosine 3′:5′-cyclic monophosphate (cyclic AMP) plays an important role as the second messenger in the positive inotropic and chronotropic effects of catecholamines (Tsien, 1977; Opie, 1982). However, when administered from outside the cells this compound does not produce positive inotropic and chronotropic effects (James, 1965; Meinertz *et al.*, 1973), presumably because it is unable to cross the cell membrane. In contrast derivatives of cyclic AMP having lipophilic side chains, e.g. dibutyryl cyclic AMP (Skelton *et al.*, 1970; Imai *et al.*, 1974) and benzyl thiobutyl cyclic AMP (Evans *et al.*, 1976; Nakazawa *et al.*, 1985), have been shown to produce positive inotropic and chronotropic effects. Positive inotropic and chronotropic effects were also observed in this laboratory with a series of 8-substituted derivatives, while 2-substituted derivatives were found to be negative inotropic and chronotropic agents (Matsubara & Imai, 1975; Yamaji *et al.*, 1980). We have found that cyclic AMP derivatives with simple alkyl side chains at the N^6 position can produce a mixed response, i.e. positive inotropic and negative chronotropic effects. This paper describes in detail the actions of N^6-substituted cyclic AMPs (N^6-R cyclic AMPs) on isolated preparations of guinea-pig right atria and papillary muscle from guinea-pig right ventricle. The ultimate aim of these studies was to find derivatives, with a potent inotropic action coupled with weak chronotropic effects, with a potential for use as cardiotonic agents in the therapy of heart failure.

Methods

Measurement of inotropic and chronotropic effects on guinea-pig right atria and papillary muscle

Male albino guinea-pigs weighing 230–530 g were anaesthetized with ether. The hearts were rapidly

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removed and the right atria and the papillary muscle of the right ventricle were dissected out in a cold bathing solution. These were suspended individually in 10 ml organ baths for recording isometric contractions. The bathing solution was a Krebs-Henseleit solution (32.0 ± 0.1°C) containing (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $NaHCO_3$ 25, $MgSO_4$ 1.2, KH_2PO_4 1.2, glucose 11 and was continuously bubbled with 95% O_2 + 5% CO_2. Initial tensions of 0.25 and 0.1 g were applied to the preparations of right atria and papillary muscle, respectively. After 60 min equilibration, the optimal resting tension at which the active tension development was the maximum, was determined and maintained thereafter. Right atria were allowed to beat spontaneously, while papillary muscles were stimulated by square wave pulses of 1 ms duration at a frequency of 1 Hz and a voltage approximately 20% above the threshold, supplied by a square-wave pulse stimulator (Nihon Kohden MSE-3) via a pair of silver plate electrodes between which the preparations were placed. The isometric contraction was measured by a force-displacement transducer (Toyo Baldwin T7-30-240) connected to a carrier-amplifier (Sanei 1829), and the atrial rate was determined by a cardiotachometer (Sanei 1321). All measurements were recorded on a thermostylus recorder (Watanabe Sokki Linear Corder Mark V). Positive inotropic effects were expressed as a percentage of the maximum response evoked by 10^{-7} M isoprenaline on each preparation and chronotropic effects as a percentage of the values before administration of the test drug. Approximate ED_{30} values were determined (assuming a linear relationship between log concentrations and response in the range 20–80% of the maximum) and used as measures of the positive inotropic potencies.

Determination of the potency of N^6-R cyclic AMPs as activators of a cyclic AMP-dependent protein kinase

The cyclic AMP-dependent protein kinase activity was assessed on the basis of phosphorylation of histone essentially as described by Miyamoto *et al.* (1969) using the bovine myocardial cyclic AMP-dependent protein kinase. Briefly, the activity of the protein kinase was determined in 0.1 ml of an incubation mixture containing 50 mM HEPES (pH 7.0), 5 mM $MgCl_2$, 100 $\mu g\,ml^{-1}$ histone, 1 μM γ-[^{32}P]-ATP, 1 mM 3-isobutyl-1-methylxanthine, 10 mM NaF, 10 $\mu g\,ml^{-1}$ protein kinase enzyme and various concentrations (5–50 nM) of cyclic AMP or cyclic AMP derivative. After 7 min incubation at 30°C, 2 ml of 10% Cl_3CCO_2H was added and the incubation mixture kept at 0°C for 10 min. The mixture was then filtered under reduced pressure through a Whatman GF/C glass-fibre filter using an Automatic Cell Harvester (Labomash LH-101; Labo Science, Tokyo, Japan) and the filters were washed twice with 3 ml of 5% Cl_3CCO_2H. The amount of ^{32}P covalenty bound to histone was determined by counting the radioactivity remaining on the filters, with a liquid scintillation spectrophotometer, after addition of 5 ml of a Triton-toluene based scintillation fluid. The activity of cyclic AMP derivatives as activators of the cyclic AMP-dependent protein kinase was measured at a minimum of seven different concentrations in duplicate and K_m and V_{max} determined from a Lineweaver-Burk plot. The relative potency was estimated using the following equation:

$$\text{Relative potency} = \frac{K_m \text{ for cyclic AMP}}{K_m \text{ for } N^6\text{-R cyclic AMP}}$$

Determination of partition coefficients

Partition coefficients of N^6-R cyclic AMPs between octanol and the aqueous phase (0.01 M phosphate buffer, pH 7.0) were determined at 37°C. Both octanol and the buffer were saturated with the other phase before use. N^6-R cyclic AMPs were dissolved initially in buffer at a concentration of 0.1 mM, except $C_{14}H_{29}$ cyclic AMP, which was dissolved in octanol at the same concentration. Equal volumes (10 ml) of both phases were mixed and agitated for 30 min. The concentrations of N^6-R cyclic AMPs in the initial solution (A) and in the solution after partition (B) were measured by u.v.-spectroscopy (292 nm), and the partition coefficients (K) were calculated using the equation:

$$K = \frac{A - B}{B}$$

(for substances initially dissolved in buffer),

or

$$K = \frac{B}{A - B}$$

(for substances dissolved in octanol).

Materials

The following chemicals were used: histone (Worthington HLY), γ-[^{32}P]-ATP (7.0 Ci $mmol^{-1}$, New England Nuclear), (−)-isoprenaline (Nikken Kagaku Co.), 8-phenyltheophylline (Sigma Chemicals) and 3-isobutyl-1-methylxanthine (Sigma Chemicals). Bovine myocardial cyclic AMP-dependent protein kinase was purchased from Sigma Chemicals as a crude lyophilized powder. The structures of the N^6-R cyclic AMPs used are shown in Figure 1. These compounds were kindly supplied by the Kikkoman Corporation. The compounds with alkyl side chains of length C_7H_{15} or longer and

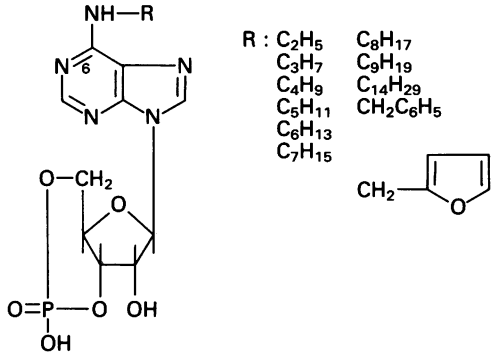


Figure 1 Chemical structures of the N⁶-R cyclic AMP derivatives used in the present study.

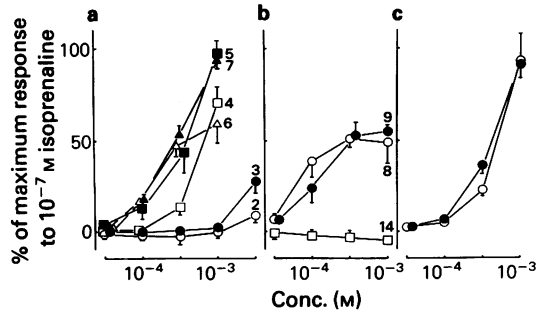


Figure 2 Inotropic effects of N⁶-R cyclic AMPs as assessed on guinea-pig papillary muscle. Points are means from five preparations with vertical lines indicating s.e.mean. In (a) (○) R = C_2H_5, (●) C_3H_7, (□) C_4H_9, (■) C_5H_{11}, (△) C_6H_{13}, (▲) C_7H_{15}. In (b), (○) R = C_8H_{17}, (●) C_9H_{19}, (□) $C_{14}H_{29}$. In (c), (○) R = furfuryl, (●) benzyl.

benzyl and furfuryl groups were supplied as water soluble sodium salts. The less soluble free acids were dissolved in Krebs-Henseleit solution and applied to the preparation by replacing less than 0.72 ml of the bathing solution.

Results

Activation of a cyclic AMP-dependent protein kinase

The relative potencies and the V_{max} of N⁶-R cyclic AMPs as activators of bovine myocardial cyclic AMP-dependent protein kinase are set out in Table 1. All the compounds except N⁶-$C_{14}H_{29}$ cyclic AMP were found to be more potent activators of the enzyme than cyclic AMP itself, while the V_{max} values were not significantly different from that of cyclic AMP.

Table 1 ED_{30} values for positive inotropic effects, relative potency and V_{max} for activation of the protein kinase, and the partition coefficient, K, of N⁶-R cyclic AMPs

		Protein kinase activation		
R	ED_{30} ($\times 10^{-4}$ M)	*Relative potency*	V_{max} *	K ($\times 10^{-3}$)
C_2H_5	> 30	3.5 ± 0.5	2.0 ± 0.1	2.2 ± 0.5
C_3H_7	> 30	2.3 ± 0.4	1.6 ± 0.1	4.8 ± 1.6
C_4H_9	4.46 ± 0.05	3.4 ± 0.1	2.2 ± 0.1	7.1 ± 0.3
	3.75 ± 0.49	2.7 ± 0.1	2.4 ± 0.2	6.0 ± 1.1
$CH_2C_6H_5$	2.48 ± 0.34	3.3 ± 1.0	2.1 ± 0.3	56 ± 2
C_5H_{11}	2.07 ± 0.52	3.5 ± 0.6	2.0 ± 0.1	63 ± 7
C_6H_{13}	1.89 ± 0.30†	2.5 ± 0.2	2.6 ± 0.1	67 ± 10
C_7H_{15}	1.60 ± 0.19	4.2 ± 0.4	2.3 ± 0.1	252 ± 8
C_8H_{17}	0.95 ± 0.21†	2.2 ± 0.9	3.6 ± 0.6	732 ± 20
C_9H_{19}	1.46 ± 0.33†	1.2 ± 0.5	4.7 ± 1.4	2620 ± 9
$C_{14}H_{29}$	> 10	**	**	77,500 ± 7900

Data shown are means ± s.e.mean. ED_{30} is the concentration required to produce 30% of the positive inotropic response of 10^{-7} M isoprenaline (n = 5).
Relative potency for activation of the cyclic AMP-dependent protein kinase is estimated using the following equation: relative potency = K_m for cyclic AMP/K_m for N⁶-R cyclic AMP.
K_m and V_{max} for cyclic AMP were 43.9 ± 7.9 nM (n = 6) and 3.5 ± 0.3 pmol ^{32}P incorporated into histone μg^{-1} protein min^{-1} (n = 6), respectively.
K is the oil/water partition coefficient (n = 4 or 6).
† partial agonist.
* pmol ^{32}P incorporated into histone μg^{-1} protein min^{-1}.
** No activation.

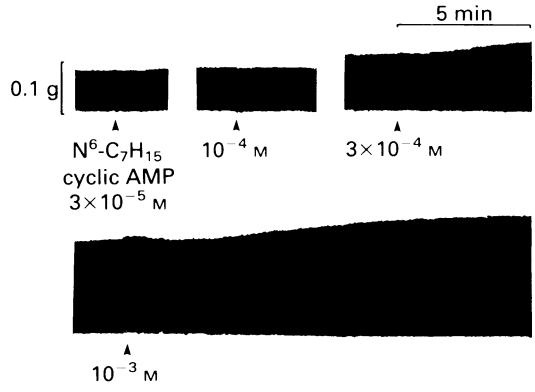


Figure 3 Time course of the positive inotropic effect of N^6-C_7H_{15} cyclic AMP on papillary muscle.

Inotropic and chronotropic effects of N^6-R cyclic AMPs

As shown in Figure 2, all the N^6-R cyclic AMP except N^6-$C_{14}H_{29}$ produced concentration-dependent positive inotropic effects in papillary muscle preparations in the dose range 3×10^{-5}–3×10^{-3} M. The maximal positive inotropic effects produced by some of these compounds equalled the maximal response evoked by 10^{-7} M isoprenaline, but N^6-C_6H_{13}, C_8H_{17} and C_9H_{19} were clearly partial agonists (Figure 2a and b). N^6-$C_{14}H_{29}$ was ineffective even at 10^{-3} M. Pretreatment of the preparation with propranolol did not modify the positive inotropic effects of these compounds. As shown in Figure 3, with a typical record obtained with N^6-C_7H_{15} cyclic AMP, the positive inotropic effects developed slowly. There were no significant differences in onset times between the compounds used. Since the positive inotropic effects of some derivatives did not attain 50% of the maximal response to isoprenaline, even at 10^{-3} M, comparisons of potency were made using the concentrations required to produce responses 30% of the maximum (ED_{30}) (Table 1). The positive inotropic effects of N^6-C_2H_5 and C_3H_7 derivatives were too weak to obtain ED_{30} values even at 3×10^{-3} M, the highest concentrations at which they could be tested.

As is evident from Table 1 and Figure 2, the potency as positive inotropic agents of N^6-R cyclic AMPs with a straight alkyl chain of C_2H_5-C_8H_{17} increased as the length of the N^6-side chain increased up to C_8H_{17}, but $C_{14}H_{29}$ had little activity.

Concentration-effect curves for chronotropic effects on right atria are shown in Figure 4. N^6-R cyclic AMPs with a straight alkyl chain of C_2H_5-C_7H_{15} produced negative chronotropic effects

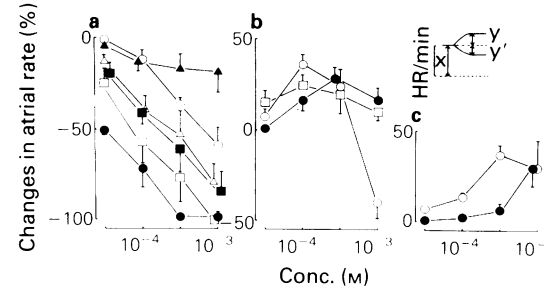


Figure 4 Chronotropic effects of N^6-R cyclic AMPs on guinea-pig right atria. As shown in the inset, the changes in the atrial rate (y or y') are expressed as a percentage of the rate before addition of the drug (x), i.e., $y/x \times 100$ or $-y'/x \times 100$. The curves are cumulative dose-response curves from four or five preparations; vertical lines show s.e.mean. In (a), (○) R = C_2H_5, (●) C_3H_7, (□) C_4H_9, (■) C_5H_{11}, (△) C_6H_{13}, (▲) C_7H_{15}. In (b), (○) R = C_8H_{17}, (●) C_9H_{19}, (□) $C_{14}H_{29}$. In (c), (○) R = furfuryl, (●) benzyl.

(Figure 4a), while N^6-R cyclic AMPs with a straight chain of C_8H_{17}-C_9H_{19} and $C_{14}H_{29}$ (Figure 4b) or a furfuryl or a benzyl groups (Figure 4c) produced positive chronotropic effects. The positive chronotropic effects of the cyclic AMP derivatives with alkyl chains of C_8H_{17}, C_9H_{19} and $C_{14}H_{29}$ became smaller at higher concentrations, and the highest concentration of N^6-C_8H_{17} cyclic AMP, 10^{-3} M, produced a negative chronotropic effect.

The effect of 8-phenyltheophylline on negative chronotropic responses

In order to examine the possible involvement of adenosine receptors in the negative chronotropic effect of some N^6-R cyclic AMPs, the effect of 3×10^{-5} M 8-phenyltheophylline, a specific antagonist of adenosine R-receptors (Griffth *et al.*, 1981), was studied. As shown in Figure 5, in the presence of 8-phenyltheophylline the negative chronotropic effects were converted to positive ones, except for N^6-C_3H_7 cyclic AMP. Although much attenuated, N^6-C_3H_7 cyclic AMP still produced a negative chronotropic effect, even after treatment of the preparation with 8-phenyltheophylline. In the absence of 8-phenyltheophylline N^6-C_3H_7 cyclic AMP was the most potent negative chronotropic agent tested (Figure 4) and induced arrest at concentrations above 10^{-4} M.

Partition coefficient between octanol and an aqueous phase

The partition coefficients, K, of N^6-R cyclic AMPs between octanol and 10 mM phosphate buffer,

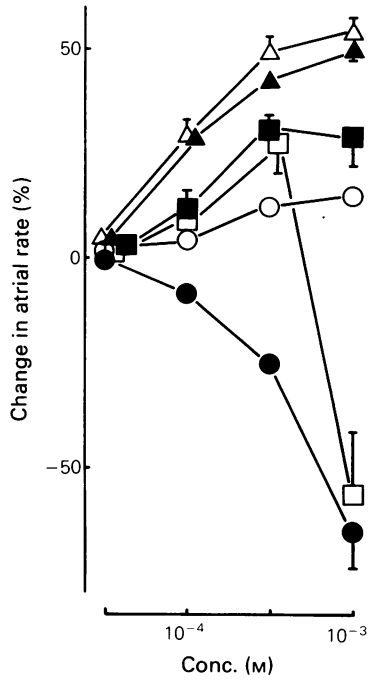


Figure 5 The effect of 8-phenyltheophylline on the negative chronotropic effects of N^6-R cyclic AMPs. The changes in atrial rate are expressed as a percentage of the rate before addition of the cyclic AMP derivative. Points are means from four or five preparations with vertical lines indicating s.e.mean. Where no error bars are shown the error was within the size of the symbol. (○) R = C_2H_5, (●) C_3H_7, (□) C_4H_9, (■) C_5H_{11}, (△) C_6H_{13}, (▲) C_7H_{15}.

pH 7.0, are given in Table 1 as a measure of lipophilicity. Lipophilicity of N^6-R cyclic AMPs tended to increase with increasing length of the alkyl chain, and with the exception of C_9H_{19}, there appeared to be a relationship between the log ED_{30} values for the positive inotropic effects and the log K. These results suggest the importance of lipophilicity as a determinant of the potency of N^6-R cyclic AMPs as positive inotropic agents.

Discussion

In the present study all the N^6-R cyclic AMPs used except N^6-$C_{14}H_{29}$ cyclic AMP produced concentration-dependent positive inotropic effects in the isolated papillary muscle preparations of the guinea-pig and were more potent activators of the bovine myocardial protein kinase than cyclic AMP itself. We used the bovine myocardial enzyme because of the easy accessibility. However, it is known that the isozyme patterns of the bovine and the guinea-pig heart enzymes are the same; both contain predominantly type II isozymes (George & Graeff, 1981).

Among the derivatives with a straight alkyl chain, N^6-C_5H_{11}, C_6H_{13}, C_7H_{15}, C_8H_{17} and C_9H_{19} cyclic AMPs were the most potent as inotropic agents. The potency as positive inotropic agents became higher as the length of N^6-alkyl chain increased up to the length of C_8H_{17}, while the potency as activators of bovine myocardial protein kinase was much the same with all the compounds (Table 1). Though N^6-C_6H_{13}, C_8H_{17} and C_9H_{19} cyclic AMPs were partial agonists as positive inotropic agents, the values of V_{max} were not different from that of cyclic AMP. As expected, the oil/water partition coefficient was greater with compounds having longer alkyl side chains, and if C_9H_{19} is excluded there appears to be a correlation between the inotropic potency and the oil/water partition coefficient, although the potency range is very limited. This may indicate that membrane penetrativeness rather than potency as activators of the protein kinase is a determinant of the potencies of N^6-R cyclic AMPs as positive inotropic agents. A similar correlation between lipophilicity and inotropic potency was obtained by Korth & Engeles (1987) for 8-R cyclic AMPs. It is known that N^6-R cyclic AMPs are resistant to phosphodiesterase (PDE) (Simon *et al.*, 1973) and some of the derivatives were shown to be inhibitors of the bovine heart PDE (Miller *et al.*, 1980), a property which might explain the positive inotropic effects of these compounds. However, as three types of PDE are now known and the positive inotropic effects have been shown to be connected with only one type of the enzymes, i.e. PDE III, further studies would have to be undertaken paying due attention to the species differences noted for the inhibition of this enzyme (Simon *et al.*, 1973; Miller *et al.*, 1980).

The negative chronotropic effects of C_2H_5-C_7H_{15} cyclic AMPs were converted to positive chronotropic effects after 8-phenyltheophylline. Thus, the negative chronotropic effects of these compounds are apparently due to the activation of adenosine receptors on the sinus node, resulting in an increase in potassium conductance and a decrease in an intracellular calcium transient not associated with changes in the cyclic AMP level (Kurachi *et al.*, 1986). It is now well established (Kurahashi & Paton, 1981) that N^6-substituted analogues of adenosine such as N^6-cyclohexyl adenosine and L-phenylisopropyladenosine are very potent agonists at the adenosine R_i-receptor (classified by Londos & Wolff, 1977; Van Calker *et al.*, 1978; Daly *et al.*, 1981). According to Daly *et al.* (1987) N^6-substituted adenosines are more potent at R_i- than at R_a-receptors. It

may be that some of the N^6-substituted cyclic AMPs are also potent agonists at the R_i-receptor.

In summary, N^6-R cyclic AMP derivatives appear to function as activators of cyclic AMP-dependent protein kinase both in right atria and in papillary muscle. However, their ability to penetrate membranes rather than potency as activators of the protein kinase could be the determinant of their potencies as positive inotropic agents. With some derivatives activation of adenosine R_i receptors leads to negative chronotropic effects. It is hoped that N^6-R cyclic AMPs such as N^6-C_7H_{15} cyclic AMP, which are positive inotropic agents with weak negative chronotropic activities, may be useful as cardiotonic agents in the therapy of heart failure.

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Negative inotropic activity of antilaminin IgG: participation of cholinergic mechanisms

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1 Antilaminin IgG decreased the dF/dt of mouse isolated atria and inhibited the mechanical effect of acetylcholine in a non-competitive fashion.

2 Inhibitors of nicotinic and muscarinic cholinoceptors impaired the negative inotropic action of antilaminin IgG in mouse isolated atria. Hemicholinium and tetrodotoxin also reduced the response while the antihistamine, pyrilamine was without effect.

3 These results suggest that antilaminin IgG modulates cholinergic function in mouse isolated atria. Possible mechanisms are discussed.

Introduction

It is known that laminin is a non-collagenous glycoprotein present in all basement membranes, with multiple biological activities that have been related to the different components of the molecule. By rotary shadow electronmicroscopy, laminin revealed a characteristic cross-shape structure composed of three short arms and a long arm held together by disulphide bonds. Globular structures are present in the ends of the long and the short arms (Timpl *et al.*, 1983).

Laminin mediates the attachment of cells to basement membranes and probably links the components of basement membranes (type IV collagen, heparin-sulphate proteoglycans, intactib, nidogen) to one another to form a supramolecular structure. Heparin binding is located at the globular end of the long chain whereas type IV collagen attaches to the globular ends of the short chains. Moreover, the binding to the cells has been ascribed to the region where the short chains interact (Yamada, 1983).

It has been demonstrated, *in vitro*, that laminin plays an important role in cell adhesion, migration, growth and differentiation (Kleinman *et al.*, 1985). These biological activities include a role of laminin in the phenomenon of metastasis (Lissitzky *et al.*, 1986). Another property of this glycoprotein is the promotion of neurite outgrowth activity (Baron Von Evercooren *et al.*, 1982); this has been related to the long chain of the molecule (Engvall *et al.*, 1986).

It is thought that the receptor on laminin to which cells bind is located in the region where the short chains interact (Horan-Hand *et al.*, 1985; Lesot *et al.*, 1983). Direct evidence for the role of the laminin receptor in the binding of cells to laminin was provided by a monoclonal antibody which inhibited almost completely the binding of radiolabelled laminin MCF-7 breast carcinoma (Rogers *et al.*, 1983).

Recently Mollenhauer & Von der Mark (1986) demonstrated that the laminin receptor is associated with or related to a 5'-nucleotidase (AMPase). This enzyme is present in many cells such as smooth muscle, liver cells, glial cells and tumour cells (Rogers *et al.*, 1983). It catalyzes the cleavage of AMP and GMP into nucleotides and phosphate on the cell surface. The physiological role of this enzyme is still unknown and it is possible that this enzyme initiates developmental changes by activating adenylate and guanylate cyclase systems.

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On the other hand, previous studies (McMahan *et al.*, 1978; Vigny *et al.*, 1983) have indicated that the asymmetrical form of acetylcholinesterase is localized in the basement membrane zone of the neuromuscular junction.

It is important to note that each skeletal muscle fibre is ensheathed by a basement membrane. This structure plays an important role in neuromuscular function and reinnervation. In fact, acetylcholine receptors present in regenerating myotubes following injury, cluster at synaptic sites on the basement membrane. Laminin is present in synaptic as well as extrasynaptic regions of the muscle fibre basement membrane while other components of the extracellular matrix are confined to only one of these areas (Sanes & Cheney, 1982).

The aim of the experiments described here was to obtain evidence for pharmacological and molecular actions of the antilaminin IgG on presynaptic and postsynaptic acetylcholine receptors in mouse heart. We conclude that antilaminin IgG is able to exert a pharmacological effect acting on nicotinic and muscarinic receptors in the isolated heart of the mouse, behaving as a cholinoceptor agonist and decreasing myocardial contractility.

Methods

Preparation of antilaminin IgG

Laminin from an Engelbreth Holm Swarm (EHS) murine sarcoma (Bethesda Res. Lab., Maryland, U.S.A.) was used as antigen. Rabbits of about 2.5 kg weight were injected with an emulsion prepared of equal parts of laminin (1 mg ml^{-1}) and Freund's complete adjuvant. Injections of 0.5 mg were repeated 3 times more at intervals of 15 days. Rabbits were bled after the last injection. IgG was isolated from the rabbit antiserum by precipitation with ammonium sulphate and passage through a DEAE-cellulose column. IgG from a normal rabbit serum was also obtained in order to use it in control experiments.

Titers of antilaminin antibodies from the total rabbit serum and from the isolated IgG were detected by the enzyme-linked immunosorbent assay (ELISA) mainly following the method described by Avila *et al.* (1984). Wells of a microtiter plate were coated with 200 μl of laminin (1 μg ml^{-1}). Goat anti-rabbit immunoglobulin conjugated with alkaline phosphatase (Sigma Chemical Co, U.S.A.) 1:1000 was used. Enzyme activity was measured at 405 nm in a Dynatech photometer. Titer of antibodies was determined from the serum dilution that reveals a ten fold difference in the absorbance values between the sera obtained from the rabbits before and after immunization.

By ELISA, a titer of 6.4×10^5 was detected either in the total rabbit antiserum or in the isolated IgG. Antilaminin antibodies were not detected in normal rabbit serum. The molecular weights of the normal and antilaminin IgG were 150,000.

Mouse isolated atria preparation

Male BALB/c mice (20–40 g) were decapitated and the entire heart was quickly removed. Atria were separated from ventricles, carefully dissected and placed in a glass chamber containing Krebs-Ringer-Bicarbonate (KRB) solution gassed with 5% CO_2 in O_2 and maintained at pH 7.4 and 30°C. The ionic composition of KRB was described by Borda *et al.* (1984a). After 1 h for equilibration, initial control values for inotropy of the isolated atria were recorded with a force transducer coupled to an ink writing oscillograph (Borda *et al.*, 1986). The preparations were paced with a bipolar electrode and Grass Stimulator. Stimuli were of 2 ms duration and 10% above threshold voltage. A preload tension of 350 mg was applied to the atria.

Inotropic effects (dF/dt) were assessed by recording the maximum rate of tension development during electrical stimulation at a fixed frequency of 300 beats min^{-1}, that is close to the resting heart rate of mice and consequently inotropy was tested within the physiological range of the force-frequency relationship.

Control values (= 100%) refer to the dF/dt before the addition of antibodies and drugs. The mean absolute value for dF/dt at the end of the equilibrium was 4.1 ± 0.3 g s^{-1}. After a period of equilibration, each preparation was exposed to graded concentrations of antilaminin IgG and acetylcholine. Cumulative dose-response curves were obtained by the method of Van Rossum (1963). Single doses were administered in volumes of 0.01 to 0.025 ml of KRB solution. The total volume added to the bath never exceeded 0.1 ml. The volume of the organ bath was 15 ml. Atria were exposed for 15 min to each concentration of the IgG to obtain the maximal effect.

Drugs

Acetylcholine chloride, atropine sulphate, hexamethonium bromide, hemicholinium-3, pyrilamine maleate and tetrodotoxin (Sigma Chemical Company, U.S.A.) were used. Stock solutions of all the drugs were dissolved in distilled water. The drugs were diluted in the bath to achieve the final concen-

trations shown in Table 3. At these concentrations the drugs had no effect *per se* before the addition of the antibodies and acetylcholine. Mice isolated atria were incubated for 30 min with cholinoceptor antagonists before adding increasing amounts of antilaminin IgG, normal IgG and acetylcholine.

Statistics

Experimental results were compared by means of Student's *t* test. Differences between means were considered significant if $P < 0.05$.

Results

Antilaminin IgG induced a concentration-dependent decrease in atrial *dF/dt* whereas normal IgG was without effect (Figure 1). The inhibitory effect of antilaminin IgG developed gradually reaching a plateau at approximately 12–18 min and for this reason atria were exposed for a period of 15 min to each antilaminin or normal IgG concentration.

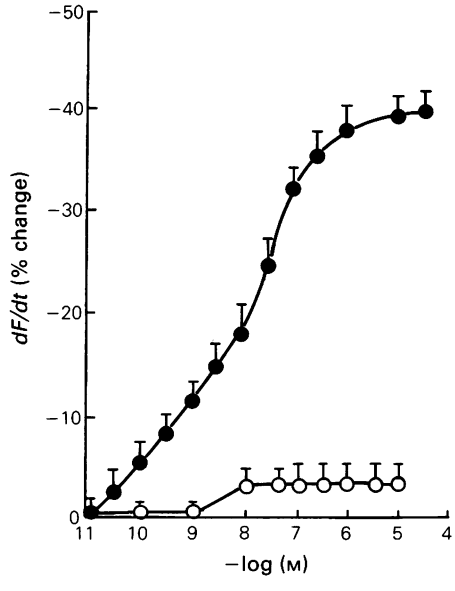


Figure 1 Effect of antilaminin or normal IgG on *dF/dt* of mouse isolated atria. Increasing amounts of antilaminin IgG (●) or normal IgG (○) were added to the bath leaving each concentration 15 min to produce its maximal effect. Values are means with s.e.mean (vertical bars) from 12 experiments.

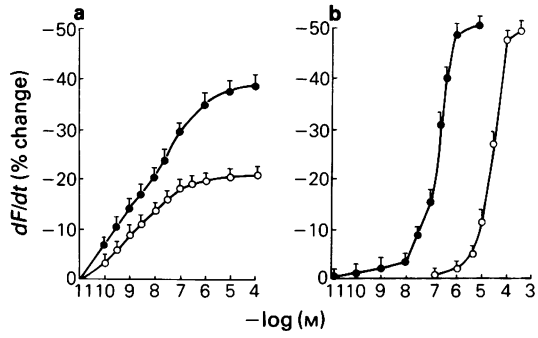


Figure 2 Comparative effect of antilaminin IgG (a) and exogenous acetylcholine (b) upon *dF/dt* of mouse isolated atria in the presence (○) or absence (●) of 3×10^{-7} M atropine. Values are means with s.e.mean (vertical bars) from 8 experiments.

Cholinoceptor participation in the effect of antilaminin IgG

The degree of inhibition induced by exogenous acetylcholine was directly proportional to its concentrations. Atropine antagonized in a competitive manner the action of acetylcholine shifting the dose-response curve for the cholinoceptor agonists to the right (Figure 2b). The negative inotropic effect of antilaminin IgG was antagonized by atropine but in a non-competitive manner (Figure 2a).

The nicotinic antagonist, hexamethonium, failed to modify the dose-response curve for exogenous acetylcholine (Figure 3b) but the inhibitory action of high concentrations of antilaminin IgG was attenuated by the presence of hexamethonium (Figure 3a).

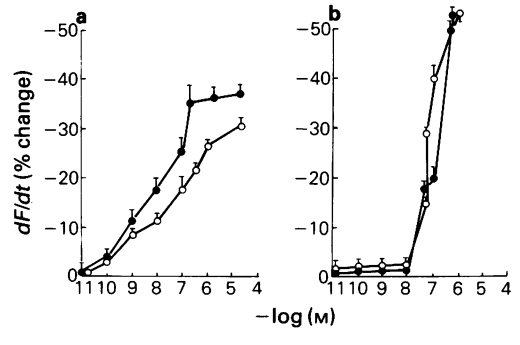


Figure 3 Effect of antilaminin IgG (a) and exogenous acetylcholine (b) upon *dF/dt* of mouse isolated atria. Increasing amounts of antilaminin IgG or exogenous acetylcholine were added in the presence (○) or absence (●) of 1×10^{-6} M hexamethonium. Values are means with s.e.mean (vertical bars) from 6 experiments.

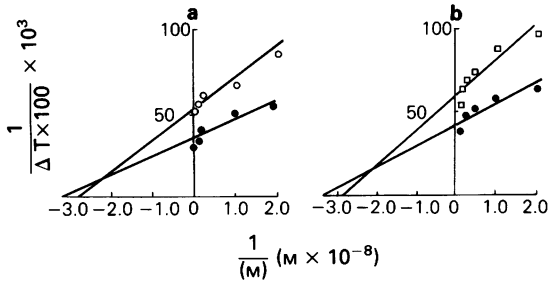


Figure 4 Inhibitory effect of atropine (○) (a) or hexamethonium (□) (b) on the antilaminin IgG action (●). The values were obtained by fitting data of Figures 2 and 3 to Lineweaver-Burk plots. A typical non-competitive inhibition is shown.

Lineweaver-Burks plots of the data presented in Figures 2a and 3a confirm the non-competitive nature of the inhibitory effects of atropine or hexamethonium on the negative inotropic action of antilaminin IgG (Figure 4).

Figure 5a shows a significant reduction of the inhibitory action of antilaminin IgG in the presence of the acetylcholine synthesis inhibitor hemicholinium. Hemicholinium did not modify the dose-response curve to exogenous acetylcholine (Figure 5b). Tetrodotoxin (5×10^{-7} M) impaired the negative inotropic action of antilaminin IgG but did not modify that of exogenous acetylcholine (Figure 5).

Antilaminin IgG (10^{-9} M), but not normal IgG, decreased the maximum effect of acetylcholine,

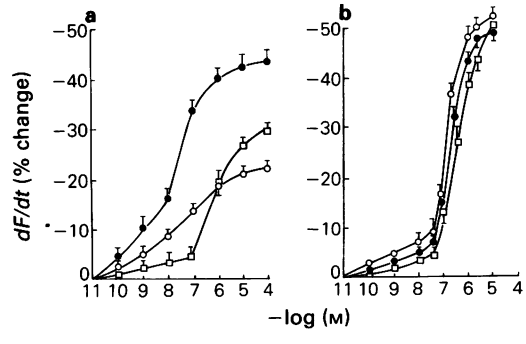


Figure 5 Effect of antilaminin IgG (a) and exogenous acetylcholine (b) upon *dF/dt* of mouse isolated atria. Increasing amounts of antilaminin IgG (●) ($n = 10$) or exogenous acetylcholine (●) ($n = 10$) were added in the presence of 2×10^{-5} M hemicholinium (○) ($n = 5$) or 5×10^{-7} M tetrodotoxin (□) ($n = 5$). Values are means with s.e.mean (vertical bar); n = number of preparations.

Table 1 Inhibitory effect of antilaminin IgG on the negative inotropic action of exogenous acetylcholine (ACh): comparison with normal IgG

Additions	E_{max} ($g s^{-1}$)	K_d (10^{-8} M)	n
ACh	2.26 ± 0.18*	19.6 ± 2.51	7
ACh + antilaminin IgG	3.21 ± 0.22*	17.8 ± 1.9	8
ACh + normal IgG	2.30 ± 0.20	18.2 ± 2.3	6

Atria were exposed to different concentrations of acetylcholine (ACh) in the absence or presence of 1×10^{-9} M antilaminin or normal IgG. E_{max}: maximum effect of ACh. K_d: effective concentration of ACh causing 50% of the maximum response. Basal value: 4.21 ± 0.28 $g s^{-1}$. Values are means ± s.e.mean. n: represents the number of experiments. *$P < 0.01$ between ACh vs ACh + antilaminin IgG.

increasing the E_{max} without affecting the K_d of the dose-response curve for acetylcholine (Table 1). Neither antilaminin IgG action nor exogenous acetylcholine action were modified by the histamine receptor blocking agent pyrilamine (Table 2).

None of the pharmacological interventions used in this study modified the magnitude of the resting *dF/dt* of mouse isolated atria nor altered the action of normal IgG (Table 3).

Discussion

A negative inotropic action of antilaminin IgG on isolated atria of the mouse is described which

Table 2 Action of pyrilamine on the negative inotropic effect of antilaminin IgG and acetylcholine (ACh)

Additions	E_{max} ($g s^{-1}$)	K_d (10^{-8} M)	n
Anti-L IgG	2.73 ± 0.16	3.5 ± 0.2	5
Anti-L IgG + Pyrilamine	2.69 ± 0.14	3.7 ± 0.3	5
ACh	2.28 ± 0.20	18.3 ± 1.9	5
ACh + Pyrilamine	2.26 ± 0.18	19.4 ± 2.0	5

Atria were exposed to different concentrations of antilaminin IgG (Anti-L) or acetylcholine (ACh) in the absence or presence of pyrilamine (1×10^{-6} M). Values are means ± s.e.mean; n: number of experiments.

Table 3 Influence of the antagonist agents on the effect of normal IgG upon *dF/dt* of mouse isolated atria

Incubation (30 min) of atria with:		dF/dt ($g s^{-1}$)* *without normal IgG*	*with normal IgG*	n
KRB alone		4.5 ± 0.5	4.1 ± 0.4	12
KRB + atropine	(3×10^{-7} M)	4.7 ± 0.6	3.8 ± 0.6	5
KRB + hexamethonium	(1×10^{-6} M)	4.4 ± 0.4	3.7 ± 0.5	6
KRB + hemicholinium	(2×10^{-5} M)	4.2 ± 0.7	3.3 ± 0.6	5
KRB + pyrilamine	(1×10^{-6} M)	4.5 ± 0.4	3.7 ± 0.6	5
KRG + tetrodotoxin	(5×10^{-7} M)	4.6 ± 0.8	3.2 ± 0.2	5

* Mean ± s.e.mean. Normal IgG added to the organ bath at a final concentration of 1×10^{-7} M. *n*: number of preparations.
KRB: Krebs Ringer bicarbonate solution.

appears to be mediated via cholinergic mechanisms. Thus the antilaminin IgG action was impaired by the nicotinic antagonist hexamethonium, the muscarinic antagonist atropine and the acetylcholine synthesis inhibitor hemicholinium, but not by the antihistamine pyrilamine. Hexamethonium impairs the negative inotropic action of antilaminin IgG but not that of exogenous acetylcholine, implicating nicotinic receptors in the action of antilaminin IgG. Nicotinic receptors on the postganglionic vagal fibres may be activated directly by antilaminin IgG or indirectly by the antibody acting on the preganglionic nerves to induce a release of endogenous acetylcholine, as hemicholinium opposes the action. However, the action of hemicholinium may be exerted at the postganglionic nerve endings. Antilaminin IgG induces firing of propagated action potentials in the postganglionic neurones as tetrodotoxin impairs its activity, as does atropine by blocking the action of the neuronally-released acetylcholine on the postsynaptic muscarinic receptors. However, antilaminin IgG decreases the maximal negative inotropic effect of acetylcholine with the implication that the antibody interacts also with the muscarinic receptor or its transduction mechanism.

Immunohistochemical studies showed (Sanes & Cheney, 1982) that acetylcholinesterase is present at synaptic sites of muscle fibre basal lamina, where it constitutes the main non-collagenous glycoprotein. It is still a matter of discussion if the collagen subunit of this enzyme interacts with laminin or other components of basement membranes. Vigny *et al.* (1983) as well as Brandan & Inestrosa (1987) demonstrated that the asymmetrical form of acetylcholinesterase binds to heparin sulphate proteoglycan and to a lesser extent to laminin and fibronectin contained in the basal lamina present at the neuromuscular junction. However, Grassi *et al.* (1983) were not able to find any binding of the enzyme to basement membrane components.

The antilaminin IgG is able to mimic the biological effects of a cholinoceptor agonist such as acetylcholine. However, there are some important differences between the action of acetylcholine and that of the antibody: (1) atropine inhibited in a non-competitive manner the inotropic action of antilaminin IgG while the same drug inhibited in a competitive way the action of acetylcholine and (2) hexamethonium and hemicholinium attenuated the action of the antibody while they were unable to modify the effect of exogenous acetylcholine. In addition none of the pharmacological agents used was able alone to block completely the action of antilaminin IgG.

We suggest that the antibody directly or indirectly stimulates nicotinic cholinoceptors that are located on the parasympathetic postganglionic nerves. Activation of these receptors excites the postganglionic neurone and causes the release of acetylcholine which then stimulates the postsynaptic muscarinic receptors, culminating in a negative inotropic response. Post-junctional muscarinic receptor activation by antilaminin IgG is also possible.

We have already described other antibodies with specificity for cardiac membranes antigen that can also trigger the pharmacological effect in isolated myocardium. In fact, we observed that alloimmune IgG against histocompatibility antigen is able to induce a positive inotropic effect on mouse isolated atria (Sterin-Borda *et al.*, 1984). The IgG fraction from sera of Chagas' disease patients also exerted a positive inotropic effect (Borda *et al.*, 1984b). Both type of antibodies (alloimmune and chagasic) bind to β-adrenoceptors of myocardium. The sera of patients with Chagas' disease have been reported to contain antibodies to laminin (Szarfman *et al.*, 1982; Avila *et al.*, 1984). These antibodies recognize a carbohydrate epitope present on mouse laminin, which was assigned to a terminal galactosyl (α1–3)-galactose group (Towbin *et al.*, 1987).

The mechanism whereby antilaminin IgG modulates cholinergic function is uncertain. Whether components of the extracellular matrix are related to the anchorage of asymmetric acetylcholinesterase to the synaptic basal lamina or to the anchorage of acetylcholine receptors to the plasma membrane is still unknown. Laminin is present in practically all tissues. It may be postulated that the binding of a ligand to laminin antigen may induce directly or indirectly the activation of cholinoceptors. Further studies will be required before any mechanism can be proposed.

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Reduction of cephalic arteriovenous shunting by ergotamine is not mediated by 5-HT_1-like or 5-HT_2 receptors

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1 The potent, antimigraine drug ergotamine has affinity for both 5-HT_1 and 5-HT_2 binding sites and constricts arteriovenous anastomoses. Since 5-HT also constricts arteriovenous anastomoses (mainly via 5-HT_1-like receptors), this study investigates the involvement of 5-HT receptors in the ergotamine-induced reduction of arteriovenous shunting in the carotid circulation of the cat and pig.

2 In the cat, ergotamine (3, 10 and 30 $\mu g\ kg^{-1}$, i.v.) reduced carotid blood flow, predominantly by a reduction in arteriovenous anastomotic blood flow. Pretreatment with ketanserin (0.5 $mg\ kg^{-1}$, i.v.) or methiothepin (1 $mg\ kg^{-1}$, i.v.) did not antagonize the effects of ergotamine.

3 In the pig, ergotamine (2.5, 5, 10 and 20 $\mu g\ kg^{-1}$, i.v.) also reduced carotid blood flow and arteriovenous shunting, which was not affected by pretreatment with methiothepin (1 $mg\ kg^{-1}$, i.v.).

4 These results suggest that the reduction by ergotamine in the shunting of carotid arterial blood via cephalic arteriovenous anastomoses is not mediated by 5-HT_1-like or 5-HT_2 receptors.

Introduction

Ergotamine is a potent and effective drug for the treatment of acute attacks of migraine headaches where, among other pathophysiological factors (for references, see Blau, 1987), an opening of arteriovenous anastomoses has been suggested (Heyck, 1969; Saxena, 1978; 1987). Indeed, ergotamine decreases blood flow in the carotid circulation in a variety of species, including man (Saxena & De Vlaam-Schluter, 1974; Spira *et al.*, 1976; Puzich *et al.*, 1983). In anaesthetized cats (Johnston & Saxena, 1978), pigs (Schamhardt *et al.*, 1979) and dogs (Saxena *et al.*, 1983), the decrease in carotid blood flow by ergotamine is almost entirely due to a reduction in the cephalic arteriovenous anastomotic ('shunt') fraction. The constrictor effect of ergotamine on arteriovenous anastomoses in the dog is not antagonized by phentolamine and/or pizotifen, thereby excluding the involvement of α-adrenoceptors and histamine H_1-receptors, as well as the 'D' 5-hydroxytryptamine (5-HT) receptors (Saxena *et al.*, 1983) which are claimed to be involved in ergotamine-induced constrictions of isolated extracranial arteries (Müller-Schweinitzer & Weidmann, 1978; Müller-Schweinitzer, 1986).

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The receptors for 5-HT have recently been subdivided into three distinct types, called 5-HT_1-like, 5-HT_2 (generally equivalent to 'D' 5-HT) and 5-HT_3 receptors (Bradley *et al.*, 1986; Humphrey & Richardson, 1988). The selective agonists at the three receptor types are, respectively, 5-carboxamidotryptamine, α-methyl-5-HT and 2-methyl-5-HT. Ketanserin and MDL 72222 selectively antagonize 5-HT_2 and 5-HT_3 receptors respectively, whilst methiothepin and methysergide are non-selective antagonists at both 5-HT_2 and 5-HT_1-like receptors. The 5-HT_1-like receptors mediate the constriction of arteriovenous anastomoses by 5-HT in the carotid circulation of the pig, since this response is mimicked by 5-carboxamidotryptamine (Saxena & Verdouw 1985a) and other selective 5-HT_1-like agonists such as BEA 1654 (Verdouw *et al.*, 1985), AH25086 (Humphrey *et al.*, 1987) and GR43175 (Feniuk *et al.*, 1987; Humphrey *et al.*, 1988), and is blocked by methiothepin but not by ketanserin or MDL 72222 (Verdouw *et al.*, 1984; Saxena *et al.*, 1986). Recent radioligand experiments show that ergotamine possesses a high affinity for 5-HT_1 binding sites (Leysen & Gommeren, 1984; Richardson *et al.*, 1986). In this investigation, therefore, we address the question whether 5-HT_1-like

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receptors mediate the ergotamine-induced constriction of arteriovenous anastomoses. For this purpose the effects of ergotamine on the carotid circulation has been compared in untreated cats and pigs with those in animals pretreated with ketanserin or methiothepin.

Methods

Experimental set-up

Cats The experimental set-up used in the cats has been described in detail (Johnston & Saxena, 1978; Saxena & Verdouw, 1982). Briefly, the animals weighing between 2.1 and 4.4 kg, were anaesthetized with a mixture of α-chloralose (60 mg kg^{-1}, i.p.) and urethane (700 mg kg^{-1}, i.p.). Bilateral cervical vagosympathectomy was performed (to reduce reflex influences on the carotid circulation) and the trachea was cannulated and connected to a respirator for intermittent positive pressure ventilation. Arterial blood gasses were monitored and kept within normal limits (pH, 7.35–7.45; Pa_{O_2}, 90–150 mmHg; Pa_{CO_2}, 35–45 mmHg). Aortic blood pressure was measured with a Statham transducer (model P23 Ac) connected to a polyethylene cannula inserted in a femoral artery. Heart rate was derived from the pressure signals. The femoral vein was cannulated for drug administration. Blood flow in the left common carotid artery was measured with electromagnetic flow probes (1.5–2 mm i.d.; Transflow 600-system, Skalar, Delft, The Netherlands). A catheter was placed in the left lingual artery to allow retrograde injection of microspheres into the carotid artery. The body temperature was maintained around 37°C by use of an electric heating pad.

Pigs As described earlier (Saxena & Verdouw, 1982), fasted young Yorkshire pigs (body weight: 19–29 kg) were sedated with azaperone (120 mg, i.m.) and metomidate (120–150 mg, i.v.), intubated and given intermittent positive pressure ventilation with a mixture (1:2) of oxygen and nitrous oxide. A continuous infusion of pentobarbitone sodium (20–26 mg kg^{-1} h^{-1}, i.v.) completed the anaesthesia. Respiratory rate and tidal volume were adjusted to keep arterial blood gases and pH within normal limits (see above). The right femoral artery and vein were cannulated for the measurement of aortic blood pressure (Statham transducer, model P23 ID) and administration of ergotamine, respectively. Heart rate was derived from the pressure signals. The common carotid arteries were dissected free and both cervical vagosympathetic trunks were severed. Blood flow in one of the common carotid arteries was measured with a precalibrated flow probe (2.5–3 mm, i.d.; Transflow 601-system, Skalar, Delft). A hub-less 0.5 mm (external diameter) needle, connected to suitable polyethylene tubing, was inserted in this common carotid artery for the administration of microspheres and methiothepin (see later). The animals were wrapped in a thermo-insulating blanket to maintain body temperature between 37–38°C.

Distribution of common carotid blood flow

The distribution of common carotid blood flow was determined with the radioactive microsphere method using 15 ± 1 (s.d.) μm diameter spheres labelled with either ^{141}Ce, ^{113}Sn, ^{103}Ru, ^{95}Nb or ^{46}Sc (NEN Company, Dreieich, West Germany). For each measurement a suspension of microspheres, labelled with one of the nuclides, was mechanically agitated and injected against the direction of the carotid blood flow to facilitate uniform mixing with the blood. At the end of the experiment the animal was killed and the heart, kidneys, lungs and the various tissues of the head were dissected out, weighed and placed in vials. The radioactivity in these vials was counted for 5–10 min in a τ-scintillation counter (Packard Minaxi Autogamma 5000) using suitable windows for discriminating the different isotopes. The amount of carotid blood flow distributed to the individual tissues ($Q_{tis[car]}$) was calculated as described earlier (Saxena *et al.*, 1980; Saxena & Verdouw, 1982) by: $Q_{tis[car]}$ (ml min^{-1}) = $(I_{tis}/I_{tot}) \times Q_{[car]}$, where I_{tis} and I_{tot} are, respectively, the radioactivity (c.p.m.) in a particular tissue and that detected in all tissues, and $Q_{[car]}$ is carotid blood flow (ml min^{-1}). Because there was complete entrapment of microspheres passing through the cranial circulation in the capillaries of the lungs (no significant amounts of radioactivity were detected in the heart or kidneys), the values determined for the lungs provide an *index* of the arteriovenous anastomotic fraction of the common carotid blood flow (see Johnston & Saxena, 1978; Saxena & Verdouw, 1982).

Experimental protocol

Cats About 45 min after completion of the surgical procedures baseline measurements of heart rate, blood pressure and common carotid blood flow and its distribution (using 20,000–50,000 radioactive microspheres) were made. The cats were then divided into three groups receiving pretreatment with either physiological saline (0.9% NaCl, w/v) (2 ml, i.v.), ketanserin (0.5 mg kg^{-1}, i.v.) or methiothepin (1 mg kg^{-1}, i.v.) This pretreatment was followed by the administration of ergotamine in cumulative doses (3, 10, 30 μg kg^{-1}, i.v.) at intervals of 15 min. The systemic and carotid haemodynamic measure-

Table 1 Effects of saline ($n = 7$), ketanserin (0.5 mg kg^{-1}, i.v.; $n = 7$) or methiothepin (1 mg kg^{-1}, i.v.; $n = 7$) and subsequent cumulative doses of ergotamine on heart rate and mean arterial blood pressure in cats

			Ergotamine (μg kg^{-1}, i.v.)		
Pretreatment	*Baseline*	*Pretreatment*	3	10	30
Heart rate (beats min^{-1})					
Saline	181 ± 20	182 ± 18	154 ± 14**	135 ± 8**	129 ± 7**
Ketanserin	212 ± 8	177 ± 8*	155 ± 8**	141 ± 9**	133 ± 9**
Methiothepin	189 ± 12	162 ± 9*	139 ± 7**	131 ± 7**	127 ± 8**
Mean arterial blood pressure (mmHg)					
Saline	90 ± 7	80 ± 3	80 ± 5	82 ± 7	104 ± 7**
Ketanserin	108 ± 8	71 ± 7*	80 ± 8	85 ± 7**	91 ± 6**
Methiothepin	95 ± 7	72 ± 7*	76 ± 8	85 ± 9**	92 ± 10**

* Significantly ($P < 0.05$) different vs baseline;
** Significantly ($P < 0.05$) different vs pretreatment.

ments were repeated at the end of each intervention (pretreatment and the three ergotamine doses).

Pigs After a post-surgical stabilization period of about 45 min, the animals were divided into two groups receiving intracarotid infusions (rate: 1 ml min^{-1} for a period of 10 min) of either physiological saline or methiothepin (total dose: 1 mg kg^{-1}). Ten minutes later, in all experiments baseline measurements of heart rate, arterial blood pressure, common carotid arterial blood flow and its distribution (with 100,000–150,000 spheres) were made. These measurements were repeated after the administration of ergotamine (2.5, 5, 10 and 20 μg kg^{-1} in cumulative doses) at intervals of 15 min.

Statistical analysis

The significance of the differences between the variables was evaluated by Duncan's new multiple range test, once an analysis of variance (randomized block design) had revealed that the samples represented different populations (Steel & Torrie, 1980). Statistical significance was accepted at $P < 0.05$ (two-tailed). All data are expressed as means ± s.e.mean.

Drugs

Apart from the anaesthetics, the drugs used in this study were: ergotamine tartrate (Gynergeen ampoules Sandoz B.V., Uden, The Netherlands), ketanserin tartrate (courtesy: Dr J.M. van Nueten, Janssen Pharmaceutica, Beerse, Belgium) and methiothepin maleate (courtesy: Dr P.J. Pijper, Hoffmann-La Roche B.V., Mijdrecht, The Netherlands). The drugs were diluted with (ergotamine) or dissolved in physiological saline. The doses of ergotamine, ketanserin and methiothepin refer to the salts.

Results

Systemic haemodynamics

In the cat experiments, pretreatment with saline did not change arterial blood pressure or heart rate. Pretreatment with ketanserin (0.5 mg kg^{-1}) or methiothepin (1 mg kg^{-1}) was followed by a decrease

Table 2 Effect of cumulative doses of ergotamine on heart rate and mean arterial blood pressure in untreated ($n = 6$) and methiothepin (1 mg kg^{-1})-treated pigs ($n = 6$)

		Ergotamine (μg kg^{-1}, i.v.)			
Pretreatment	*Baseline*	2.5	5.0	10.0	20.0
Heart rate (beats min^{-1})					
Saline	104 ± 10	91 ± 7*	84 ± 5*	79 ± 5*	76 ± 4*
Methiothepin	101 ± 12	96 ± 10	87 ± 9*	81 ± 8*	77 ± 7*
Mean arterial blood pressure (mmHg)					
Saline	76 ± 4	86 ± 5	86 ± 5	86 ± 5	80 ± 7
Methiothepin	79 ± 5	83 ± 5	89 ± 8	85 ± 10	84 ± 11

* Significantly different ($P < 0.05$) vs baseline.

in both variables. Irrespective of the type of pretreatment, cumulative doses of ergotamine (3, 10 and 30 $\mu g kg^{-1}$, i.v.) caused a decrease in heart rate, whereas arterial blood pressure showed a marked increase (Table 1). In the pig experiments ergotamine had no effect on mean arterial blood pressure in either the saline- or methiothepin-treated animals. Heart rate decreased dose-relatedly after ergotamine in both groups (Table 2).

Carotid haemodynamics

Cats In the saline group of animals, where saline-treatment itself had no effect on carotid blood flow or its distribution, cumulative doses of ergotamine (3, 10 and 30 $\mu g kg^{-1}$, i.v.) caused a decrease in total carotid blood flow. This decrease was predominantly in the arteriovenous anastomotic fraction (non-nutrient) but the nutrient (arteriolar; capillary) fraction was also reduced (Figure 1). Pretreatment with ketanserin (0.5 $mg kg^{-1}$) or methiothepin (1 $mg kg^{-1}$) caused a decrease in total carotid blood flow which was similar in the arteriovenous anastomotic and nutrient fractions. Neither pretreatment modified the effects of ergotamine on total carotid and arteriovenous anastomotic blood flows though the decrease in nutrient flow seemed attenuated (Figure 1). The decrease in the nutrient blood flow by ergotamine in the animals pretreated with saline was distributed over a number of cephalic tissues, particularly the ears, skin, muscles and salivary glands, but not the brain (data not shown). Pretreatment with ketanserin reduced blood flow, especially to the muscles, ears and skin. Methiothepin also reduced blood flow to the muscles and ears. Both drugs seemed to prevent ergotamine-induced decreases in nutrient blood flow, but the pre-ergotamine blood flow values were less after ketanserin and methiothepin when compared to saline (Figure 1).

Since both potential antagonist drugs, ketanserin and methiothepin, decreased mean arterial blood pressure, the effects of these drugs on blood flow may be related to reduced perfusion pressure; hence vascular conductances within the carotid bed were calculated as ratios of blood flow and mean arterial blood pressure values. The data reveal that the decrease in blood flow by ketanserin and methiothepin was clearly related to the drop in blood pressure, probably caused by vasodilatation in other vascular beds. Furthermore, it is observed that ergotamine-induced decreases in vascular conductances in total carotid, arteriovenous anastomotic and nutrient beds were not much altered by either ketanserin or methiothepin (Figure 2). The examination of individual tissues shows that vascular conductance decreased with ergotamine in the saline pretreated animals in the ears, skin, eyes, muscles, bones and

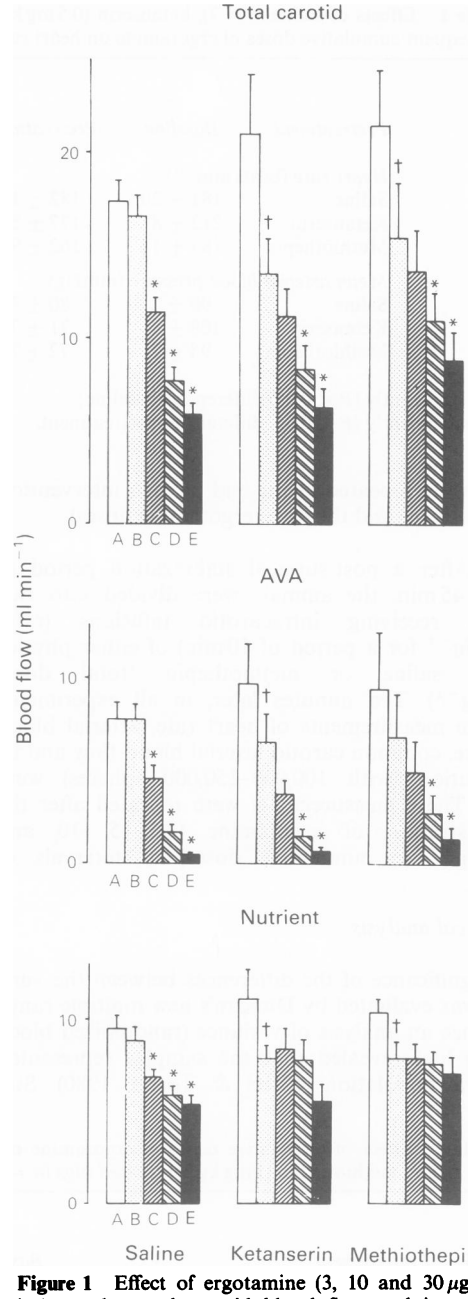


Figure 1 Effect of ergotamine (3, 10 and 30 $\mu g kg^{-1}$, i.v.) on the total carotid blood flow and its arteriovenous anastomotic (AVA) and nutrient (tissue) fractions in 6 cats each pretreated with either physiological saline, ketanserin (0.5 $mg kg^{-1}$, i.v.) or methiothepin (1.0 $mg kg^{-1}$, i.v.). Baseline, A; pretreatment, B; ergotamine 3 $\mu g kg^{-1}$, C; 10 $\mu g kg^{-1}$, D and 30 $\mu g kg^{-1}$, E. † Significantly ($P < 0.05$) different vs baseline; * significantly ($P < 0.05$) different vs pretreatment.

salivary glands, but not in the brain. In antagonist pretreated animals the effects of ergotamine were apparently either less or absent in several tissues; however, as with blood flow values, the pre-ergotamine conductance values were lower than in the saline group (data not shown).

Pigs As in the cats, ergotamine dose-dependently reduced common carotid blood flow in the saline-pretreated pigs by decreasing arteriovenous anastomotic blood flow; the nutrient (arteriolar; capillary) fraction was unaffected. Pretreatment with methiothepin (1 mg kg^{-1}) did not much modify the effects of ergotamine (Figure 3). After administration of ergotamine, blood flow to some tissues (brain, eyes, fat, bones or salivary glands) did not change, but in others there was a tendency to increase (ears, skin and tongue) or decrease (muscles). The increases in tissue blood flow seemed to be attenuated in animals pretreated with methiothepin (data not shown).

Discussion

Shunting of microspheres

The present results confirm the observations that a large fraction (cat: 35–45%; dog: 40–45%; pig: 75–80%) of 15 μm microspheres, injected into the carotid circulation of anaesthetized animals, is not trapped in the arteriolar bed of the cephalic tissues; these spheres pass via arteriovenous anastomoses and veins to the right side of the heart to be sieved by the pulmonary capillary bed (Johnston & Saxena; 1978; Saxena & Verdouw, 1982; Saxena *et al.*, 1983). The simultaneous use of microspheres of four different diameters (10, 15, 25 and 35 μm) has shown that the large arteriovenous anastomoses in the cephalic circulation are mainly present in the skin and ears (Saxena & Verdouw, 1985b), but such 'shunt' vessels have also been located in the nasal mucosa (Änggård, 1974), tongue (Krönert *et al.*, 1980), dura mater (Rowbotham & Little, 1965) and rete mirabile (Gillilan & Markesbery, 1963). The opening of arteriovenous anastomoses, possibly secondary to a decrease in blood 5-HT concentrations (Lance, 1982), may be involved in the pathophysiology of migraine (see Heyck, 1969; Saxena, 1978; 1987).

Effects of ergotamine

Ergotamine decreased heart rate in both cats and pigs. In the cat the ergotamine-induced bradycardia is due to an agonist action on presynaptic dopamine receptors located on the cardiac sympathetic nerve terminals (Saxena & Cairo-Rawlins, 1983), but in the rat presynaptic α_2-adrenoceptors have been implicated (Roquebert & Grenié, 1986).

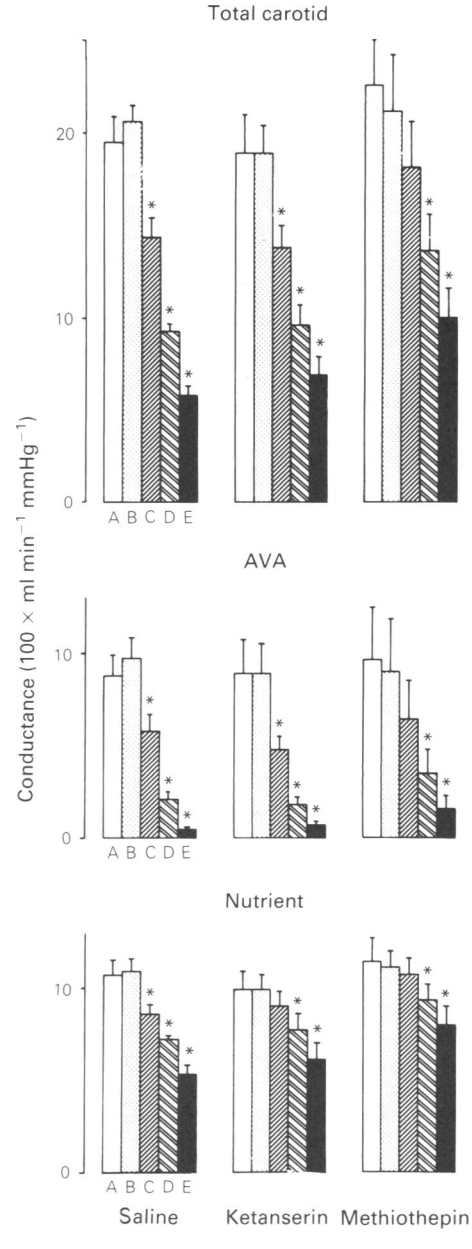


Figure 2 Effect of ergotamine (3, 10 and 30 μg kg^{-1}, i.v.) on vascular conductances in the total carotid bed and its arteriovenous anastomotic (AVA) and nutrient (tissue) fractions in 6 cats each pretreated with either physiological saline, ketanserin (0.5 mg kg^{-1}, i.v.) or methiothepin (1.0 mg kg^{-1}, i.v.). Baseline, A; pretreatment, B; ergotamine 3 μg kg^{-1}, C; 10 μg kg^{-1}, D and 30 μg kg^{-1}, E. * Significantly ($P < 0.05$) different vs. pretreatment.

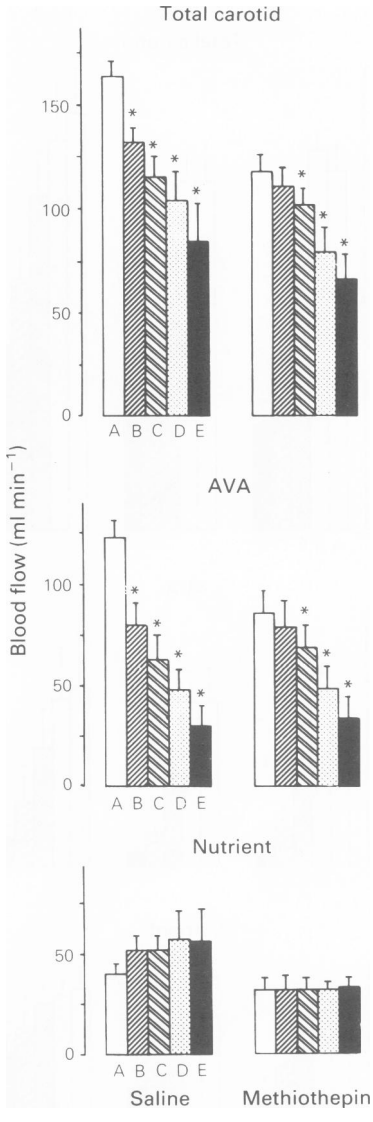


Figure 3 Effect of ergotamine (2.5, 5, 10 and 20 $\mu g kg^{-1}$, i.v.) on the total carotid blood flow and its arteriovenous anastomotic (AVA) and nutrient (tissue) fractions in 6 pigs each pretreated with either physiological saline or methiothepin (1.0 $mg kg^{-1}$, i.a.). No ergotamine, A; ergotamine 2.5 $\mu g kg^{-1}$, B; 5 $\mu g kg^{-1}$, C; 10 $\mu g kg^{-1}$, D and 20 $\mu g kg^{-1}$, E. * Significantly ($P < 0.05$) different vs baseline (pre-ergotamine) values.

In both species studied, ergotamine caused a dose-related decrease in carotid blood flow, predominantly by affecting the arteriovenous anastomotic fraction. Pretreatment with the 5-HT_2 receptor antagonist ketanserin or the mixed 5-HT_1-like and 5-HT_2 receptor antagonist methiothepin did not modify these effects. The doses of ketanserin (0.5 $mg kg^{-1}$) and methiothepin chosen (1 $mg kg^{-1}$) were more than adequate, since they antagonize several cardiovascular responses mediated by 5-HT_1-like receptors (e.g.: 5-HT-induced hypotension in the rat and cat, tachycardia in the cat and arteriovenous anastomotic constriction and arteriolar dilatation in the porcine carotid bed; Saxena & Lawang, 1985; Saxena *et al.* 1985; 1986; Connor *et al.*, 1986) or by 5-HT_2 receptors (e.g.: 5-HT-induced hypertension in the rat; Saxena & Lawang, 1985). Therefore, despite demonstration of a high affinity for both 5-HT_1 and 5-HT_2 binding sites (Leysen & Gommeren, 1984) and for 5-HT_2 receptors in 'large' conducting arteries (Müller-Schweinitzer & Weidmann, 1978; Müller-Schweinitzer, 1986), the reduction in the carotid blood flow following constriction of cephalic arteriovenous anastomoses by ergotamine does not appear to be mediated by either 5-HT_1-like or 5-HT_2 receptors. As shown earlier in the dog, phentolamine and pizotifen were also unable to modify the responses to ergotamine, excluding the involvement of α-adrenoceptors as well as histamine H_1-receptors (Saxena *et al.*, 1983). A possible mediation by 5-HT_3 receptors can not be ruled out from the present experiments, but neither 5-HT_3 receptors mediate constriction of arteriovenous anastomoses (Saxena *et al.*, 1986) nor does ergotamine have an agonist action on these receptors. Thus receptors involved in the ergotamine-induced reduction of arteriovenous anastomotic blood flow in the carotid vascular bed are yet to be characterized. In this context it may be recalled that 5-HT can act on receptors unrelated to 5-HT_1-like, 5-HT_2 or 5-HT_3 types in isolated papillary muscle of kitten (Kaumann, 1985) and in the pig heart (Bom *et al.*, 1988) and that vasodilator dopamine receptors have been described on arteriovenous anastomoses in the canine paw pad (Bell *et al.*, 1978; Bell & Lang, 1979). Whether ergotamine can act on these receptors is not yet known. Another mechanism explaining the action of ergotamine could be related to prostaglandin formation. In canine vein strips the constrictor response to ergotamine is in part mediated by production of a prostaglandin-like substance (Müller-Schweinitzer & Weidmann, 1978) and prostaglandin $F_{2\alpha}$ can increase vascular resistance in the external carotid bed of the monkey (Spira *et al.*, 1978).

The vasoconstrictor action of ergotamine in some tissues was attenuated in both ketanserin- and methiothepin-pretreated animals. It may suggest that the vasoconstriction by ergotamine in the nutrient circulation may be partly mediated by α-adrenoceptors and/or 5-HT_2 receptors (see Saxena & De Vlaam-Schluter, 1974; Müller-Schweinitzer & Weidmann, 1978).

In conclusion this study shows that the effects of ergotamine on arteriovenous anastomotic blood flow in cat and pig are not mediated by 5-HT_1-like or 5-HT_2 receptors. The involvement of α-adrenoceptors and histamine H_1-receptors has been excluded in earlier studies.

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Involvement of the protein kinase C system in calcium-force relationships in ferret aorta

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1 Intracellular Ca^{2+}-force relationships were investigated in ferret aortic smooth muscle by the simultaneous measurement of aequorin luminescence and isometric force. Complete calcium-force curves were constructed by plotting calibrated aequorin luminescence versus force, while intracellular $[Ca^{2+}]$ was made to change by increasing degrees of K^+ depolarization or decreasing extracellular $[Ca^{2+}]$.

2 The steady state calcium-force curve in response to K^+ depolarization exhibited maximal force generation at an intracellular $[Ca^{2+}]$ of approximately 4×10^{-7} M. Further increases in intracellular $[Ca^{2+}]$ did not yield additional increments in force.

3 Protein kinase C activation with the phorbol ester, 12-deoxyphorbol-13-isobutyrate 20 acetate (DPBA) produced contractions accompanied by no detectable increases in aequorin luminescence. DPBA significantly shifted the control $[Ca^{2+}]$-force relationship leftward to lower intracellular $[Ca^{2+}]$ with an increase in the magnitude of maximal generated force.

4 In aorta maximally precontracted by K^+ depolarization, the addition of DPBA resulted in a significant increase in force in the absence of further increases in intracellular $[Ca^{2+}]$. Conversely, in muscles maximally precontracted with DPBA, responses to K^+ depolarization resulted in subsequent increases in force in the presence of simultaneous sustained increases in intracellular $[Ca^{2+}]$.

5 The relatively specific protein kinase C antagonist H-7 caused a significant decrease in intrinsic myogenic tone in the absence of any statistically significant decrease in intracellular $[Ca^{2+}]$.

6 These results suggest that protein kinase C may be an important regulator of vascular smooth muscle contractility by: (1) providing a mechanism by which the apparent $[Ca^{2+}]$ sensitivity of the contractile apparatus during agonist-induced contractions is increased, and (2) maintaining intrinsic myogenic tone by a mechanism the $[Ca^{2+}]$ requirement of which is satisfied by the resting $[Ca^{2+}]_i$.

Introduction

Tumour-promoting phorbol esters, which presumably activate protein kinase C, have been shown to cause sustained contractions of vascular smooth muscle (Rasmussen *et al.*, 1984; Danthuluri & Deth, 1984; Baraban *et al.*, 1985) in the absence of increasing levels of $[Ca^{2+}]_i$ (Jiang & Morgan, 1987). Protein kinase C activation has also been shown to modulate the phosphorylation of heavy meromyosin (Nishikawa *et al.*, 1984). However, this protein kinase C-mediated myosin phosphorylation occurred at sites different from those phosphorylated by myosin light chain kinase, suggesting a mechanism of myosin regulation in addition to the Ca^{2+}-calmodulin dependent pathway. This phosphorylation resulted in an inhibition of actomyosin ATPase activity. Therefore, the mechanism of protein kinase C-mediated contraction remains unknown.

In the present study we have determined the $[Ca^{2+}]_i$-force relationships in ferret aortic smooth muscle in the presence and absence of protein kinase C activation and have investigated the degree to which protein kinase C activation contributes to α-adrenoceptor-mediated contractions as well as to the

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phenomenon of additivity of agonists (Somlyo & Somlyo, 1968).

Methods

Adult male ferrets were anaesthetized with an overdose of chloroform by procedures approved by the Institutional Committee on Animal Research. Strips of ferret aorta were prepared in a manner similar to that previously described (DeFeo & Morgan, 1985). Experiments were performed 37°C in a modified Krebs solution of the following composition (mM): NaCl 120, KCl 5.9, $MgCl_2$ 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25, NaH_2PO_4 1.2 and dextrose, 11.5; aerated with 95% O_2:5% CO_2. Solutions containing elevated K^+ were obtained by isotonic replacement of NaCl by KCl and therefore resulted in solutions which were somewhat Na-deficient. 'Isometric' contractions were recorded simultaneously with aequorin signals from circular strips, 1–2 mm wide, 5–7 mm long and approximately 200 μm thick. Stress was determined by dividing the force of contraction by the cross sectional area. The cross sectional area of the strips was calculated by weight by the method of Herlihy & Murphy (1973), assuming that area = (mass/(density × length) and that density is 1.05 g ml^{-1}.

The viability of each strip was assessed at room temperature with a maximally effective dose of phenylephrine before the bioluminescent protein, aequorin, was loaded into the smooth muscle cells. Aequorin was loaded into the vascular smooth muscle cells by a chemical loading procedure previously described (Morgan & Morgan, 1982; Bradley & Morgan, 1987). Briefly, this procedure consists of incubating the muscle in a series of EGTA- and ATP-containing solutions at 2°C. As previously shown (Morgan & Morgan, 1984; Jiang & Morgan, 1987), this procedure does not alter the responsiveness of aortic smooth muscle when compared before and after the aequorin loading. Aequorin luminescence was monitored using a light-tight enclosure for the muscle bath and photomultiplier tube (Bradley & Morgan, 1987). All experiments were performed at 37°C.

Aequorin luminescence was calibrated in terms of absolute $[Ca^{2+}]_i$ by the method of fractional luminescence (Allen & Blinks, 1979) as adapted to vascular muscle by DeFeo & Morgan (1985). A time constant of 0.4 s was used in the calculation of L_{max}. The ratio of L/L_{max} was then converted to $[Ca^{2+}]_i$ by the use of a standard *in vitro* calibration curve constructed at a free $[Mg^{2+}] = 0.5$ mM (Jiang & Morgan, 1987). The choice of 0.5 mM $[Mg^{2+}]$ was based on NMR measurements of $[Mg^{2+}]$ in bladder and uterine smooth muscle (Kushmerick *et al.*, 1986). For a discussion of the effect of errors in the estimation of $[Mg^{2+}]_i$ on the calculation of $[Ca^{2+}]_i$ from aequorin luminescence in ferret aorta, see DeFeo & Morgan, 1985.

The aequorin used in this study was obtained from Professor J.R. Blinks of the Mayo Clinic. The following drugs were used as aqueous solutions: 1-(5-isoquinolinesulphonyl)-2-methylpiperazine dihydro-chloride (H-7) (Seikagaku Kogyo), N-(6-aminohexyl)-5-chloro-1-naphthalenesulphonamide hydrochloride (W-7) (Seikagaku Kogyo), phenylephrine (Sigma). 12-Deoxyphorbol-13-isobutyrate 20-acetate (DPBA) (LC Services) was dissolved in dimethylsulphoxide.

Data are reported as mean values with standard errors of the mean. Statistical significance was accepted for comparisons at $P < 0.05$ using Student's t test.

Results

Resting $[Ca^{2+}]_i$

The quantitation of $[Ca^{2+}]_i$ from aequorin signals requires a knowledge of the intracellular Mg^{2+} concentration ($[Mg^{2+}]_i$) (Blinks *et al.*, 1978), which is still controversial in smooth muscle. A value of $[Mg^{2+}]_i = 0.5$ mM was assumed in these studies. This assumption is based on n.m.r. measurements of $[Mg^{2+}]_i$ in smooth muscle cells (Kushmerick *et al.*,

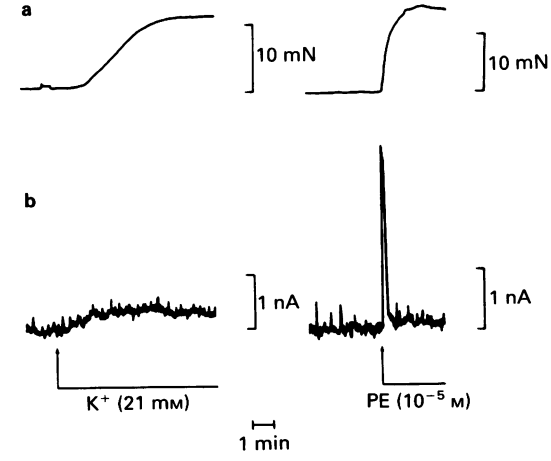


Figure 1 Simultaneously recorded tension in mN (102 mg = 1 mN) (a) and light in nA (b) in ferret aorta at 37°C. Left pair of traces, 21 mM K^+. Right pair of traces, 10^{-5} M phenylephrine (PE).

1986). Using this value for $[Mg^{2+}]_i$, resting $[Ca^{2+}]_i$ was $2.60 \pm 0.09 \times 10^{-7}$ M ($n = 33$ preparations, 33 animals) in ferret aortic smooth muscle cells at 37°C.

Concentration-response relationships

Morgan & Morgan (1984) have previously shown that different contractile stimuli produce very different temporal changes in $[Ca^{2+}]_i$. However, the concentration-dependence of these responses was not previously determined in ferret aorta. Figure 1 illustrates typical light and tension responses of the ferret aorta at 37°C to the addition of 21 mM K^+ and 10^{-5} M phenylephrine. In the presence of 10^{-5} M phenylephrine light rises rapidly but the increase is transient, quickly declining toward resting levels while isometric tension continues to increase to a steady state level. When the complete concentration-response relationships were determined (Figure 2) it was found that 10^{-5} M phenylephrine is a maximally effective concentration. The mean 50% effective concentration (EC_{50}) for phenylephrine was $4.4 \pm 0.5 \times 10^{-8}$ M ($n = 13$ preparations, 9 animals). The aequorin response to 21 mM K^+ (a near EC_{50} concentration) stimulation rises gradually with force and both responses plateau at a steady state level. The maximally effective concentration of K^+ was approximately 51 mM (Figure 2) and the mean EC_{50} was 19.6 ± 1 mM ($n = 17$ preparations, 7 animals). Paired analysis of the amplitude of the maximal contractions to the two stimuli ($n = 15$ preparations, 7 animals) revealed that maximal phenylephrine responses (mean stress, 0.755 ± 0.03 N m$^{-2} \times 10^5$) were significantly larger (9%) than maximal K^+ stimulated responses (mean stress, 0.695 ± 0.03 N m$^{-2} \times 10^5$).

Control $[Ca^{2+}]_i$-force relationships

$[Ca^{2+}]_i$-force curves were generated in intact aequorin-loaded cells by causing graded degrees of K^+ depolarization of the muscle. Maintaining $[Ca^{2+}]$ constant in the Krebs solution at 2.5 mM, $[K^+]$ was increased from 5.9 mM to 81 mM. The K^+-stimulated force response increased in a concentration-dependent fashion achieving a maximum at 51 mM K^+. Luminescence increased in parallel with force up to 51 mM K^+ and further increased in the presence of 81 mM K^+ in the absence of a further increase in generated force. A mean $[Ca^{2+}]_i$-force curve is shown in Figure 3. Values for $[Ca^{2+}]_i$ and force below resting levels were obtained by depletion of extracellular calcium with EGTA (4 mM) in the presence of 24 mM K^+.

In order to obtain an index of the steepness of the $[Ca^{2+}]_i$-force relationship the data were fitted to the Hill equation: relative force = $100\%/(1 + 10^{N(pCa - pK)})$ where $pCa = -\log_{10}[Ca^{2+}]$, pK = the pCa at the half maximal force level of the Hill fit, and N = the Hill coefficient, a measure of the slope of the curve at the pK. The mean pK was 6.50 ± 0.03 which is $3.15 \pm 0.2 \times 10^{-7}$ M [Ca] with $N = 12.2 \pm 2.2$.

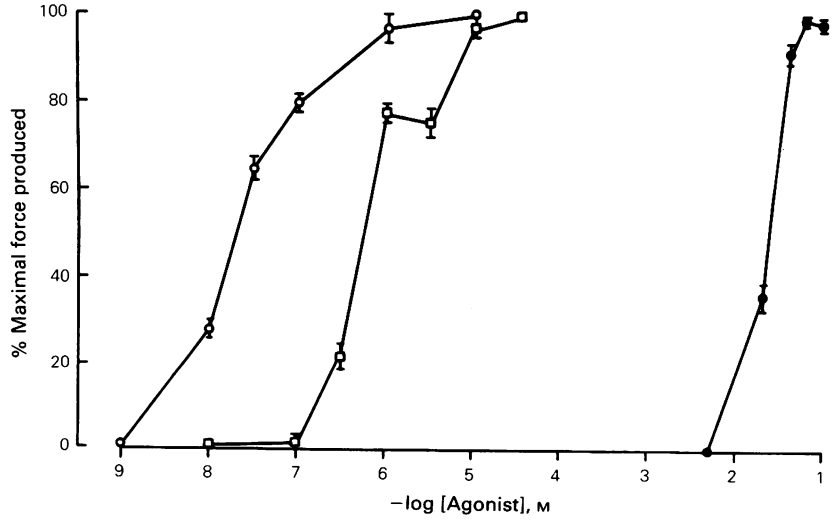


Figure 2 Mean concentration-force relationships for phenylephrine (○), DPBA (□), and K^+ depolarization (●) in ferret aorta at 37°C. The mean EC_{50} values given in the text were determined from the mean of the EC_{50} values determined for each individual curve.

Dependence of phenylephrine contraction on presence of Ca^{2+} transient

The transient light responses associated with phenylephrine-induced contractions were most pronounced upon the addition of a single high concentration ($>10^{-6}$ M) of the agonist and were generally not demonstratable upon cumulative addition of the agonist. This was in spite of the fact that essentially identical force was obtained with a maximal concentration of phenylephrine applied alone or in a cumulative protocol. In 8 preparations from 6 animals, in a cumulative protocol, 10^{-5} M phenylephrine caused no detectable Ca transient but produced 98 ± 2.3% of the contractile force to a challenge of 10^{-5} M phenylephrine. These results suggest that the calcium spike may be merely coincidental with and may not be directly related to the generation and maintenance of the contraction.

Additivity of responses

As has been previously reported for portal vein smooth muscle (Johansson & Somlyo, 1980; Morgan & Morgan, 1984), we have found that the addition of phenylephrine (10^{-5} M) to ferret aortic smooth muscle which was maximally pre-contracted with respect to K^+ stimulation (81 mM), produced a further sustained increase in force. This subsequent increase in force (0.19 ± 0.01 N m^{-2} × 10^5 or 23% of the K^+ generated precontraction; cf. Table 1) occurred in the presence of only a transient increase in the $[Ca^{2+}]_i$ level over that due to the initial K^+ stimulation. The steady state $[Ca^{2+}]$ was not significantly changed (Table 1).

A further sustained contraction (0.14 ± 0.02 N m^{-2} × 10^5 or 25% of the force generated by the precontraction; Table 1) was also generated when 81 mM K^+ was added on top of a maximally effective concentration of phenylephrine (10^{-5} M), with a concurrent sustained increase in $[Ca^{2+}]_i$ above the $[Ca^{2+}]_i$ observed during the phenylephrine-induced steady state contraction. The increase in $[Ca^{2+}]_i$ induced by 81 mM K^+ under these conditions was not significantly different from that observed under control conditions.

To investigate whether the increase in maintained force associated with the addition of phenylephrine in the presence of maximal K^+ stimulation could be attributed to a protein kinase C-mediated pathway, we determined the degree to which the effects of the active phorbol ester DPBA, a putative protein kinase C agonist, could mimic the actions of phenylephrine. At a maximally effective concentration of DPBA (10^{-5} M; Figure 2) the mean generated contraction was larger than either maximal K^+ or phenylephrine contractions (mean stress, 0.91 ± 0.07 N m^{-2} × 10^5).

The addition of DPBA (10^{-5} M) to a muscle maximally precontracted with K^+ resulted in a further slowly developing sustained contraction in the absence of any significant change in $[Ca^{2+}]_i$ (Table 1). The mean increase in force caused by the addition of a maximally effective concentration of DPBA on top of maximum K^+ was 0.21 ± 0.03 N m^{-2} × 10^5 (23% of the precontraction; cf. Table 1). This increase in force was not significantly different from the increase in force observed upon addition of phenylephrine to a maximally K^+-contracted tissue

Table 1 Effects of stimulant combinations on isometric force generation in ferret aorta

Initial stimulus	*Secondary stimulus*	*Additional force (as a % of the precontraction)*	*Steady state $[Ca^{2+}]_i$*	
			Precontraction	*Secondary contraction*
$[K^+]_{max}$	$[PE]_{max}$	23 ± 2 (15,8)	421 ± 28 nM (11,11)	389 ± 88 (4,4)
	$[DPBA]_{max}$	23 ± 2 (15,8)		420 ± 51 (3,3)
$[PE]_{max}$	$[K^+]_{max}$	25 ± 3 (16,10) *	304 ± 43 (6,5)	405 ± 32 (6,5)
	$[DPBA]_{max}$	11 ± 4 (15,8)		307 ± 21 (4,3)
$[DPBA]_{max}$	$[K^+]_{max}$	30 ± 4 (17,12) *	263 ± 12 nM (13,12)	416 ± 24 nM (13,12)
	$[PE]_{max}$	7 ± 4 (12,7)		282 ± 32 (7,7)

PE = phenylephrine; DPBA = 12-deoxyphorbol-13-isobutyrate 20 acetate.
$[K^+]_{max}$ = 81 mM; $[PE]_{max}$ = 10^{-5} M; $[DPBA]_{max}$ = 10^{-5} M. *$P < 0.01$ between values above and below asterisk.
The number of preparations used, followed by the number of animals used are given in parentheses.

(see Table 1). However, the increase in force observed upon addition of DPBA to tissues maximally precontracted with phenylephrine was far less, $0.08 \pm 0.02\,N\,m^{-2} \times 10^5$ or 11% of the precontraction (Table 1).

DPBA (10^{-5} M) contracts ferret aortic smooth muscle in the absence of any significant rise in $[Ca^{2+}]_i$, as previously reported (Jiang & Morgan, 1987) and confirmed in this study. Addition of 81 mM K^+ to a maximally DPBA-precontracted muscle resulted in a further significant increase in force ($0.22 \pm 0.02\,N\,m^{-2} \times 10^5$; or 30% of the precontraction; cf. Table 1) concurrent with a sustained increase in $[Ca^{2+}]_i$. The steady state $[Ca^{2+}]_i$ level reached during this subsequent addition of 81 mM K^+ was not significantly different from that observed in the presence of 81 mM K^+ alone. Moreover, the magnitude of the combined force generated by maximal concentrations of K^+ and DPBA ($1.05 \pm 0.07\,N\,m^{-2} \times 10^5$) was not significantly different from the combined force generated by maximal concentrations of K^+ and phenylephrine ($1.02 \pm 0.05\,N\,m^{-2} \times 10^5$).

Addition of 10^{-5} M phenylephrine to a maximally DPBA-contracted muscle strip caused a small increase in force which reached significance in a paired t test. The transient spike in light normally associated with phenylephrine-induced responses was also abolished in the presence of 10^{-5} M DPBA.

The relative differences in forces generated by the different combination protocols employed are summarized in Table 1. These results, taken together, demonstrate an additivity of responses between K^+ and phenylephrine contractions and between K^+ and DPBA contractions but little or no additivity between DPBA and phenylephrine contractions.

$[Ca^{2+}]_i$-force relationship and C kinase activation

It has been suggested that phorbol esters, via protein kinase C activation, contract vascular smooth muscle, in part, by increasing the apparent sensitivity of the contractile apparatus to calcium (Miller *et al.*, 1986; Jiang & Morgan, 1987). However, the actual effect of protein kinase C activation on the $[Ca^{2+}]_i$-force relationship has not been demonstrated. To evaluate further the calcium-dependence of DPBA-induced contractions of ferret aorta, mean calcium-force curves were constructed in the presence and absence of DPBA (10^{-5} M). As mentioned previously, these curves were generated by causing graded degrees of K^+ depolarization in the presence and absence of the phorbol ester. In each case, steady state active force was plotted against steady state $[Ca^{2+}]_i$. As shown in Figure 3, under control conditions (absence of phorbol ester) active force increased with $[Ca^{2+}]_i$ from a sub-resting level of 200 nM

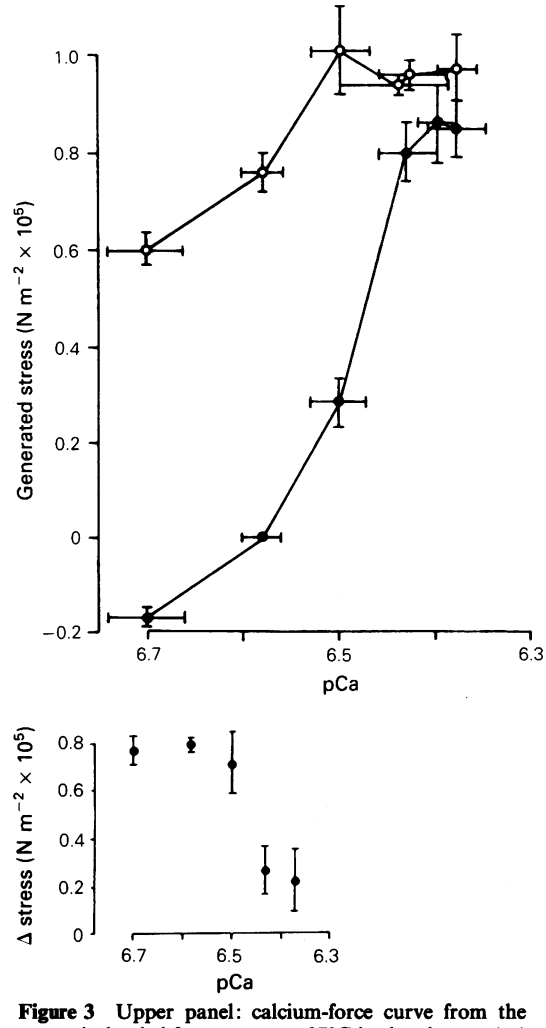


Figure 3 Upper panel: calcium-force curve from the aequorin-loaded ferret aorta at 37°C in the absence (●) and presence of 10^{-5} M DPBA (○). Zero force is arbitrarily defined as the resting level of force at body temperature in Krebs solution. Lower panel: Net DPBA-mediated calcium-force relationship. See text for details.

(achieved by depletion of extracellular calcium with 4 mM EGTA and 24 mM K^+) to approximately 390 nM after which further increases in $[Ca^{2+}]_i$ did not result in further significant increases in force. When $[Ca^{2+}]_i$ was lowered from resting levels by this protocol, active resting force decreased and was recorded as negative generated force. DPBA (10^{-5} M) was found to cause a leftward shift in the calcium-force relationship along with an increase in the magnitude of the maximal generated force relative to the control condition. In the presence of the phorbol ester, maximal force was reached at lower $[Ca^{2+}]_i$

(320 nM) with further increases in $[Ca^{2+}]_i$ not generating further force.

It is apparent from Figure 3 that, if there are two force generating systems in this tissue, their contributions overlap (and consequently add) in the range of pCa's between 6.7 and 6.5. If the 'control' curve is obtained solely by depolarization-induced increases and no other pharmacological or receptor mediated actions, it would be unlikely that there would be significant Ca^{2+}-dependent activation of protein kinase C under these conditions. Therefore, if this 'control' curve is subtracted from the curve obtained in the presence of the phorbol ester, the true $[Ca^{2+}]_i$-force relationship utilized by the phorbol ester should become evident. In fact, the 'net' DPBA-induced $[Ca^{2+}]_i$ force curve (Figure 3, lower panel) shows that there does not appear to be a $[Ca^{2+}]_i$-dependence of DPBA-mediated force production over a range of $[Ca^{2+}]_i$ from 200 nM (sub-resting) to 320 nM. At higher $[Ca^{2+}]_i$ less force was obtained, probably, as a result of a ceiling on the total amount of force the muscle could generate, preventing complete additivity of the two stimuli.

Effects of inhibition of C kinase and myosin light chain kinase

Recently, Hidaka & Hagiwara (1987) described a group of novel naphthalenesulphonamide enzyme inhibitors with relative selectivity for either protein kinase C or calmodulin. The isoquinoline derivative, H-7, was reported to be a direct inhibitor of protein kinase C with a 2–20 fold selectivity over cyclic nucleotide-dependent protein kinases and myosin light chain kinase, respectively. Another structurally related compound, W-7, has been described as a direct calmodulin inhibitor (Kanamori *et al.*, 1981) which inhibits protein kinase C only indirectly at higher concentrations (Hidaka & Hagiwara, 1987), allowing for the possible discrimination of protein kinase C and Ca^{2+}-calmodulin (myosin light chain kinase)-mediated responses (Wright & Hoffman, 1987).

As shown in Figure 4, 30 μM H-7 caused a significant decrease in resting active tone of the ferret aorta ($0.10 \pm 0.01\,N\,m^{-2} \times 10^5$ or 20%; $n = 15$ preparations, 11 animals) in the absence of any significant decreases in $[Ca^{2+}]_i$ at 37°C; this compares with a control active resting tone of $0.48 \pm 0.03\,N\,m^{-2} \times 10^5$ ($n = 38$ preparations, 29 animals). The magnitude of resting tone was measured as the difference between the force on the transducer after stretching the muscle to the optimal length (L_M) for force production and cooling to 2°C and the force with the muscle at L_M but warmed to 37°C. Lowering of $[Ca^{2+}]_i$ to sub-resting levels in the presence of H-7, achieved by depletion of extracellular calcium

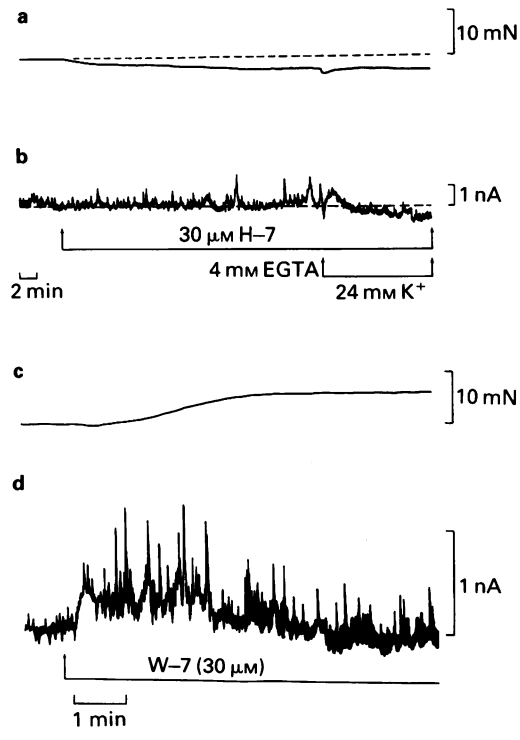


Figure 4 Upper pair of traces: force (a) and light (b) in response to the addition H-7. The dashed lines indicate baseline levels. Lower pair of traces: force (c) and light (d) in response to the addition of W-7.

with EGTA under mildly depolarizing conditions (in presence of 24 mM K^+), produced a detectable decrease in luminescence (Figure 4) but failed to cause a further decrease in resting tone. This ruled out the possibility that the lack of a fall in luminescence during H-7 exposure was a result of being below the range of $[Ca^{2+}]_i$ to which aequorin is sensitive.

In contrast, 30 μM W-7 caused a small but significant rise ($0.09 \pm 0.02\,N\,m^{-2} \times 10^5$ or 18%; $n = 10$ preparations, 8 animals) in resting active tone (Figure 4). In the presence of this agent, $[Ca^{2+}]_i$ did not appear to rise in a sustained manner but rather, frequent oscillations of rapid transient light spikes were observed in aequorin-loaded muscle strips. Additionally, these changes in $[Ca^{2+}]_i$ did not temporally parallel observed changes in resting tone.

To evaluate further the role of protein kinase C in K^+ and phenylephrine-mediated contractile responses, the effects of both compounds, H-7 and W-7, were compared and contrasted. Since both agents also affected baseline tone (see above) the determination of the net effects of the inhibitor on agonist-mediated contractions is reported after com-

Table 2 Effect of protein kinase C inhibition (+30 μM H-7) and calmodulin inhibition (+30 μM W-7) on agonist-stimulated isometric force

Stimulus	*Generated stress* ($N m^{-2} \times 10^5$)	*% inhibition*
K^+ (21 mM)	0.299 ± 0.01 (16,9)	
PE (10 nM)	0.370 ± 0.02 (16,9)	
DPBA (0.7 μM)	0.373 ± 0.04 (15,8)	
K^+ + H-7	0.262 ± 0.04 (8,4)*	12
PE + H-7	0.302 ± 0.07 (8,4)*	18
DPBA + H-7	0.181 ± 0.05 (8,3)*	51
K^+ + W-7	0.323 ± 0.06 (8,5)*	−8
PE + W-7	0.268 ± 0.08 (8,5)*	28
DPBA + W-7	0.247 ± 0.06 (8,5)*	34

Forces generated are given after compensating for the effects of H-7 or W-7 (see text for details). The number of preparations used, followed by the number of animals used are in parentheses.
* Indicates significant ($P < 0.05$) change from corresponding control stress in the absence of inhibitors.
PE = phenylephrine; DPBA = 12-deoxyphorbol-13-isobutyrate 20 acetate; H-7 = 1-(5-isoquinolinesulphonyl)-2-methylpiperazine dihydrochloride; W-7 = N-(6-amino-hexyl)-5-chloro-1-naphthalene sulphonamide hydrochloride.

pensating for the effects on resting active tone in the absence of an agonist. This compensation was made by adding an amount equal to the expected mean decrease in basal tone ($0.10 N m^{-2} \times 10^5$) onto the observed generated force in the presence of H-7 and subtracting the expected mean rise in basal tone ($0.09 N m^{-2} \times 10^5$) in the presence of W-7. H-7 at an approximately mean inhibitory concentration of 30 μM (Hidaka *et al.*, 1984), inhibited EC_{50} K^+-phenylephrine- and DPBA-induced contractions. DPBA contractions were most sensitive to inhibition by H-7 (51%) whereas phenylephrine responses were affected much less (18%) and K^+ were affected least of all (12%; Table 2).

In contrast, the calmodulin inhibitor, W-7, exhibited a very different profile against these responses. At a mean inhibitory concentration (30 μM), W-7 inhibited both DPBA- (34%) and phenylephrine-mediated responses (28%) whereas K^+-stimulated contractions were slightly but significantly potentiated (8%; Table 2). These responses are not consistent with the expected inhibition of force as a result of inhibition of calcium-calmodulin-mediated myosin light chain phosphorylation, but were found to be consistent with the observed changes in $[Ca^{2+}]_i$ (see below).

In a separate series of studies, the effects of W-7 on K^+ and phenylephrine contractions were determined in aequorin-loaded preparations. The potentiation of K^+ contractile responses by W-7 occurred in the presence of further small transient increases in $[Ca^{2+}]_i$ in addition to that produced by K^+ stimulation (Figure 5). The inhibition of phenylephrine-generated force by 30 μM W-7 was accompanied by complete abolition of the characteristic $[Ca^{2+}]_i$ spike response associated with maximal phenylephrine (10^{-5} M) stimulation.

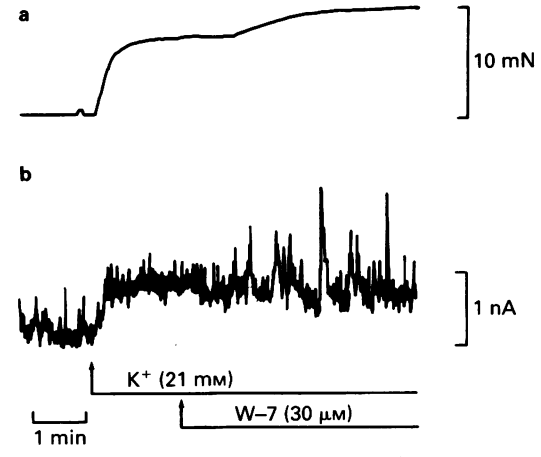


Figure 5 Force (a) and light (b) from ferret aorta at 37°C.

Discussion

The main finding of the present study is that there appear to exist at least two mechanisms mediating intrinsic tone maintenance in intact aortic smooth muscle of the ferret. One, requires a higher $[Ca^{2+}]_i$, and can be distinguished by a characteristic $[Ca^{2+}]_i$-force relationship with a p*K* of 6.50. This p*K* value is similar to that reported by Sobieszek (1977) for calcium-dependent myosin phosphorylation in myofibrils by myosin light chain kinase. Thus this first mechanism may utilize the Ca^{2+}-calmodulin-dependent activation of myosin phosphorylation by myosin light chain kinase for force production. The second mechanism appears to have a lower $[Ca^{2+}]_i$ requirement that may in fact be below resting $[Ca^{2+}]_i$. Such a pathway was demonstrated in the presence of phorbol esters, and may also be involved in the generation of intrinsic tone and the phenylephrine-induced contraction.

One of the more interesting findings of this study is that we were able to demonstrate that additional force could be generated under conditions where a calcium-dependent force production pathway was apparently at its maximal capacity (further increases in $[Ca^{2+}]_i$ could not produce further force). This was observed during maximal K^+ contractions subsequently challenged with either phenylephrine or a

phorbol ester. These results indicate that the additivity occurs at a step in the excitation-contraction coupling sequence beyond the calcium-dependent step and point to the operation of a second force generating pathway in addition to the first calcium-dependent pathway.

In this tissue, the addition of phenylephrine to a ferret aorta precontracted with a high level of KCl, produced both a further contraction and a transient increase in $[Ca^{2+}]_i$. These observations are somewhat different from those reported in ferret portal vein from the same laboratory (Morgan & Morgan, 1984) in that the phenylephrine transient calcium signal was absent when added to a 96 mM K^+ response in portal vein. This probably reflects tissue specific differences in calcium mobilization processes. Even though a transient $[Ca^{2+}]_i$ increase was observed with the addition of phenylephrine (but not phorbol ester) in maximally K^+-stimulated tissues, the phenylephrine-induced calcium spike was not thought to cause the further sustained force since it was observed in the presence of an apparent saturation of the $[Ca^{2+}]_i$-dependent force production pathway (by the K^+ precontraction). Similarly, we have shown in cumulative phenylephrine concentration-response experiments that this transient calcium increase is probably only coincidental with and is not necessary for phenylephrine-mediated force production.

The generation and maintenance of phorbol ester-mediated vascular tone in the absence of detectable elevations in $[Ca^{2+}]_i$ (Jiang & Morgan, 1987) or in the presence of low levels of myosin light chain phosphorylation (Chatterjee & Tejada, 1986; Jiang & Morgan, 1988) have suggested a role for endogenous phospholipid hydrolysis products (diacylglycerols) in contractile signal transduction. Phorbol ester-induced contractions in intact (Jiang & Morgan, 1987) and skinned vascular smooth muscle (Chatterjee & Tejada, 1986; Miller *et al.*, 1986) were maintained at a $[Ca^{2+}]_i$ which could not support other contractions (K^+ stimulated) requiring myosin light chain phosphorylation. These findings indicate that protein kinase C activation can result in an apparently increased sensitivity of the contractile apparatus to calcium.

The $[Ca^{2+}]_i$-force relationships reported in this study are consistent with the hypothesis that protein kinase C activation, in the presence of agonists which apparently release diacylglycerol, may cause the apparent increase in the sensitivity of the contractile apparatus to calcium observed in the presence of other agonists (Bradley & Morgan, 1987). In the presence of DPBA, the $[Ca^{2+}]_i$-force relationship was shifted significantly leftward (to lower $[Ca^{2+}]_i$) relative to the control curve. The presence of an increased level of maximal force in comparison to the control curve, however, suggests the existence of more than a simple change in sensitivity. Additionally, there is an indication that a large part of this force producing mechanism may be calcium independent. This became apparent when the 'control' $[Ca^{2+}]_i$-force curve was subtracted from the curve determined in the presence of protein kinase C activation (inset, Figure 3). With this manipulation two distinct force generating mechanisms are apparent, with one being activated by calcium over the same general range as myosin light chain kinase and the other appearing to be calcium-independent over the physiological range of pCa's in this tissue. A similar calcium independence has been suggested for some agonist-induced platelet function (Rink & Hallam, 1984). These observations may in part be explained by reports of protein kinase C-mediated phosphorylation of other proteins such as the actin binding protein, caldesmon (Umekawa & Hidaka, 1985).

The Hill coefficient for the 'control' $[Ca^{2+}]_i$-force relationship in the presence of K-depolarization is 12. This value is relatively high compared to values reported for skinned skeletal muscle preparations (Brandt *et al.*, 1982) but in intact (aequorin loaded) preparatons a value as high as 9.1 has been reported (Yue *et al.*, 1986). Since there were relatively few points in the critical middle portion of the curve, the results should be interpreted with caution but they do suggest a relatively high degree of cooperativity and complexity in the $[Ca^{2+}]_i$-force relationship in the intact muscle strip. This relationship agrees in general with the previously determined $[Ca^{2+}]_i$-force relationship in ferret aorta (DeFeo & Morgan, 1985), to the extent that the p*K*'s appear similar, but the complete $[Ca^{2+}]_i$-force curve was not previously determined.

Interestingly, the relatively specific C kinase inhibitor H-7 caused a significant (20%) decrease in intrinsic myogenic tone in the absence of any statistically significant decrease in $[Ca^{2+}]_i$, in spite of the fact that further detectable decreases in $[Ca^{2+}]_i$ were observed on addition of EGTA in the absence of further decreases in active force. This finding is consistent with recent studies by Laher & Bevan (1987) indicating an important direct role of protein kinase C in the modulation of vascular myogenic tone and providing evidence that this pathway may be critical to the expression of subsequent agonist-stimulated vascular contractility. Conversely, the calmodulin inhibitor W-7 caused a significant rise in intrinsic myogenic tone with the appearance of frequent transient oscillations in $[Ca^{2+}]_i$. Thus the expected inhibition of the calcium-calmodulin-dependent enzyme myosin light chain kinase was not the dominant effect of this agent. The mechanism of these oscillations is not currently understood. This

may relate to the disruption of the functioning of various calmodulin-dependent calcium pumps on the sarcoplasmic reticulum or sarcolemma (Yamaguchi, 1987). The temporal dissociation of force generation from the observed increase in $[Ca^{2+}]_i$ may also reflect an alteration in calcium-force relationships at the level of the contractile proteins.

Attempts to distinguish the force maintaining mechanisms of phenylephrine- and K^+-induced contractions with these antagonists of protein kinase C (H-7) and calmodulin (W-7) yielded somewhat confusing results. Whereas DPBA-induced contractions were significantly more sensitive to inhibition by H-7 than W-7, a significant amount (34%) of inhibition by the calmodulin inhibitor was observed. This result can most probably be attributed to the lack of total specificity for calmodulin of this inhibitor, a structural congener of H-7 (Hidaka & Hagiwara, 1987). Alternatively, protein kinase C may interact with calmodulin in exerting its cellular actions. However, in contrast to previous experiments suggesting phenylephrine responses are mediated through protein kinase C, submaximal phenylephrine responses were found to be slightly more sensitive to inhibition by W-7 than H-7. It is difficult to say whether this suggests a co-involvement of the Ca^{2+}-calmodulin pathway in α-adrenoceptor-mediated contractions in this tissue or reflects a lack of specificity of the inhibitor.

In conclusion, we have shown that protein kinase C activation can not only cause a state of increased apparent calcium sensitivity of vascular smooth muscle contractile apparatus but may also regulate intrinsic myogenic tone in a calcium-requiring but not necessarily calcium-dependent manner. The α-adrenoceptor agonist phenylephrine may contract ferret aorta by multiple mechanisms involving both protein kinase C- and calmodulin-dependent pathways. It is possible that other Ca-dependent mechanisms may also play a role, such as caldesmon (Marston, 1982), or Ca-dependent regulation of phosphatases (Ingebristen & Cohen, 1983). With respect to the latter possibility, however, it appears that there is little Ca-dependence of the main phosphatases present in smooth muscle (DiSalvo *et al.*, 1985).

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Defibrotide reduces infarct size in a rabbit model of experimental myocardial ischaemia and reperfusion

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1 Defibrotide, a single-stranded polydeoxyribonuncleotide obtained from bovine lungs, has significant anti-thrombotic, pro-fibrinolytic and prostacyclin-stimulating properties.

2 The present study was designed to evaluate the effects of defibrotide on infarct size and regional myocardial blood flow in a rabbit model of myocardial ischaemia and reperfusion.

3 Defibrotide ($32\,mg\,kg^{-1}$ bolus + $32\,mg\,kg^{-1}\,h^{-1}$, i.v.) either with or without co-administration of indomethacin ($5\,mg\,kg^{-1} \times 2$, i.v.) was administered 5 min after occlusion of the left anterior-lateral coronary artery and continued during the 60 min occlusion and subsequent 3 h reperfusion periods.

4 Defibrotide significantly attenuated the ischaemia-induced ST-segment elevation and abolished the reperfusion-related changes (R-wave reduction and Q-wave development) in the electrocardiogram. In addition, defibrotide significantly improved myocardial blood flow in normal and in ischaemic, but not in infarcted sections of the heart. The improvement in blood flow in normal perfused myocardium, but not in the ischaemic area was prevented by indomethacin.

5 Although the area at risk was similar in all animal groups studied, defibrotide treatment resulted in a 51% reduction of infarct size. Indomethacin treatment abolished the reduction of infarct size seen with defibrotide alone.

6 The data demonstrate a considerable cardioprotective effect of defibrotide in the reperfused ischaemic rabbit myocardium. This effect may be related, at least in part, to a stimulation of endogenous prostaglandin formation. Other possible mechanisms are discussed.

Introduction

Defibrotide is the sodium salt of polydeoxyribonucleotide obtained by controlled depolymerization of deoxyribonucleic acid of bovine lungs. The active fraction, corresponding to about 60 nucleotides with a defined ratio of purine and pyrimidine bases, has a molecular weight of approximately 20 kDa. However, the precise chemical structure of the compound is not yet known. Defibrotide has significant profibrinolytic (Pescador *et al.*, 1983) and plasminogen-activating properties (Mussoni *et al.*, 1979), eventually resulting in the dissolution of preformed arterial and venous platelet thrombi *in vivo* (Niada *et al.*, 1981; Grodzinska *et al.*, 1987). Most notably, Löbel & Schrör (1985) have demonstrated an increase of coronary vascular prostacyclin formation in the presence of platelets and defibrotide. Furthermore, in cat (Niada *et al.*, 1985; Thiemermann *et al.*, 1985) and rabbit (Berti *et al.*, 1987) models of acute myocardial ischaemia and reperfusion, defibrotide exerts considerable beneficial effects on biochemical, electrocardiographic and functional indices of ischaemia- and reperfusion-induced tissue injury. However, an improvement of biochemical indicators of ischaemic tissue injury, such as the loss of cytosolic marker enzymes from the ischaemic myocardium is not necessarily associated with a reduction of infarct size (Smith *et al.*, 1984a).

The present study was designed to evaluate the effects of defibrotide on infarct size in an open-chest rabbit model of acute myocardial ischaemia and reperfusion. A further aim was to determine if the cardioprotective effects of defibrotide are related to

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an increased formation of endogenous prostaglandins and/or associated with changes in myocardial collateral blood flow.

An account of some of these results was presented to the British Pharmacological Society in Dublin, (Thiemermann *et al.*, 1988).

Methods

Animal selection and exclusion criteria

The study was carried out on male rabbits (New Zealand White SPF Rabbits, Regal, Great Bookham, U.K.) weighing 2.0 to 2.8 kg receiving a standard diet and water *ad libitum*. All data obtained from rabbits which died during the course of the experiment due to either ventricullar fibrillation or cardiac failure were excluded from the study.

Surgical procedures

Ten min before surgery, all animals were premedicated with 0.1 ml kg^{-1} Hypnorm i.m. (containing 0.315 mg ml^{-1} fentanyl citrate and 10 mg ml^{-1} fluanisone; Janssen Pharmaceutical Ltd.). General anaesthesia was then induced with sodium pentobarbitone (30 mg kg^{-1}, i.v.; Sagatal, May & Baker) and anaesthesia was maintained with supplementory doses of sodium pentobarbitone as required. The rabbits were intubated and ventilated with air from a Harvard ventilator at a rate of 36–40 strokes per min and a tidal volume of 18–20 ml. Arterial blood gasses and pH were checked periodically on a Corning 168 pH blood gas analyzer. When necessary, the ventilation rate and/or oxygen flow rate were adjusted to maintain physiological blood gas values (Po_2: 90–130 mmHg, Pco_2: 30–35 mmHg, and pH 7.38–7.42). Body temperature was maintained at 38 ± 1°C by means of a rectal probe thermometer attached to a homeothermic blanket control unit (CFP 8185). The left femoral vein was cannulated for drug administration. The right femoral artery was catheterized for phasic aortic blood pressure recordings made from a Bell & Howell 40-422-0001 pressure transducer. The left femoral artery was cannulated for the withdrawal of a reference blood sample during the microsphere injection. Another catheter was placed in the left ventricle via the right common carotid artery for measurement of left ventricular systolic pressure (LVP) and subsequent injection of microspheres and Evans blue dye solution. A 2–3 cm left intercostal thoracotomy (4th intercostal space) was performed and the heart was suspended in a temporary pericardial cradle. A snare occluder was placed around the first antero-lateral branch of the left coronary artery (LAL) 1 cm distal from its origin. In contrast to other species, the rabbit LAL supplies most of the left ventricle and apex of the left ventricular myocardium (Flores *et al.*, 1984). Care was taken not to include any veins draining blood from this area. Upon completion of the surgical procedure, fluid loss was replaced with 3 ml of physiological saline i.v. The animals were then allowed to stabilize for 30 min before LAL ligation.

The coronary artery was occluded at time 0 by a two-stage tightening of the occluder. The first stage was a tightening of the snare-occluder until a blue hue appeared on the epicardium. One min later this procedure was followed by a complete occlusion of the coronary artery, which was associated with the typical electrocardiographic and haemodynamic changes of myocardial ischaemia. After 60 min of acute myocardial ischaemia (MI), the occluder was re-opened to allow a 3 h reperfusion period. The presence of reperfusion was confirmed by the appearance of an 'epicardial blush'.

Three experimental groups were studied: controls (vehicle, $n = 10$); defibrotide ($n = 8$) and indomethacin-defibrotide ($n = 11$).

Drug regimens

Defibrotide (Procyclide; loading dose of 32 mg kg^{-1}; followed by a continuous infusion of 32 mg kg^{-1} h^{-1}) or saline (vehicle) were infused i.v. at a rate of 0.6 ml h^{-1} starting 5 min after LAL occlusion and maintained for the remainder of the experiment. In indomethacin-defibrotide-treated animals, indomethacin (5 mg kg^{-1}; Sigma, Poole, Dorset) was given as an i.v. bolus injection 10 min before coronary artery occlusion and 1 h after the start of reperfusion. Bult *et al.* (1980) have shown that even lower doses of indomethacin (2.5 mg kg^{-1}) are sufficient to inhibit prostacyclin formation (measured as 6-keto-$PGF_{1\alpha}$ from extracted plasma samples by radioimmunoassay) in the rabbit. Even when the rabbits were subjected to endotoxin (a potent stimulus of endogenous prostacyclin formation), this low dose of indomethacin abolished the endotoxin-induced rise in plasma 6-keto-$PGF_{1\alpha}$ levels (as demonstrated in control animals) for at least 3 h. In the present study, indomethacin-treated animals were subjected to the same defibrotide protocol as that described for the defibrotide alone group. Defibrotide was a generous gift of Crinos Research Laboratories, Villa Guardia, Como, Italy.

Haemodynamic measurements and electrocardiogram

Haemodynamic parameters, including heart rate (HR), systolic and diastolic pressure and left ven-

tricular systolic pressure (LVP) were continuously recorded on a 4-channel Grass 7D polygraph recorder (Quincy, Mass., U.S.A.). However, detailed data analysis was only performed at time 0 (baseline measurement), 20, 40, 60 min (occlusion period) and every hour during the subsequent reperfusion period. Arterial blood gasses were measured 10 min before and at 30 min intervals after occlusion. Lead II electrocardiograms (ECGs) were recorded from subdermal platinum electrodes on a 7P4H Grass ECG-amplifier attached to Grass 4-channel recorder (Grass, Mass., U.S.A.) R-wave and Q-wave amplitude together with ST-segment changes were calculated as absolute differences with respect to the J-point of the QRS complex (of at least 10 cardiac cycles) and expressed as delta mV. The heart rate was automatically calculated from left ventricular systolic pulse curves by means of a Grass 7P4H tachograph. The pressure-rate index, a relative indicator of myocardial oxygen consumption (Baller *et al.*, 1981) was calculated as the product of mean arterial blood pressure and heart rate.

Measurement of regional myocardial blood flow

^{113}Sn-labelled microspheres (New England Nuclear, Boston, Mass., U.S.A.) were used to determine regional myocardial blood flow (RMBF) at the end of the 3 h reperfusion period. Details of this procedure have been previously described (Heymann *et al.*, 1977) and validated for the rabbit (Maxwell *et al.*, 1987). Microspheres $15 \pm 3\,\mu$m diameter were suspended in physiological saline solution containing 0.01% Tween 80. To ensure adequate dispersion, the microsphere suspension, prewarmed to 38 ± 1°C, was sonicated for 10 s immediately before use. The LAL was then re-occluded and 150,000 to 200,000 microspheres (0.6 ml total volume) were injected into the left ventricle over a period of 30 s. This was followed by 1 ml of physiological saline solution over 15 s. Concurrently, blood was withdrawn from the left femoral artery at a rate of 2 ml min^{-1} (Braun, Melsungen, F.R.G.) and continued for 90 s after the saline flush.

Measurements of area at risk and infarct size

Subsequent to microsphere injection, Evans blue dye solution (4 ml of 1% w/v; Sigma, Dorset) was injected into the left ventricle to distinguish between perfused and non-perfused (myocardium at risk) sections of the heart. The Evans blue solution stains the perfused myocardium while the occluded vascular bed remains uncoloured. The dose of Evans blue dye was well within the range reported for nearly exclusive binding to plasma albumin or other proteins in the rabbit (Lindner & Heinle, 1982). The rabbits were killed with an overdose of pentobarbitone sodium. The heart was excised and left ventricular weight determined after removing the right ventricular wall, valves, papillary muscle and epicardial fat deposits. Area at risk and non-ischaemic myocardium were separated by following the line of demarcation between blue stained and unstained tissue. To distinguish between ischaemic and infarcted tissue, the area at risk was sectioned into 2–3 mm thick slices, which were incubated in 0.5 mg ml^{-1} *p*-nitroblue tetrazolium (NBT) (Sigma, Poole, Dorset) for 20 min at 37 ± 1°C. In the presence of intact dehydrogenase enzyme systems (normal myocardium), NBT forms blue coloured precipitates, while areas of necrosis lack dehydrogenase activity and therefore fail to stain (Nachlas & Shnitka, 1963).

Regional myocardial blood flow

Non-ischaemic left ventricular myocardium, area at risk and infarcted tissue were blotted dry and placed in separate vials for weighing and isotope counting. Additionally, the reference blood sample and any radioactive material remaining in the injection syringe or cannula were counted. Furthermore, both kidneys were removed, cleared of adhering connective tissue, blotted dry, weighed and counted. Subsequently, renal flow values were used as a control for adequate microsphere dispersion and mixing. All tissue samples and blood probes were counted in a 1282 LKB gamma counter (Wallak Oy, Turku, Finland) with a 2″ × 2″ NaI(T1) well type detector. Regional myocardial blood flow (RMBF) was calculated by using the reference blood flow method (Heymann *et al.*, 1977) according to the formula,

$$Qm = \frac{Qr \times Cm}{Cr}$$

where Qm = myocardial blood flow (ml min^{-1}); Qr = reference blood flow (ml min^{-1}); Cm = counts min^{-1} in myocardial sample; Cr = counts min^{-1} in reference sample. RMBF was expressed as ml min^{-1} 100 g^{-1} wet weight. Additionally, the NBT solution was checked for the presence of microspheres lost from the heart tissue as a result of the staining technique. Radioactivity in the NBT solution did not exceed background activity ($n = 3$).

Statistical comparison

All values in the text, figures and tables are expressed as the mean ± s.e.mean of n observations. Where repeated measures were taken over the course of the experimental period the results were analysed by a

Table 1 Haemodynamic data for left ventricular pressure (LVP), heart rate (HR), pressure-rate-index (PRI) in vehicle (Veh), defibrotide (Def) and indomethacin-defibrotide (Indo-Def)-treated rabbits subjected to LAL-occlusion and reperfusion

Time (min)	LVP (mmHg) Veh	Def	Indo-Def	HR (beats min^{-1}) Veh	Def	Indo-Def	PRI (mmHg min^{-1} 10^2) Veh	Def	Indo-Def
0	99 ± 3	102 ± 5	93 ± 4	248 ± 5	254 ± 5	248 ± 7	197 ± 11	200 ± 9	196 ± 5
20	91 ± 5	91 ± 6	85 ± 4	227 ± 5	240 ± 7	239 ± 9	170 ± 11	171 ± 9	171 ± 11
40	96 ± 3	94 ± 4	89 ± 3	243 ± 5	254 ± 8	240 ± 7	186 ± 10	192 ± 6	180 ± 5
60	98 ± 3	98 ± 3	91 ± 5	241 ± 6	255 ± 7	241 ± 6	197 ± 8	199 ± 5	189 ± 8
120	99 ± 6	100 ± 3	85 ± 4*	251 ± 6	251 ± 7	238 ± 13	209 ± 18	205 ± 9	176 ± 10*
180	107 ± 6	104 ± 3	84 ± 3*	250 ± 4	254 ± 5	248 ± 7	228 ± 15	214 ± 9	177 ± 10*
240	107 ± 3	106 ± 5	85 ± 3*	250 ± 5	254 ± 4	246 ± 9	236 ± 11	220 ± 11	181 ± 9*

Values are given as mean ± s.e.mean of 5–7 observations for each group. *$P < 0.05$, when compared to vehicle at the same time-point.

two-way analysis of variance, while endpoint experiments were analysed by a one-way analysis of variance. Analyses of variance were followed by a least significance difference procedure (LSD) to determine the nature of the response (SPSS Inc., Chicago, Il., U.S.A.). A P value of less than 0.05 was considered statistically significant.

Results

Haemodynamic data

Values for LVP, HR and PRI measured during the course of the experiment are given in Table 1. Baseline (time 0) haemodynamic data were similar in all 3 animal-groups studied. Coronary artery occlusion resulted in a consistant but not significant 8–11 mmHg decrease in LVP. In comparison to the vehicle-control group, defibrotide infusion, starting 5 min after LAL ligation, did not influence LVP or HR. Furthermore, the PRI, an index of myocardial oxygen consumption (Baller *et al.*, 1981), was similar in vehicle- and defibrotide-treated groups. In the indomethacin-defibrotide-treated animal group, LVP and PRI were lower ($P < 0.05$), but HR was not significally different from vehicle-treated animals.

Reduction of myocardial infarct size

A total of 29 rabbits were used to determine the effects of defibrotide on myocardial infarct size resulting from ligation of the LAL for 60 min followed by subsequent reperfusion. Two rabbits of the control group, 1 rabbit of the defibrotide-treated group and 6 rabbits of the indomethacin-defibrotide-treated group were excluded from the study because of ventricular fibrillation. Furthermore, one rabbit of the control group having an area at risk and infarct size of 65 and 48% of the left ventricle, was excluded due to cardiac failure during the reperfusion period (3 h).

Area at risk expressed as a percentage of left ventricle and myocardial infarct size expressed as a percentage of the area at risk are given in Figure 1. The percentage of the left ventricle subjected to ischaemia and constituted the area at risk was similar in all 3 groups studied ($P > 0.05$). Defibrotide-treatment resulted in a 51% reduction of the amount of infarcted myocardium within the area at risk when compared with the vehicle-treated control group ($P < 0.05$, Figure 1). Furthermore, infarct expressed as a percentage of the total left ventricle (IN/LV) was similarly decreased by defibrotide-treatment: control ($n = 7$), IN/LV 26 ± 3%; defibrotide IN/LV 13 ± 2%; $P < 0.05$. In contrast, infarct size of the indomethacin-defibrotide-treated animal group was not different from the vehicle-treated control group (Figure 1). Thus, indomethacin treatment ablated the infarct size reduction seen with defibrotide.

Electrocardiogram alterations

In vehicle-treated control animals, LAL occlusion was associated with a rapid increase in the ST-segment amounting to 0.28 ± 0.02 mV after 60 min of permanent coronary artery ligation. Defibrotide infusion significantly attenuated this ischaemia-induced ST-segment elevation to 0.12 ± 0.01 mV at 60 min ($P < 0.05$). In comparison to control, defibrotide-indomethacin treatment did not influence the ischaemia-induced rise in the ST-segment (0.24 ± 0.02 mV at 60 min), indicating that the beneficial effects of defibrotide on ST-segment elevation were largely prevented by indomethacin (Figure 2).

Upon reperfusion, a rapid and continuous decrease of the ST-segment was observed in all animal groups studied, reaching control levels at the

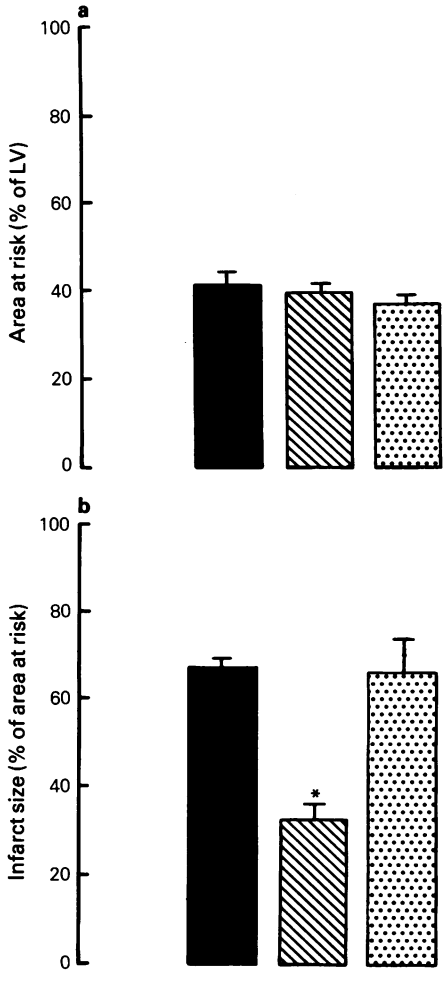


Figure 1 Area at risk expressed as percentage of left ventricle (LV) (a) and infarct size expressed as percentage of area at risk (b) of vehicle-($n = 7$, solid columns), defibrotide-($n = 7$, hatched columns) and indomethacin-defibrotide-($n = 5$, stippled columns) treated rabbits after 1 h of LAL-occlusion, followed by 3 h of reperfusion. Values are mean of n observations, vertical lines show s.e.mean. $*P < 0.05$, when compared to vehicle.

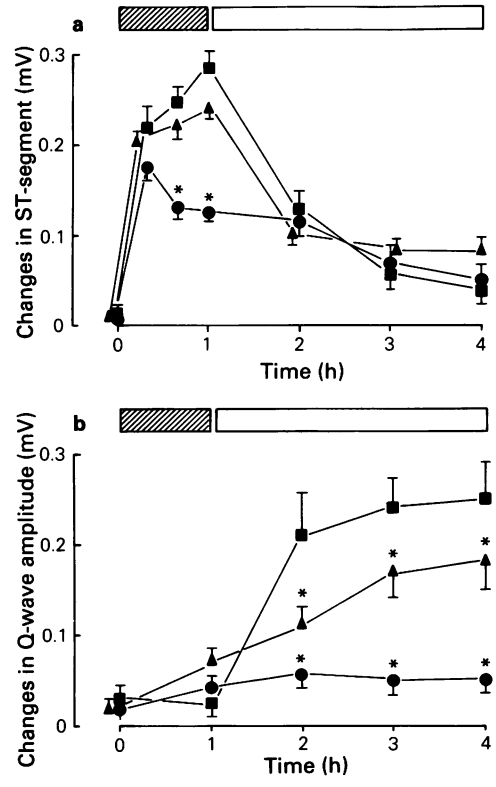


Figure 2 Changes in ST-segment (a) and Q-wave amplitude (b) in vehicle-(■), defibrotide-(●) and indomethacin-defibrotide-(▲) treated rabbits, subjected to occlusion (hatched bar) and reperfusion (open bar) of the LAL-coronary artery. Values are mean of 5–7 observations, vertical lines show s.e.mean. $*P < 0.05$, when compared to vehicle at the same time point.

end of the 4 h experimental period (Figure 2). In vehicle-treated controls, reperfusion was associated with a change of the QRS-complex, namely a reduction of R-wave amplitude and the concomitant development of a pathological Q-wave. The R-wave amplitude was reduced by more than 40% from 0.64 ± 0.08 mV at 1 h to 0.36 ± 0.08 mV at 4 h ($P < 0.05$; Table 2), while the Q-wave magnitude increased from 0.02 ± 0.01 mV (1 h) to 0.25 ± 0.05 mV at the end of the reperfusion period ($P < 0.05$; Figure 2). These reperfusion-related changes of the QRS-complex were abolished by defibrotide-infusion. In comparison to vehicle-treated controls, indomethacin-defibrotide treatment did not affect the loss in R-wave amplitude, but partly reduced the development of a pathological Q-wave during reperfusion ($P < 0.05$). Thus, indomethacin reversed the beneficial effects of defibrotide on ST-segment elevation, R-wave and partially on Q-wave development.

Microsphere studies

The data on changes in regional myocardial blood flow are given in Figure 3. In vehicle-treated controls, coronary artery occlusion was associated with a reduction of RMBF from 214 ± 34 ml min^{-1} 100 g^{-1} (in the non-ischaemic myocardium) to 35 ± 6 ml min^{-1} 100 g^{-1} in the ischaemic and 7 ± 2 ml min^{-1} 100 g^{-1} in the infarcted sections of the area at risk. In comparison, defibrotide treatment

Table 2 Changes of R-wave amplitude after LAL-occlusion and reperfusion in vehicle-(Veh), defibrotide-(Def) and indomethacin-defibrotide-(Indo-Def) treated rabbits

	R-wave-amplitude (mV)		
Time (min)	*Veh*	*Def*	*Indo-Def*
0 (CO)	0.54 ± 0.07	0.47 ± 0.05	0.59 ± 0.08
20 (CO)	0.74 ± 0.12	0.68 ± 0.05	0.82 ± 0.06
40 (CO)	0.69 ± 0.12	0.58 ± 0.04	0.71 ± 0.05
60 (CO)	0.64 ± 0.08	0.57 ± 0.03	0.61 ± 0.07
120 (REP)	0.40 ± 0.08	0.56 ± 0.02*	0.44 ± 0.06
180 (REP)	0.41 ± 0.07	0.56 ± 0.03*	0.37 ± 0.07
240 (REP)	0.36 ± 0.08	0.53 ± 0.03*	0.38 ± 0.07

CO: LAL-occlusion period; REP: LAL-reperfusion period
Values are expressed as mean ± s.e.mean of 5–7 observations for each group. Statistical significance from vehicle-controls was determined by two-way analysis of variance followed by a least square difference analysis. *$P < 0.05$ when compared with vehicle at the same timepoint.

significantly increased RMBF to the non-ischaemic myocardium by 64% and to the ischaemic part of the area at risk by 300% ($P < 0.05$). However, RMBF to the actual infarcted myocardium was not affected by defibrotide treatment (7 ± 2 ml min^{-1} 100 g^{-1}). Indomethacin abolished the defibrotide-induced increase in RMBF in the normal perfused myocardium, but did not affect the defibrotide-induced increase in RMBF in the ischaemic sections of the area at risk. Furthermore, indomethacin-defibrotide treatment did not influence blood flow in the infarcted myocardium, when compared to either vehicle or defibrotide-treated animal groups.

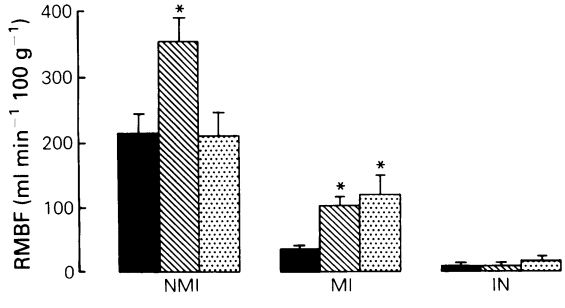


Figure 3 Regional myocardial blood flow (RMBF) distribution in non-ischaemic (NMI), ischaemic (MI) and infarcted (IN) sections of the myocardium after coronary artery occlusion in rabbits treated with vehicle (solid columns), defibrotide (hatched columns) or indomethacin-defibrotide (stippled columns). Values are mean of 5–7 observations, vertical lines show s.e.mean *$P < 0.05$, when compared to vehicle.

Discussion

Defibrotide reduced infarct size in an occlusion-reperfusion model of myocardial ischaemia in the rabbit. Other indications of the anti-ischaemic effect of defibrotide were attenuation of ST-segment elevation, a widely accepted indicator of ischaemic injury (Kjekshus *et al.*, 1972; Ross, 1976) and abolition of the pathological changes in the R- and Q-waves during reperfusion. Such changes in the QRS-complex have also been related to myocardial tissue injury (Hinohara *et al.*, 1984).

The mechanism by which defibrotide protects the ischaemic reperfused myocardium is still largely unknown. Maroko *et al.* (1971) suggested that a reduction in blood pressure would protect the ischaemic heart by reducing oxygen demand. However, defibrotide did not affect left ventricular pressure, mean arterial blood pressure or heart rate. Additionally, defibrotide did not alter the pressure-rate-index, so that a reduction in myocardial oxygen consumption is unlikely to play a role in the mechanism of action of the compound.

Alternatively, an improvement of myocardial oxygen supply, i.e. by enhancement of collateral perfusion, should also protect against ischaemia (Maroko *et al.*, 1971). Our results clearly show an improvement in blood flow to the ischaemic myocardium in defibrotide treated rabbits, which was associated with a reduction in infarct size. However, blood flow to the infarcted area was not improved, but this was to be expected because blood flow was greatly reduced (by 96%), as also found by Maxwell *et al.* (1987) and Chiariello *et al.* (1988) in the rabbit.

What mechanisms, then, can account for the limitation in infarct size and the increase in myocardial blood flow seen with defibrotide? Niada *et al.* (1981) have suggested that an increase of prostacyclin formation contributes to the anti-ischaemic effects of defibrotide. Interestingly, Löbel & Schrör (1985) have shown that defibrotide released prostacyclin in the isolated, platelet-perfused guinea-pig heart in sufficient quantities to cause an increase in platelet cyclic AMP as well as a reduction in coronary perfusion pressure. Surprisingly, in our experiments, defibrotide increased blood flow to the *normal* myocardium and this effect was abolished by indomethacin. Thus, a cyclo-oxygenare product, possibly prostacyclin, may well bring about this effect. Also in agreement with a prostacyclin-dependent mechanism is our result in which indomethacin abrogated the beneficial effects of defibrotide on infarct size, on ST-segment elevation and partly on Q-wave development. In support of a prostacyclin-dependent mechanism, Berti *et al.* (1987) showed that the ability of defibrotide to release prostacyclin and to prevent ischaemia-induced myocardial contracture in the

ischaemic rabbit heart *in vitro* were both completely abolished by indomethacin.

Indomethacin pretreatment appears to be associated with a higher incidence of fatal ventricular fibrillation. An aggravation of ischaemic myocardial tissue injury by indomethacin has been shown in experimental myocardial ischaemia in dogs (Kirmser *et al.*, 1976; Jugdutt *et al.*, 1979) and rabbits (Berti *et al.*, 1988) as well as in patients suffering from ischaemic heart disease (Friedman *et al.*, 1981). Berti *et al.* (1988) concluded that the deleterious effects of indomethacin in ischaemic Langendorff-hearts of the rabbit were related to the ablation of vasodilator prostaglandins, such as prostacyclin.

The one result which is difficult to attribute to a cyclo-oxygenase dependent mechanism is the improvement in blood flow brought about by defibrotide in the ischaemic border zone, for this was insensitive to indomethacin. This result is in accord with the lack of effect of prostacyclin in improving collateral blood flow in the infarcted centre and in the ischaemic border zone of the occluded vascular bed as demonstrated in cat (Smith *et al.*, 1984a), dog (Simpson *et al.*, 1987) and rabbit (Chiariello *et al.*, 1988) hearts *in vivo*.

The improvement in blood flow brought about by defibrotide in the ischaemic border zone could also be related to an effect of the compound on the microvasculature rather than a direct action on collateral blood supply. Reperfusion of the ischaemic microvasculature is often associated with a 'no-reflow' phenomenom (Kloner *et al.*, 1974) mainly due to plugging of the capillaries with neutrophils (Engler *et al.*, 1983) and/or platelets (Vic-Mo, 1978). Since defibrotide prevents platelet aggregation *in vivo* (Thiemermann *et al.*, 1985) and inhibits neutrophil activation *in vitro* (Di Perri *et al.*, 1987), defibrotide may well interfere with some of the initial events leading to an impaired perfusion of the microvasculature.

However, our results indicate that the limitation of infarct extension seen with defibrotide (and abolished by indomethacin) is a prostacyclin-dependent mechanism unrelated to changes in myocardial collateral blood flow. Perhaps this improvement is associated with the so-called 'cytoprotective' activity of prostacyclin, which has been proposed not only in whole animals (Ogletree *et al.*, 1979; Schrör *et al.*, 1981), but also in a variety of isolated tissues, in which the effects of prostacyclin must be due to factors other than blood flow (Araki & Lefer, 1980; Robert, 1984; Smith *et al.*, 1984b).

Defibrotide inhibits neutrophil function *in vitro* (Di Perri *et al.*, 1987) and so another possibility which deserves further investigation is that the compound may well reduce the accumulation and/or the activation of polymorphonuclear cells in the ischaemic myocardium *in vivo*. Certainly, neutropenia induced with specific neutrophil antibodies (Romson *et al.*, 1983) or with nitrogen mustard (Mullane *et al.*, 1984) is associated with myocardial salvage in animal models of regional ischaemia and reperfusion. Furthermore, prostacyclin has also been shown to reduce neutrophil accumulation and activation in the ischaemic reperfused dog heart (Simpson *et al.*, 1987).

In conclusion, defibrotide substantially salvaged the ischaemic myocardium and decreased infarct size in a rabbit model of regional myocardial ischaemia and reperfusion. The mechanism of the cardioprotective action of defibrotide may be related to an enhanced formation of prostacyclin, which may also contribute (Utsunomiya *et al.*, 1980; Dembinska-Kieć *et al.*, 1982) to the fibrinolytic activity of the compound.

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The cardiovascular pharmacology of ICI 170777 ((6**RS**)-6-methyl-5-(pyrid-4-yl)-3H,6H-1,3,4-thiadiazin-2-one) a novel compound with positive inotropic and vasodilator effects

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1 This paper describes the cardiovascular effects of ICI 170777, a novel compound which enhances cardiac contractility and causes arterial and venous dilatation.

2 The positive inotropic effects of ICI 170777 on the heart were demonstrated by an increase in left ventricular dP/dt_{max} in the anaesthetized and conscious dog, and by an increase in tension development in isolated papillary muscles from the cat.

3 In the anaesthetized dog, the positive inotropic effects of ICI 170777 and of isoprenaline were attenuated by atenolol ($5\,mg\,kg^{-1}$, i.v.). Atenolol displaced the dose-response curve to ICI 170777 to the right by 4 fold but displaced the isoprenaline dose-response curve to the right by 247 fold. *In vitro*, however, atenolol ($10\,\mu M$) had no significant effect on the positive inotropic response to ICI 170777. In the ganglion-blocked anaesthetized dog, infusion of a low dose of ICI 170777 which had no effect on the basal left ventricular dP/dt_{max}, selectively potentiated the positive inotropic effects of isoprenaline. These results indicate that ICI 170777 has both a non-adrenoceptor-mediated positive inotropic effect on the heart and also facilitates the β-adrenoceptor-mediated control of contractility.

4 In the denervated and perfused hind-limb of the dog, ICI 170777 reduced arterial perfusion pressure and increased limb circumference at a constant arterial flow and venous pressure. This indicates that ICI 170777 has direct dilator actions on both arterial and venous vessels. In this preparation, diazoxide exerted an arterial selective vasodilator effect and sodium nitroprusside was a relatively selective venous dilator. ICI 170777 exhibited a balanced arterial and venous dilator effect which was intermediate in profile between that of diazoxide and that of sodium nitroprusside.

5 In the conscious dog, low doses ($2–5\,mg\,kg^{-1}$, orally) of ICI 170777 evoked an increase in left ventricular dP/dt_{max} with no significant effect on heart rate or blood pressure. At a higher dose ($10\,mg\,kg^{-1}$, orally) it also reduced blood pressure and caused a significant increase in heart rate. The duration of the positive inotropic effect of $5\,mg\,kg^{-1}$ (orally) of ICI 170777 was 10–12 hours. This response did not diminish following repeated administration of the compound.

6 The positive inotropic action and balanced arterial and venous dilator effect of ICI 170777 indicate that the compound may be useful in the treatment of congestive heart failure, a disorder that is characterized by decreased cardiac contractility and enhanced arterial and venous constrictor tone.

Introduction

Congestive heart failure is characterized by a depression in ventricular contractility and by an inappropriate increase in arterial and venous constrictor tone (Harris, 1982; Vanhoutte, 1983). It therefore appears logical to treat the disorder with agents that reduce or reverse these haemodynamic abnormalities. The orally active positive inotropic agents that are currently available, the cardiac glycosides, are not ideal since they have the ability to provoke life threatening arrhythmias (Mason *et al.*, 1971). Arterial and

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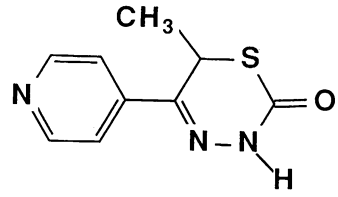


Figure 1 Structure of ICI 170777 ((6RS)-6-methyl-5-(pyrid-4-yl)-3H,6H-1,3,4-thiadiazin-2-one).

venous vasodilatation can be achieved by co-administration of hydralazine and isosorbide dinitrate; however, a high incidence of side-effects is observed with this combination (Cohn *et al.*, 1986). Recent clinical results suggest that novel β_1-adrenoceptor partial agonists and angiotensin converting enzyme inhibitors exert clinically useful positive inotropic and vasodilator effects, respectively, with a low incidence of side effects (Pouleur *et al.*, 1982a; Lipkin & Poole-Wilson, 1985; The German and Austrian Xamoterol Study Group, 1988).

The development of novel pharmacological agents which combine arterial and venous dilatation with positive inotropic effects on the heart seems logical on haemodynamic grounds and could yield drugs with a high degree of efficacy in treating congestive heart failure (Cohn & Franciosa, 1978). The present paper describes the cardiovascular pharmacology of ICI 170777 (Figure 1), a novel, orally active compound which evokes these therapeutically advantageous haemodynamic effects.

Methods

Cat isolated papillary muscle

Cat right ventricular papillary muscles were mounted for isometric tension recording in organ baths containing Krebs solution (37°C) bubbled with 95% O_2, 5% CO_2. The composition of the Krebs solution was (mM): NaCl 118.3, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 2.5, disodium edetate 0.026, glucose 5.5. An initial tension of 1 g was applied to the tissues which were stimulated electrically via punctate electrodes (5 ms, 20% supra-threshold voltage, 1 Hz). A 1 h equilibration period was allowed before the addition of drugs. Dose-response curves were made to ICI 170777 and to isoprenaline before and after incubation of the tissue with atenolol (10 μM, 30 min incubation time). ICI 170777 was prepared at 10 mM in 20% polyethylene glycol 400, 80% distilled water. This vehicle had no effect on force development by the papillary muscle.

Anaesthetized dog preparations

Alderley Park beagle dogs of either sex (10–15 kg) were anaesthetized with either sodium pentobarbitone (30–40 mg kg^{-1}, i.v.) or α-chloralose (100 mg kg^{-1}, i.v. after pre-medication with morphine sulphate, 1 mg kg^{-1}, s.c.). The trachea was intubated and the dogs were artificially ventilated (18–24 cycles min^{-1}, tidal volume 10–15 ml kg^{-1}) with a mixture of room air and oxygen. In those dogs in which the thorax was opened, expiration was against a fixed resistance (3–5 cmH_2O) to prevent collapse of the lungs. The pH, P_{CO_2} and P_{O_2} of arterial blood were measured with a Corning 175 pH and blood gas system. These parameters were maintained within the normal range by adjustment of the ventilator and by i.v. injection of sodium bicarbonate. Body temperature was monitored via a rectal probe and maintained at 37°C by a heating blanket.

A femoral vein was cannulated for drug administration. A femoral artery was cannulated and systemic blood pressure was measured with a Bell and Howell L221 pressure transducer. Heart rate was derived from the blood pressure signal. Lead 2 electrocardiogram was recorded. In most animals, the left carotid artery was exposed and a Millar Mikro-tip pressure transducer (model PC470) was passed down the artery, across the aortic valves and into the left ventricle. The Mikro-tip transducer was calibrated *in situ* from the pressure recorded from the saline-filled lumen of the catheter for comparison. The first differential of left ventricular pressure (dP/dt) was derived electronically.

In animal preparations in which ICI 170777 was likely to cause a decrease in systemic blood pressure, the chest was opened and a snare was placed around the descending aorta. This was tightened for short periods (10–20 s) when necessary, in order to compensate for a decrease in diastolic pressure of greater than 10% evoked by ICI 170777. In this way the effects of ICI 170777 on dP/dt_{max} could be measured without interference from changes in afterload evoked by the compound (Furnival *et al.*, 1970).

In some animals, a hind-limb was surgically denervated and perfused via the femoral artery by use of a constant flow roller pump (Watson-Marlow HRE200). The circulation to the paw was occluded by a ligature and the perfusion pressure of the limb was monitored and matched to the systemic pressure by adjustment of the pump output. In certain animals, hind-limb circumference was also measured by use of a mercury in rubber strain gauge (Whitney, 1953). In these animals, femoral vein pressure was measured. Changes in limb circumference which occurred in the absence of changes in venous pressure were taken as indicative of changes in venous compliance. In order to standardize this measure-

ment between different dogs, the increase in circumference evoked by vasodilators was expressed as a percentage of the maximum increase evoked by sodium nitroprusside (6–8 μg) in that limb.

ICI 170777 was prepared for use in *in vivo* by dissolving it in 100% polyethylene glycol 400 at 10 mg ml^{-1} and subsequent dilution in 0.9% saline. In most experiments, ICI 170777 was administered i.v. on a cumulative dosing cycle: 5 min was allowed between doses as this allowed a maximum expression of the haemodynamic response. The vehicle used had no effect on haemodynamic parameters measured 5 min after administration. ICI 170777 was also administered by infusion in some experiments. In experiments where the effects of ICI 170777 on hind-limb perfusion pressure and limb circumference were evaluated simultaneously in the same animal, the compound was administered by injection into the extracorporeal arterial circuit that passed through the perfusion pump.

Conscious animal experiments

Alderley Park Beagle dogs of either sex, (12–15 kg) were trained to sit in a shallow box; they were otherwise unrestrained. When they had been adequately trained the dogs were anaesthetized (thiopentone 25 mg kg^{-1} i.v. for induction, and after tracheal intubation, maintenance with halothane in oxygen). All subsequent surgical procedures were performed under sterile conditions. The chest was opened in the fourth intercostal space. An electromagnetic flow probe was placed around the root of the aorta and a minature pressure transducer was implanted through the apex of the left ventricle into the ventricular cavity. A tripolar recording electrode was implanted in the lower part of the interventricular sulcus. The thorax was closed in layers, and the leads from the transducers were tunnelled to the dorsal surface of the neck, where they were brought out via transcutaneous button sockets. Analgesia (as required) and antibiotics were given throughout recovery. When the animals had recovered from this procedure and showed normal heart rate and sinus arrhythmia they were anaesthetized again and 1 mm bore polyethylene catheters were implanted in the aortic arch via the right carotid artery, and in the right atrium via a branch of the jugular vein. These catheters were also exteriorized on the dorsal surface of the neck.

From the implanted probes the following parameters were derived by analogue techniques: beat by beat stroke-volume; systolic, diastolic and mean aortic pressures; peak of the first differential of left ventricular pressure with respect to time (dP/dt_{max}) and instantaneous heart rate. Over each 1 min period, the following were derived by integration: mean systolic and diastolic aortic pressures; mean aortic pressure, heart rate and dP/dt_{max}. Mean stroke volume and cardiac output (as minute integrated aortic flow) and total peripheral resistance were determined as percentages of control values as the aortic flow probe was not calibrated.

The protocol of the experiments was as follows. The animals sat quietly in boxes to which they had become accustomed. At least 30 min of control recording was taken for each experiment. During this period it was required that the heart rate was stable and not in excess of 120 beats min^{-1}. The animals were then dosed with ICI 170777 given orally in a gelatine capsule followed by 20 ml of water. Recording of haemodynamic parameters was then continued for the duration of the experiment.

Drugs and compounds used

ICI 170777: (6**RS**)-6-methyl-5(pyrid-4-yl)-3H,6H- 1,3, 4-thiadiazin-2-one was prepared in the Chemistry department at ICI by Drs R. Hargreaves, S.D. Mills and B.J. McLoughlin (European Patent Specification No. 85227) and used as a free base. Because ICI 170777 (in aqueous solution) slowly decomposes in normal light conditions all solutions of the compound were protected from light. Atenolol (ICI), sodium nitroprusside (Sigma), diazoxide (Allen and Hanburys) and mecamylamine (M.S.D.) were also used.

Statistical analysis of the results was by Student's t test. A P value of <0.05 was regarded as significant. Data are expressed as mean $\pm$ s.e.mean except in the case of mean doses when geometric mean with 95% confidence limits, in parentheses, are given.

Results

Direct inotropic and vasodilator effects of ICI 170777 in the anaesthetized dog in which cardiovascular reflexes had been prevented

Dogs were anaesthetized and prepared for the measurement of cardiovascular parameters. Atenolol (5 mg kg^{-1}, i.v.) was administered and both vagi were cut. (The effectiveness of the β-blockade was assessed by repeating dose-response curves to isoprenaline before and after administration of atenolol, this gave a dose-ratio in excess of 100). In this preparation the effects of ICI 170777 on cardiac function and hind-limb vascular tone could be examined in the absence of autonomic reflexes. ICI 170777 (50–10,000 μg kg^{-1}) evoked dose-related increases in heart rate, dP/dt_{max} and decreased hind-limb perfusion pressure (Figure 2). These changes occurred over similar dose-ranges. A decrease in systemic blood pressure also occurred at the higher doses of the compound (data not shown) and was compensated for during short periods for the measurement

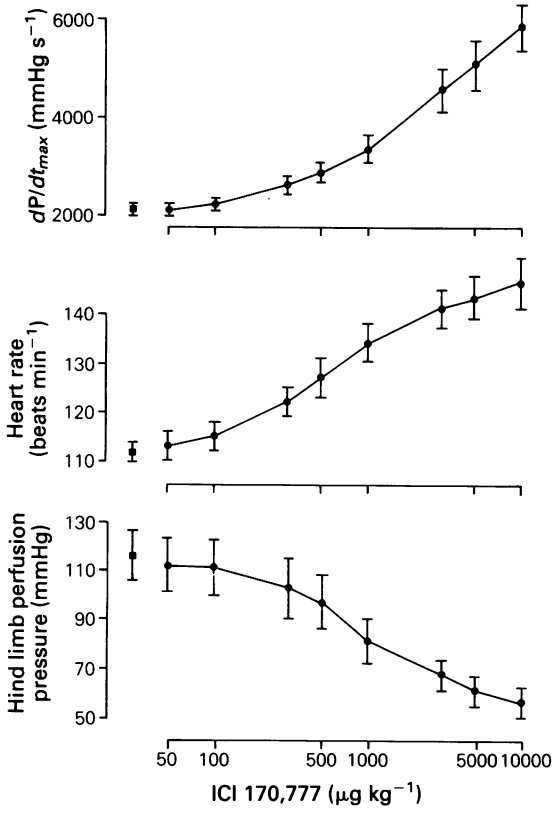


Figure 2 The effects of ICI 170777 on left-ventricular dP/dt_{max}, heart rate and hind-limb perfusion pressure in the anaesthetized areflexic dog: (■) control value; (●) value in presence of ICI 170777. All three parameters were significantly different ($P < 0.05$) from control after administration of 100–10,000 $\mu g\,kg^{-1}$ of ICI 170777 ($n = 5$).

of dp/dt_{max} by tightening of the aortic snare. A 50% increase in dP/dt_{max} was evoked by a mean dose of 0.81 (0.44–1.40) $mg\,kg^{-1}$.

Comparison of the effects of ICI 170777 with those of isoprenaline on heart rate and dP/dt_{max} was made by regression analysis of the two parameters. This analysis demonstrated that ICI 170777 evoked an increase of 119.5 ± 28.6 $mmHg\,s^{-1}$ in dP/dt_{max} for a unit increase in heart rate ($n = 5$), which was significantly greater than the increase of 46.1 ± 7.0 $mmHg\,s^{-1}$ per unit increase in heart rate for isoprenaline ($n = 9$, $P < 0.05$).

Haemodynamic effects in the chloralose-anaesthetized dog

Dogs were anaesthetized with chloralose and prepared for measurement of dP/dt_{max} (closed chest). ICI

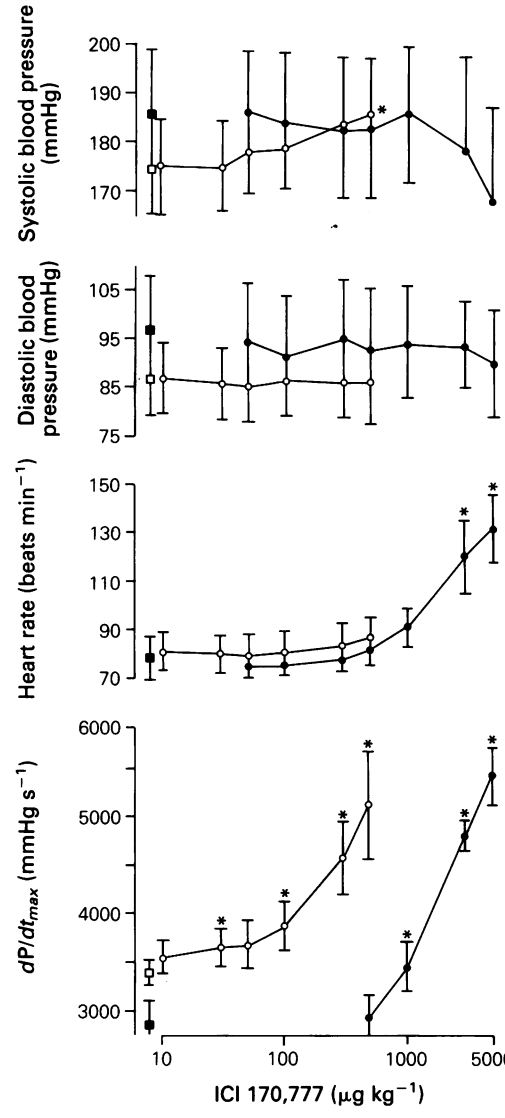


Figure 3 The effects of ICI 170777 on left-ventricular dP/dt_{max}, heart rate, systolic and diastolic blood pressure in dogs anaesthetized with chloralose: (□) control value; (○) value in presence of ICI 170777. Open symbols indicate value in the absence of atenolol ($n = 5$): closed symbols indicate value in presence of atenolol (5 $mg\,kg^{-1}$, $n = 3$). *Indicates significant difference from control ($P < 0.05$).

170777 (10–500 $\mu g\,kg^{-1}$) evoked a dose-related increase in dP/dt_{max}. In 5 dogs a 50% increase occurred at a mean dose of 0.46(0.31–0.64) $mg\,kg^{-1}$. Heart rate and diastolic blood pressure were not significantly affected, systolic pressure showed a small

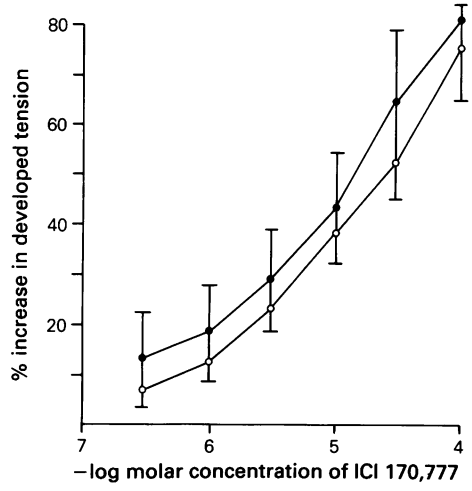


Figure 4 The effect of ICI 170777 on isometric force development in cat isolated papillary muscles in the absence (○, $n = 5$) and presence (●, $n = 5$) of atenolol (1 μM). Control tension development was 0.60 ± 0.16 g in the absence of atenolol, and 0.5 ± 0.16 g in the presence of atenolol ($P > 0.05$). Atenolol had no significant effect on the positive inotropic effect of ICI 170777.

increase (Figure 3). In a further 3 dogs, the effects of ICI 170777 and isoprenaline were examined in the presence of atenolol (5 mg kg^{-1}). The mean doses of isoprenaline required to increase dP/dt_{max} by 50% before and after administration of 5 mg kg^{-1} atenolol were 0.0134 and 3.31 μg kg^{-1} respectively (a dose-ratio of 247). In animals which had been treated with atenolol, the effect of ICI 170777 on dP/dt_{max} was attenuated but not abolished (Figure 3) when compared to the effects of the compound in the absence of atenolol. In atenolol-treated animals, a mean dose of 1.82(1.32–2.41) mg kg^{-1} of ICI 170777 was required to evoke a 50% increase in dP/dt_{max} (a dose-ratio of 4 compared to control). In atenolol-treated dogs, doses of ICI 170777 in excess of 0.5 mg kg^{-1} increased heart rate but had little effect on diastolic blood pressure. Systolic pressure tended to decrease at the highest doses of ICI 170777 (Figure 3).

Effect of ICI 170777 on isolated papillary muscles

Since the positive inotropic effect of ICI 170777 was attenuated by β-adrenoceptor blockade in the chloralose-anaesthetized dog, the interaction of the compound with atenolol was examined in isolated papillary muscles. ICI 170777 (0.3 to 100 μM, $n = 5$) evoked an increase in the tension development of papillary muscles. This positive inotropic response was repeatable but was not significantly altered in the presence of atenolol (1 μM, $n = 5$, Figure 4).

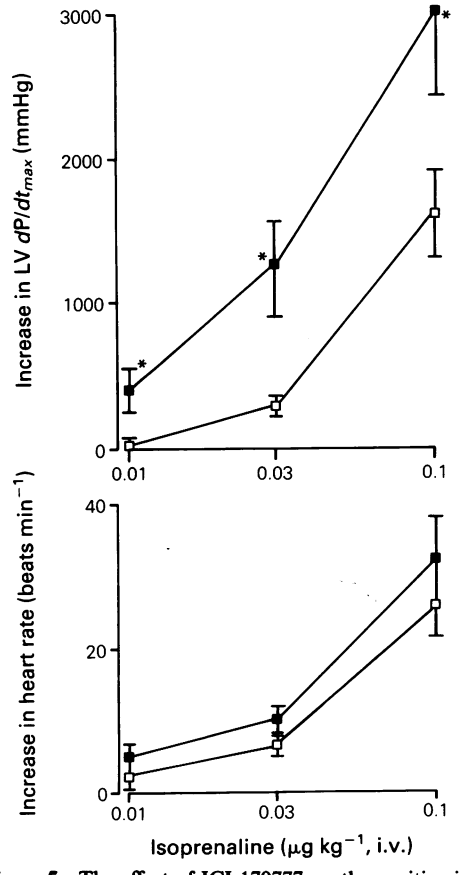


Figure 5 The effect of ICI 170777 on the positive inotropic and chronotropic effects of isoprenaline in the ganglion-blocked dog: (□) responses to isoprenaline in the absence of ICI 170777 ($n = 6$); (■) responses to isoprenaline in presence of an infusion of ICI 170777 (20 μg kg^{-1} min^{-1}, $n = 6$). *Indicates a significant ($P < 0.05$) difference from the response in the absence of ICI 170777.

Responses evoked by isoprenaline in this preparation were significantly antagonized by atenolol with a log dose-ratio = 1.34 ± 0.16 ($n = 6$) but were not significantly altered by the β_2-selective antagonist ICI 118551.

Effect of ICI 170777 on inotropic and chronotropic responses to isoprenaline in the anaesthetized ganglion-blocked dog

Dogs were anaesthetized with pentobarbitone and prepared for measurement of left ventricular

dP/dt_{max}. The ganglion blocking agent mecamylamine (5 mg kg^{-1}, i.v.) was administered. (This dose of mecamylamine abolished the tachycardia evoked by a 30 s carotid occlusion). Isoprenaline (0.01–0.1 μg kg^{-1}) was administered before and during infusion of ICI 170777 (20 μg kg^{-1} min^{-1} i.v., begun 10 min before the second dose-response curve to isoprenaline). In some dogs calcium chloride was administered (0.1 ml kg^{-1}, i.v., of a 1 M solution) before and during infusion of ICI 170777. In 6 dogs, isoprenaline evoked dose-related increases in heart rate and in dP/dt_{max}. Infusion of ICI 170777 at the low dose of 20 μg kg^{-1} min^{-1} had no significant effect on dP/dt_{max} (control = 2158 ± 318, ICI 170777 = 2250 ± 304 mmHg s^{-1}) or on blood pressure but caused a small yet significant increase in heart rate (control = 112.7 ± 5.4, ICI 170777 = 118.3 ± 6.6 $P < 0.05$). Infusion of ICI 170777 significantly enhanced the positive inotropic effects of isoprenaline but had no significant effect on the chronotropic response (Figure 5). The positive inotropic response evoked by calcium chloride was unaffected by ICI 170777 (control = 2544 ± 122, ICI 170777 = 2755 ± 235 mmHg s^{-1}).

Effects of ICI 170777 on arterial resistance and venous compliance in the dog perfused hind-limb

ICI 170777 (10–100 μg), diazoxide (200–2000 μg) and sodium nitroprusside (1–8 μg) were administered into the arterial perfusion circuit in random order. Diazoxide caused predominantly arterial dilatation decreasing arterial perfusion pressure whilst causing only small increases in venous compliance (Figure 6). Sodium nitroprusside caused large increases in venous compliance but evoked small decreases in arterial perfusion pressure. ICI 170777 had an intermediate profile having more effect in increasing venous compliance than diazoxide and more effect in decreasing arterial perfusion pressure than sodium nitroprusside (Figure 6).

Effect of ICI 170777 on haemodynamic parameters in the conscious dog

In 4 control animals, (dosed with an empty gelatine capsule) there was little significant alteration in haemodynamic parameters over a 5 h recording period. Heart rate tended to decrease with time, showing a 16 ± 5% decrease after 3 h, $P < 0.05$, but by 5 h this decrease was not significant (−8 ± 10%). Cardiac output also decreased slightly (−14 ± 4% at 3 h, $P < 0.05$; −7 ± 11% at 5 h).

ICI 170777 (2, 5 and 10 mg kg^{-1}, orally) administered to groups of 4 dogs, caused a dose-dependent

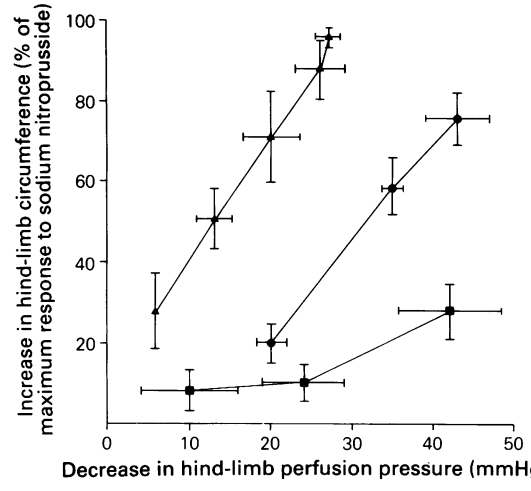


Figure 6 The effect of ICI 170777 (●, 10, 50, 100 μg), diazoxide (■, 200, 800, 2000 μg) and sodium nitroprusside (▲, 1, 2, 4, 6, 8 μg) on arterial perfusion pressure and circumference of the dog perfused hind-limb preparation ($n = 3$). (Increases in limb circumference are indicative of an increase in venous capacitance. The initial arterial perfusion pressures in the hind-limb of the three dogs were 100, 100 and 105 mmHg).

increase in dP/dt_{max} which, at all doses, was significantly different from data in untreated animals for at least one time point (Figure 7). Heart rate was also increased but this effect was less pronounced than the effect on dP/dt_{max} and was only statistically significant at the highest dose (Figure 7). At 2 and 5 mg kg^{-1} systolic and diastolic blood pressure were not significantly altered; at 10 mg kg^{-1} both showed a significant reduction (Figure 7). Stroke volume was not significantly altered by ICI 170777, cardiac output showed a tendency to increase which was significant at some time points after the 10 mg kg^{-1} dose (Figure 7). Total peripheral resistance was decreased by 5 and 10 mg kg^{-1} doses of ICI 170777 (Figure 7).

In a different group of three dogs, 5 mg kg^{-1} of ICI 170777 was administered orally and intravenously on separate occasions. The peak increase in dP/dt_{max} evoked by ICI 170777 given by the oral route (control = 2747 ± 297 mmHg s^{-1}, ICI 170777 = 5514 ± 292 mmHg s^{-1}) was only slightly less than that evoked by the compound administered i.v. (control = 2744 ± 316 mmHg s^{-1}, ICI 170777 = 6459 ± 438 mmHg s^{-1}). Thus ICI 170777 appears to have a good bio-availability.

In order to assess the potential of ICI 170777 for induction of tachyphylaxis, a different group of four

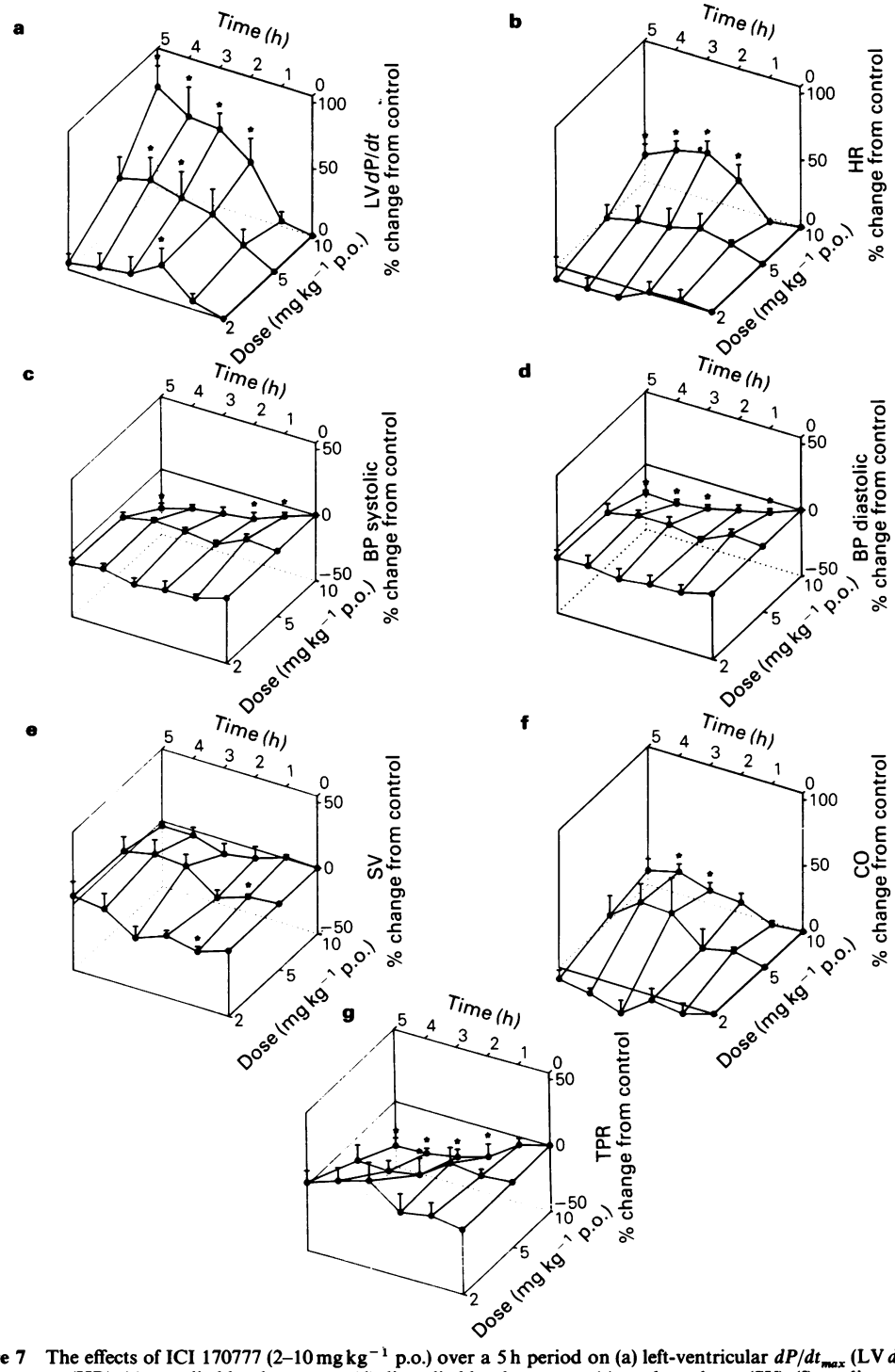


Figure 7 The effects of ICI 170777 (2–10 mg kg^{-1} p.o.) over a 5 h period on (a) left-ventricular dP/dt_{max} (LV dP/dt), (b) heart rate (HR), (c) systolic blood pressure, (d) diastolic blood pressure, (e) stroke volume (SV), (f) cardiac output (CO), and (g) calculated peripheral resistance (TPR), in a group of 4 conscious dogs.
*Indicates value is significantly different from that in a control group of 4 dogs.

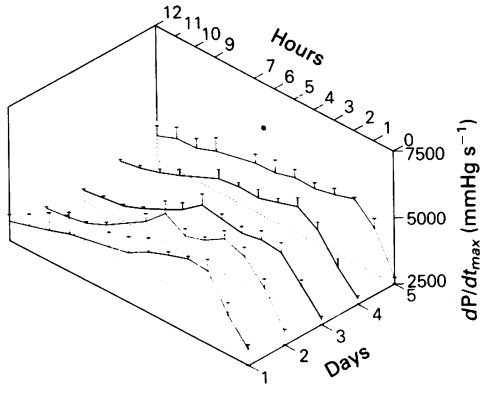


Figure 8 The effect of repeated administration of ICI 170777 (5 mg kg^{-1}, orally) on left-ventricular dP/dt_{max} in a group of 4 conscious dogs for 5 days. ICI 170777 was administered at 12 h intervals and the effect on dP/dt_{max} was recorded between doses for one 12 h period each day.

dogs were given the compound twice daily for 5 days at 5 mg kg^{-1}. Haemodynamic parameters were recorded continuously for 12 h each day immediately following administration of the compound. ICI 170777 produced a marked increase in dP/dt_{max} on each day of dosing (Figure 8). There was no evidence for a diminution of the response with repeated doses and the duration of action ranged from 10 h to in excess of 12 h.

Discussion

The results of this study demonstrate that ICI 170777 is an orally active compound which has positive inotropic and vasodilator effects. The positive inotropic effect of ICI 170777 was demonstrated by an increase in dP/dt_{max} in both anaesthetized and conscious animals, and by an increase in force development in isolated ventricular muscle.

The precise mechanism by which ICI 170777 exerts its positive inotropic effect cannot be elucidated from the present results. The compound however, appears to interact with the β-adrenoceptor control of cardiac inotropic state but also has positive inotropic effects which are independent of the β-adrenoceptor system. This latter effect was demonstrated by the inotropic effects of the compound in the presence of β-adrenoceptor blockade both *in vivo* and *in vitro*. The former effect was deduced from the effects of the compound in the chloralose anaesthetised dog. In this preparation the positive inotropic response curve to ICI 170777 was shifted to the right by β-blockade, but this displacement was of much smaller magnitude than that seen for isoprenaline. One possible explanation for these results is that part of the positive inotropic effect of ICI 170777 is due to an increase in sympathetic drive to the ventricle, as a reflex response to the effects of the compound on the peripheral circulation. Although the chloralose anaesthetized animal is known to have relatively intact cardiovascular reflexes (Greishmeir, 1965) a reflex component of the response is unlikely as ICI 170777 did not lower blood pressure and heart rate was not augmented. A localized release of catecholamines in ventricular tissue by the compound could also explain the results, however the absence of an effect of atenolol on responses to ICI 170777 in the isolated papillary muscle argues against this. The most feasible explanation of the results is that ICI 170777 selectively enhances the positive inotropic effects of β-adrenoceptor stimulation on the ventricle. This explanation is supported by experiments performed in ganglion-blocked dogs (to avoid reflex influences) which showed such a selective potentiating action. A similar selective potentiation of positive inotropic responses to β-adrenoceptor stimulation has been noted *in vitro* with the phosphodiesterase inhibitor SK&F 94120 (Blakemore *et al.*, 1985).

ICI 170777 had significantly less effect on heart rate than on indices of cardiac inotropic state. The most pronounced effect on heart rate was observed in the areflexic dog preparation. However, in this preparation ICI 170777 exhibited a significantly greater positive inotropic effect for a given increase in heart rate than isoprenaline. The relationship between positive inotropic and positive chronotropic effects for isoprenaline found in the present study is similar to that previously reported in the dog (Furnival *et al.*, 1971; Nuttall & Snow, 1982).

An effect of ICI 170777 on heart rate was much less pronounced in the chloralose-anaesthetized and conscious dog than in the areflexic preparation. It may be that the compound evokes an increase in vagal tone in the former preparations as the positive inotropic effects could increase the ejection velocity from the left ventricle and consequently the rate of change of pressure at the carotid sinus (Schmidt *et al.*, 1972). Another possibility is that venous dilatation evoked by ICI 170777, unloads cardiac mechanoreceptors with a resulting reduction in chronotropic drive to the heart (Ledsome & Linden, 1964; Donald & Shepherd, 1978). Whatever the explanation for the limited tachycardic effect of ICI 170777 in the reflexic animal, it allows an increase of approximately 50% in dP/dt_{max} to be achieved without a significant increase in heart rate. Such a

haemodynamic profile will have important consequences for a compound to be used in heart failure, as an increase in heart rate may be deleterious in terms of the concomitant increase in myocardial oxygen consumption and the reduced diastolic interval for perfusion of the coronary arteries (Evans *et al.*, 1982).

The vasodilator effect of ICI 170777 influenced both arterial and venous tone. The results do not allow speculation concerning the mode of action of the compound; however, it cannot be due to a reduction in sympathetic vasoconstrictor tone since it occurred in denervated preparations. The relative selectivity of the vasodilator effect of ICI 170777 for arterial and venous vessels was compared with that of sodium nitroprusside and diazoxide. Diazoxide and sodium nitroprusside are known respectively to be relatively arterial and venous selective in man (Robinson & Collier, 1979). The results of the present study confirm this relative selectivity, validating the model, and indicate an intermediate profile for ICI 170777. A balanced vasodilator effect on arterial and venous vessels should be advantageous in the treatment of heart failure as both arterial and venous vasoconstrictor tone is increased (Vanhoutte, 1983).

The effects of ICI 170777 in conscious dogs illustrate a number of its properties. The compound is absorbed orally and has an onset of action within 1–2 h of administration. The peak effects of the compound are seen 2–5 h after dosing and the duration of action after a $5\,mg\,kg^{-1}$ oral dose is 10–12 h. There was no evidence of the development of tachyphylaxis after repeated administration.

The haemodynamic profile in the conscious dog (positive inotropy, decrease in peripheral resistance and only minor effects on blood pressure and heart rate, except at high doses) is consistent with its effects in anaesthetized preparations. However, stroke volume was not enhanced and cardiac output only increased at the highest dose level, concomitant with an increase in heart rate. This latter observation is in agreement with other studies which have shown that positive inotropic stimulation does not enhance stroke volume in the non-failing heart (Dage *et al.*, 1982; Pouleur *et al.*, 1982b). An additional factor contributing to the lack of increase in stroke volume in the case of ICI 170777 may have been its venodilator effect which could cause pooling of blood in the veins and a reduction in end-diastolic volume.

In conclusion, the results of this study indicate that ICI 170777 is an orally active compound with good bio-availability, which exerts positive inotropic effects on the heart and can dilate both arteries and veins. These properties suggest that the compound may be useful in the treatment of congestive heart failure in man since it should acutely reverse the major haemodynamic abnormalities associated with this disorder.

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Pressor effects of the α_2-adrenoceptor agonist B-HT 933 in anaesthetized and haemorrhagic rats: comparison with the haemodynamic effects of amidephrine

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1 Blood pressure responses to single and multiple bolus doses of the α_2-adrenoceptor agonist B-HT 933 were analysed in intact anaesthetized rats which were either normotensive or hypotensive as a result of haemorrhage. Single bolus doses of B-HT 933 in normotensive rats induced a fall in blood pressure, whilst further doses induced dose-dependent pressor responses which were inhibited by the α_2-adrenoceptor antagonist yohimbine and unaffected by the α_1-adrenoceptor agonist prazosin. In the haemorrhagic, hypotensive animals, single bolus doses of B-HT 933 induced immediate dose-dependent pressor responses; the maximum pressor responses to the bolus of B-HT 933 and its ED_{50} values were the same in both the normotensive and hypotensive, haemorrhagic animals.

2 Cardiac output, its distribution and tissue blood flows were determined with tracer microspheres in intact anaesthetized normotensive and haemorrhagic, hypotensive rats during depressor (normotensive) and pressor (normotensive and hypotensive) responses to B-HT 933. Haemodynamics were also determined during pressor responses to the α_1-adrenoceptor agonist amidephrine.

3 In control normotensive rats, a single dose of B-HT 933 ($1\,mg\,kg^{-1}$) reduced blood pressure by reducing cardiac output (through a decrease in heart rate). It increased the fractional distribution of cardiac output to the spleen and stomach, reduced it to the heart and liver and reduced cardiac and hepatic blood flow. A further dose of B-HT 933 ($1\,mg\,kg^{-1}$ bolus followed by $100\,\mu g\,min^{-1}$ infusion) increased blood pressure by increasing total peripheral resistance, which was accompanied by decreased proportions of cardiac output passing to the heart, liver and testes. There was also increased fractional distribution of cardiac output to the lungs, spleen, kidneys and stomach but blood flows through the liver and testes were reduced. Amidephrine ($6\,\mu g\,kg^{-1}$ bolus followed by $0.5\,\mu g\,min^{-1}$ infusion) increased blood pressure by increasing cardiac output through an increased stroke volume. It increased cardiac output distribution to the kidneys and brain, increasing blood flow through the heart, lungs, brain, testes, epididimides, skin and large intestine.

4 Haemorrhage caused a fall in blood pressure which resulted from decreased total peripheral resistance and cardiac output (the latter due to decreases in both heart rate and stroke volume). It reduced the proportion of cardiac output distributed to the lungs, spleen, kidneys, testes and pancreas/mesentery and decreased blood flow through these organs as well as through the heart, liver, brain, epididimides, skin and the gastrointestinal tract. Haemorrhage also caused mild acidosis, hypocapnia and hyperoxia. B-HT 933 administration to the haemorrhagic, hypotensive rats partially restored blood pressure, by increasing stroke volume, and increased the fractional distribution of cardiac output to the spleen, kidneys and stomach whilst decreasing it to the heart. B-HT 933 restored blood flow through the spleen, kidneys, brain, testes, epididimides, skin, stomach and large intestine to control values. Amidephrine restored blood pressure in the haemorrhagic, hypotensive rats by increasing stroke volume; it also increased the proportion of cardiac output sent to the liver and restored blood flow through the heart, liver, kidneys, brain, testes, skin, stomach and large intestine. Haemorrhage potentiated the pressor responses to infusions of amidephrine whilst inhibiting those to infusions of B-HT 933.

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5 It is concluded that single doses of B-HT 933 induce a fall in blood pressure, probably due to its known central action on α_2-adrenoceptors, whilst further doses induce pressor responses through activation of peripheral vascular α_2-adrenoceptors. In haemorrhagic, hypotensive rats, B-HT 933 only induces pressor responses and both B-HT 933 and amidephrine restore deficits in the circulation through vital organs. Selective α-adrenoceptor agonists may, therefore, be of therapeutic value for the treatment of haemorrhagic shock.

Introduction

Single doses of α_2-adrenoceptor agonists decrease blood pressure in anaesthetized rats through stimulation of central α_2-adrenoceptors (Kobinger, 1978). In the pithed rat, peripheral post-junctional α_2-adrenoceptors and α_1-adrenoceptors mediate pressor responses (Docherty *et al.*, 1979; Drew & Whiting, 1979; Docherty & McGrath, 1980). Previously we have observed that, in anaesthetized rats, following an initial depressor i.v. dose of the α_2-adrenoceptor agonist, B-HT 933, subsequent administration of B-HT 933 induced dose-dependent pressor responses (MacLean *et al.*, 1988) similar in haemodynamic profile to those described in the pithed rat by Hiley & Thomas (1987). α-Adrenoceptor agonists induce systemic pressor responses in pithed rats by increasing both cardiac output and total peripheral resistance (Kalkman *et al.*, 1984; Hiley & Thomas, 1987; MacLean & Hiley, 1988a,b). The α_1-adrenoceptor agonist phenylephrine and the α_2-adrenoceptor agonists B-HT 933 and xylazine induce these pressor responses without reducing blood flow through the renal vascular bed, indeed phenylephrine was seen to increase renal blood flow (Hiley & Thomas, 1987).

Haemorrhagic shock can comprise arterial hypotension, low cardiac output and low peripheral resistance (see Hardaway, 1985, for review) and blood flows through the heart, lungs, liver, gut and kidney are severely compromised. This is due largely to the sudden production of catecholamines by the adrenal medulla (Carrière, 1969; Grandchamp *et al.*, 1971; Hardaway, 1985). Hock *et al.* (1982) showed that haemorrhage caused a 30% decrease in total renal blood flow in anaesthetized dogs and restoration of renal blood flow is one of the major aims in the therapeutic approach to haemorrhage.

Sympathomimetic agents have been used in cases of hypovolaemic shock but noradrenaline exacerbates the already existing renal vasoconstriction (Rao *et al.*, 1968) and isoprenaline induces unfavourable stimulation of the heart (Rona *et al.*, 1963). Dopamine has received much attention as a potential therapeutic agent in shock states (Goldberg, 1972) as it increases cardiac output and can selectively dilate renal and splanchnic vascular beds in haemorrhagic, hypotensive dogs (Carvalho *et al.*, 1969; Goldberg, 1972). Even so, dopamine is not effective at increasing survival in experimental haemorrhagic shock (Gifford *et al.*, 1968; Dagher *et al.*, 1970) and may only be of use in certain clinical circumstances depending upon the aetiology of the shock state (Chernow & Roth, 1986). Hence, the search for a more suitable drug for the treatment of shock states continues.

In the light of our previous observations that the pressor responses in pithed rats to selective α_1-adrenoceptor and α_2-adrenoceptor agonists are not accompanied by renal vasoconstriction, this study was designed to characterize the haemodynamic changes induced by single and multiple doses of B-HT 933 in anaesthetized normotensive and haemorrhagic, hypotensive rats. The haemodynamic changes induced by B-HT 933 were also compared with those induced during pressor responses to the selective α_1-adrenoceptor agonist, amidephrine.

Methods

Animals

Male Wistar rats, weighing 250–300 g (Bantin & Kingman Ltd, Hull), were used throughout this study. The rats were anaesthetized with sodium thiobutabarbitone (120 mg kg^{-1}, i.p.; Inactin, Byk Gulden, Konstanz, F.R.G.) and respired with air through a tracheal cannula by means of a respiratory pump (BioScience, Sheerness, U.K.) operating at 54 cycles min^{-1} with a volume of 15 ml kg^{-1}. All pressure measurements were made by Bell & Howell type 4-422-0001 pressure transducers connected to a Grass 7D polygraph; heart rate was determined from the systemic blood pressure trace by a Grass 7P44 cardiotachograph. Animals were maintained at 37°C by a homeothermic blanket system (Bioscience, Shearness, Kent).

Determination of dose-response curves

Experimental procedure The right common carotid artery of the rat was cannulated for systemic arterial blood pressure monitoring. The left external jugular vein was cannulated for drug administration and the

left femoral artery cannulated for withdrawal of blood. When the blood pressure had stabilized after the surgery, a period of 30 min was allowed to elapse before administration of drugs or saline.

Drug administration All drugs were administered i.v. in 0.9% saline. In the B-HT 933 groups, an initial bolus injection of 0.7 mg kg^{-1} B-HT 933 (2-amino-6-ethyl-5,6,7,8-tetrahydro-4H-oxazolo-[4,5-d]-azepine dihydrochloride; azepexole; Boehringer Ingelheim, Bracknell, U.K.) was given and any effect on blood pressure allowed to stabilize (which took approximately 10 min). This first dose was then followed by bolus doses of B-HT 933 ranging from 0.18 mg kg^{-1} to 2.14 mg kg^{-1} (the doses given in random order, at 5 min intervals and in 0.1 ml to 0.3 ml saline). An injection of either 0.3 mg kg^{-1} or 1 mg kg^{-1} yohimbine (Sigma Chemical Co., Poole, Dorset) or 50 μg kg^{-1} prazosin (Pfizer, Sandwich, U.K.) was then administered and a further dose-response curve determined to doses of B-HT 933 ranging from 0.68 mg kg^{-1} to 2.21 mg kg^{-1}.

Determination of cardiac output and its distribution

Experimental procedure The rats were prepared by cannulating the right femoral artery, for the measurement of systemic arterial blood pressure and the left femoral artery, for the withdrawal of blood by means of a Braun Perfusor IV pump (Melsungen, F.R.G.). Then, with the aid of pressure monitoring, a cannula was passed down the right common carotid artery into the left ventricle. Drugs and saline were administered, 30 min after basal blood pressure had stabilized (10–15 min), through a cannula previously placed in the left external jugular vein. When a sustained pressure response to the agonists or saline (see below) had been obtained, 60,000–80,000 ^{113}Sn labelled microspheres (15 ± 3 μm diameter; NEN, Boston, MA, U.S.A.), suspended by ultrasonication in saline containing 0.01% Tween 80, were injected into the ventricle over 20 s. Blood was withdrawn from the left femoral artery at a rate of 0.5 ml min^{-1} during, and for 70 s after, the microsphere injection. The circulation was stopped with an air embolism and the organs dissected out, weighed and placed in scintillation vials for counting in a Packard Autogamma 500 γ-scintillation counter.

The number of counts in the blood sample was also determined in order that cardiac output and tissue blood flow could be measured as described by McDevitt & Nies (1976): the cardiac output (ml min^{-1}) is given by ([counts injected × blood withdrawal rate]/[counts in blood sample]); the fraction of the cardiac output passing to an organ by ([counts in organ]/[counts injected]); and the organ blood flow (ml min^{-1} g^{-1} tissue) by ([cardiac output × fraction of cardiac output to organ]/[organ weight]).

Drug administration All drugs were administered i.v. in 0.9% saline. In one group of rats ($n = 8$ for all groups), once basal blood pressure had stabilized, 1 mg kg^{-1} B-HT 933 was administered and, after 30 min, the microspheres were injected during the infusion phase of a bolus saline injection (0.2 ml) followed immediately by a saline infusion (0.1 ml min^{-1}); this will be subsequently referred to as the B-HT 933/saline group. In the other control B-HT 933 treated rats (the B-HT 933/B-HT 933 group), the initial B-HT 933 dose was followed, after 30 min, by a microsphere injection during the constant pressor response to a 1 mg kg^{-1} bolus of B-HT 933 followed by a 100 μg min^{-1} infusion.

In haemorrhagic, hypotensive animals (B-HT 933/haemorrhage group), the first bolus dose of B-HT 933 (1 mg kg^{-1}) administered after production of haemorrhage (see below), induced an immediate pressor response; it was followed 10 min later by a second bolus (1 mg kg^{-1}) and infusion of B-HT 933 (100 μg min^{-1}) and the microspheres were injected during the stable phase of the infusion.

Amidephrine (Mead Johnson & Co., Evansville, IN, U.S.A.) was administered as a 6 μg kg^{-1} bolus followed by a 0.5 μg min^{-1} infusion in both haemorrhagic and non-haemorrhagic rats (these are referred to as the amidephrine/haemorrhage and amidephrine/control groups). The pressor response to amidephrine, as with other α_1-adrenoceptor agonists is biphasic (McGrath & O'Brien, 1987), with an initial peak pressor phase followed by a lower, though more sustained, secondary pressor response during which the microspheres were injected.

For the normotensive and haemorrhagic, hypotensive saline control groups, an initial bolus of 0.2 ml saline was followed by a second 0.2 ml bolus and the microsphere injection was given during an 0.1 ml min^{-1} infusion of saline (these are referred to as the saline/saline and haemorrhage/saline groups).

Production of haemorrhagic shock

After basal blood pressure had stabilized following the cannulations, blood was slowly withdrawn from the left femoral artery until a mean arterial pressure of approximately 40–45 mmHg was achieved. This was generally done by reducing arterial pressure in 25 mmHg steps. After the required mean arterial blood pressure was achieved, this was maintained for 30 min before drug (or saline) and microsphere injections. The blood was collected and heparinized (10 iu, Paines & Byrne Ltd, Greenford, U.K.), kept at 37°C and reinfused if mean arterial blood pressure fell below 40 mmHg. The total volume of blood removed was determined for each animal.

Determination of arterial blood gases and pH

These were analysed in all animals by removing 125 μl of blood from the right femoral artery, before agonist or saline administration, both before and after haemorrhage. The sample was analysed with a Corning 166 micro blood gas analyzer and the values ($n = 24$) obtained were as follows: before haemorrhage; pH = 7.44 ± 0.02, $Paco_2$ = 35.1 ± 1.5, Pao_2 = 70.8 ± 2.7: after haemorrhage; pH = 7.36 ± 0.02, $Paco_2$ = 31.4 ± 1.8, Pao_2 = 86.3 ± 1.9. For all these parameters, the values before haemorrhage were significantly different from those after ($P < 0.01$; Student's paired t test). Within these overall means there were no significant differences between either the 4 haemorrhagic or the 3 non-haemorrhagic groups.

Statistical comparison and calculation of ED_{50}

All results are given as the mean ± s.e.mean. The statistical significance between groups was assessed by one-way random block analysis of variance followed by the least significant difference procedure (Snedecor & Cochran, 1980). Within group comparisons for heart rate and blood pressure changes were made by Student's paired t test.

In the dose-response study, for each individual rat, the dose of B-HT 933 causing 50% of the maximum response was calculated (ED_{50}). These values were combined for each group and the ED_{50} values for B-HT 933 in the control, normotensive group and the haemorrhagic, hypotensive group were compared by the use of Student's unpaired t test.

Results

Blood pressure responses to B-HT 933 in normotensive and haemorrhagic, hypotensive rats

In the dose-response study, for each individual rat, the dose of B-HT 933 causing 50% of the maximum response was calculated (ED_{50}). These values were combined for each group and the ED_{50} values for B-HT 933 in the control, normotensive group and the haemorrhagic, hypotensive group were compared by the use of Student's unpaired t test.

pressure of 38 ± 5 mmHg and the ED_{50} for B-HT 933 in these rats was 0.59 ± 0.08 mg kg^{-1}. Figure 1 also shows that the pressor responses to B-HT 933 were inhibited by the selective α_2-adrenoceptor antagonist yohimbine in a dose-dependent fashion, whilst they were unaffected by the selective α_1-adrenoceptor antagonist prazosin. In the haemorrhagic, hypotensive rats ($n = 10$), 4.3 ± 0.3 ml of blood was removed, inducing a fall in mean arterial blood pressure (from 102 ± 5 mmHg) of 54 ± 5 mmHg. A single dose of B-HT 933 (1 mg kg^{-1}) did not induce a further fall in blood pressure but, instead, an increase in mean arterial blood pressure was observed. After these bolus doses of B-HT 933, mean arterial pressure returned, over 1–2 min, to the same level as observed before its administration (48 ± 2 mmHg, before; 45 ± 2 mmHg, after); that is, at no time was a decrease in mean arterial blood pressure observed in haemorrhagic, hypotensive rats. Repetition of these pressor responses to B-HT 933 showed them to be dose-dependent (Figure 2). The maximum pressor response to B-HT 933 in the haemorrhagic, hypotensive rats was 34 ± 7 mmHg and the ED_{50} was 0.59 ± 0.09 mg kg^{-1}; these values were not significantly different from those obtained in the normotensive control rats.

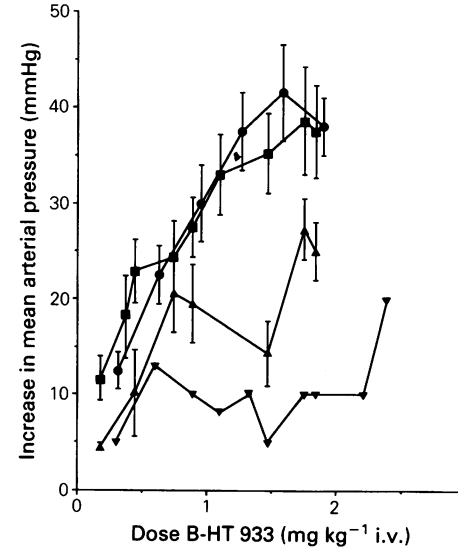


Figure 1 Dose-response curves for the increases in mean arterial pressure induced by B-HT 933 in the anaesthetized rat. (■) B-HT 933 alone, $n = 10$; (●) B-HT 933 in the presence of 50 μg kg^{-1} prazosin, $n = 4$; (▲) B-HT in the presence of 0.3 mg kg^{-1} yohimbine, $n = 4$; (▼) B-HT in the presence of 1 mg kg^{-1} yohimbine, $n = 3$. Vertical lines represent ±1 s.e.mean.

Haemodynamic effects of B-HT 933 and amidephrine in normotensive and haemorrhagic, hypotensive rats

Table 1 shows the effects of B-HT 933 and amidephrine on the systemic haemodynamics of anaesthetized rats. Basal mean blood pressure and diastolic blood pressure did not differ between any of the study groups ($P > 0.05$). In the B-HT 933/saline

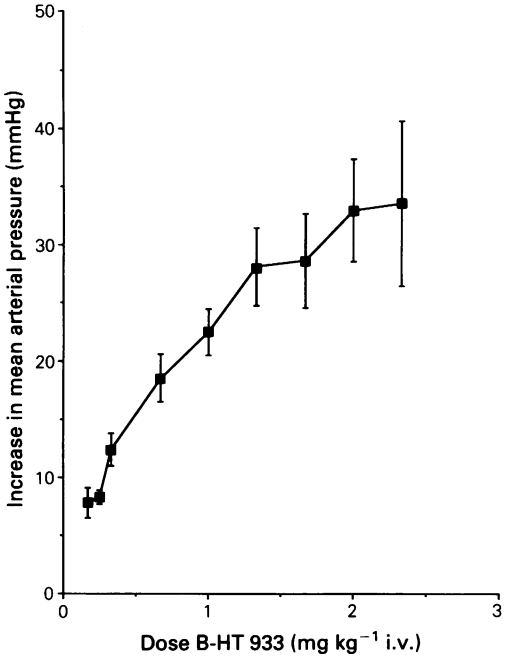


Figure 2 Dose-response curve for the increases in mean arterial pressure induced by B-HT 933 in the anaesthetized, haemorrhagic rat. Vertical lines represent ±1 s.e.mean, $n = 10$.

group of rats, the α_2-adrenoceptor agonist induced falls in both mean blood pressure and diastolic blood pressure of 27% ($P < 0.001$, within group); subsequent saline administration did not affect blood pressure further. This fall in blood pressure was due to an 18% lower cardiac output, relative to the saline/saline controls, which in turn resulted from a 17% lower heart rate since stroke volume was the same in the two groups during the microsphere injection. Total peripheral resistance was unchanged by this single dose of B-HT 933. In the B-HT 933/B-HT 933 group, the initial bolus injection of the α_2-adrenoceptor agonist again induced falls in mean blood pressure and diastolic blood pressure (of 34%) and a 23% reduction in heart rate (changes relative to the basal values in this group, $P < 0.001$). Administration of the subsequent bolus dose and infusion of B-HT 933 then induced increases in both mean blood pressure and diastolic blood pressure which were 50% and 55%, respectively, greater ($P < 0.001$, within group) than the pressures immediately before the microsphere injection. The increase in blood pressure was due to a 30% increase in total peripheral resistance relative to the normotensive group given saline. Cardiac index was not significantly altered relative to the saline control group since the effects of the 18% increase in stroke volume were counteracted by the pre-existing 23% fall in blood pressure induced by the first dose of B-HT 933. Amidephrine caused increases in mean blood pressure and diastolic blood pressure of 18% above the basal blood pressures in this group and these increases were due to a 16% greater cardiac output than in the saline/saline controls. In turn, the greater cardiac output resulted from a 19% increase in stroke volume relative to the saline control group despite a 3% fall in heart rate compared to the saline control group. Total peripheral resistance was unaffected by this dose of amidephrine.

Table 1 also shows the effect of haemorrhage on cardiovascular haemodynamics and the influence of subsequent administration of either B-HT 933 or amidephrine. The total volumes of blood removed from the rats in the haemorrhage/saline, B-HT 933/haemorrhage and amidephrine/haemorrhage groups were 4.2 ± 0.2 ml, 4.0 ± 0.3 ml and 4.0 ± 0.5 ml, respectively. Haemorrhage induced falls in mean blood pressure and diastolic blood pressure of 60% and 65% respectively, the mean blood pressures after haemorrhage being 44 ± 3 mmHg, 39 ± 2 mmHg and 44 ± 3 mmHg, respectively in the haemorrhage/saline, B-HT 933/haemorrhage and amidephrine/haemorrhage groups. From comparison of the data for the haemorrhage/saline group and the saline/saline groups shown in Table 1, it can be seen that this fall in systemic blood pressure was due to both a 52% lower cardiac output and a 27% lower total peripheral resistance. The decrease in cardiac output was due to both a 9% decrease in heart rate ($P < 0.001$; compared to the basal heart rate in this group) and a 52% lower stroke volume than in the saline control group.

After haemorrhage, the initial dose of B-HT 933 did not further decrease either mean arterial or diastolic blood pressures which were reduced by 63% from the basal values in this group. The subsequent dose of B-HT 933 increased mean and diastolic blood pressures by 38% and 28%, respectively, above those recorded immediately before the second dose of B-HT 933, i.e. after the haemorrhage. Total peripheral resistance was not significantly different between the B-HT 933/haemorrhage and saline/haemorrhage groups. The increase in blood pressure was probably due to a change in cardiac performance since, although cardiac output was not significantly increased over that in the haemorrhage/saline group, stroke volume was 56% increased compared to the latter group.

Following haemorrhage, amidephrine also increased mean systemic and diastolic blood pressures (by 72% and 103%, respectively, above those values seen immediately before amidephrine administration; $P < 0.001$). The increased blood pressure was due to a 56% increase in cardiac output com-

Table 1 The effects of B-HT 933 and amidephrine on blood pressure, cardiac index and heart rate in anaesthetized normotensive and haemorrhagic rats

	Saline/saline	*B-HT 933/ saline*	*B-HT 933/ B-HT 933*	*Amidephrine/ control*	*Haemorrhage/ saline*	*B-HT 933/ haemorrhage*	*Amidephrine/ haemorrhage*
Basal mean arterial pressure (mmHg)	111 ± 5	112 ± 4	121 ± 8	122 ± 5	116 ± 7	105 ± 6	101 ± 10
Change in mean arterial pressure	−9 ± 3	−30 ± 6 −1 ± 3	−41 ± 7 43 ± 4***	22 ± 2***	−70 ± 7 −4 ± 2	−66 ± 7 15 ± 2***,†††	−51 ± 8 36 ± 5***,†††
Basal diastolic pressure (mmHg)	85 ± 7	91 ± 4	99 ± 6	101 ± 5	99 ± 7	86 ± 7	78 ± 10
Change in diastolic pressure	3 ± 3	−25 ± 5 1 ± 3	−34 ± 5 36 ± 4***	19 ± 2***	−64 ± 7 −3 ± 1	−54 ± 7 9 ± 2*,†	−46 ± 9 33 ± 7***,†††
Basal heart rate (beats min^{-1})	431 ± 16	415 ± 15	455 ± 12	423 ± 5	450 ± 9	438 ± 9	455 ± 18
Change in heart rate	5 ± 4	−70 ± 14 3 ± 3	−105 ± 15 −1 ± 4	−13 ± 4***	−41 ± 12 3 ± 3	−78 ± 13 −10 ± 5	−54 ± 17 −6 ± 5
Cardiac index (ml min^{-1} 100 g b.wt.)	24.2 ± 1.4	19.9 ± 1.5*	23.1 ± 1.1	28.1 ± 1.3*	11.7 ± 1.2***	15.3 ± 1.0***	18.3 ± 1.7*,††
Stroke volume (μl)	168 ± 11	170 ± 16	199 ± 6*	200 ± 8*	81 ± 7***	126 ± 8*,††	133 ± 10††
TPR (mmHg, ml^{-1} min 100 g b.wt.)	4.4 ± 0.4	4.2 ± 0.3	5.7 ± 0.3*	5.2 ± 0.3	3.2 ± 0.2*	3.6 ± 0.3	5.1 ± 0.7

For all groups, $n = 8$. Data are for saline-treated control rats (saline/saline), saline-treated haemorrhagic rats (haemorrhage/saline), normotensive (B-HT 933/saline) and haemorrhagic, hypotensive (B-HT 933/haemorrhage) rats given one dose of B-HT 933, normotensive rats injected with microspheres during a second pressor dose of B-HT 933 (B-HT 933/B-HT 933), normotensive (amidephrine/control) and haemorrhagic, hypotensive (amidephrine/haemorrhage) rats given amidephrine. For the changes in blood pressure and heart rate, the upper values are changes induced by the initial dose of B-HT 933 and/or haemorrhage; the lower values are the changes induced by the administration of saline, amidephrine or the final dose of B-HT 933 and are the differences between values immediately before drug or saline administration and those at the mid-point of the microsphere injection. Statistical differences were assessed by analysis of variance: between all the drug-treated control and haemorrhagic groups and the saline/saline group, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$: between the drug-treated haemorrhagic groups and the haemorrhage/saline group, $\dagger P < 0.05$, $\dagger\dagger P < 0.01$, $\dagger\dagger\dagger P < 0.001$.

pared to the haemorrhage/saline group; this increase was due to a 64% increase in stroke volume. The total peripheral resistance after amidephrine administration to the haemorrhagic, hypotensive rats was not significantly different from that in the haemorrhage/saline control group. It is noteworthy that, after amidephrine administration, the stroke volume in haemorrhagic, hypotensive animals was no longer significantly different from that in the saline/saline controls and, also, that after either B-HT 933 or amidephrine, the total peripheral resistance in the haemorrhagic, hypotensive animals was no longer significantly different from that observed in the non-haemorrhagic saline control group. Further, the increases in both mean arterial and diastolic blood pressures induced by the B-HT 933 infusion in the haemorrhagic, hypotensive rats were significantly lower ($P < 0.001$) than the increases in blood pressures that the same dose produced in the normotensive rats. Conversely, the increases in mean arterial pressure and diastolic blood pressure induced by the amidephrine infusion were significantly higher ($P < 0.05$) in the haemorrhagic, hypotensive rats than those observed in the normotensive rats. The initial peak pressor responses to the amidephrine bolus injection (i.e. the difference in blood pressure immediately before and after the bolus injection, before the onset of the sustained response to the amidephrine infusion) were 43 ± 4 mmHg (mean arterial pressure change) and 34 ± 2 mmHg (diastolic blood pressure change). These changes were not significantly different from the equivalent peak pressor responses to the amidephrine bolus injection observed in the haemorrhagic, hypotensive rats (48 ± 5 mmHg, mean arterial blood pressure change; 36 ± 5 mmHg, diastolic blood pressure change). Therefore, only the secondary, sustained pressor response to the infusion of amidephrine was potentiated by haemorrhage.

Figure 3 shows the effects of B-HT 933 and amidephrine on the distribution of cardiac output to the organs in normotensive anaesthetized rats. A single, depressor, dose of B-HT 933 reduced the fractions of cardiac output distributed to the heart (by 38%) and liver (by 34%) and increased those passing to the spleen and stomach by 146% and 60% respectively (compared to the saline/saline control group). The subsequent second dose of B-HT 933, which induced a pressor response, decreased (relative to the saline control group) the proportions of cardiac output passing to the heart, liver and testes by 43%, 51% and 36% respectively, and increased those to the lungs (by 151%), spleen (106%), kidneys (37%) and stomach (53%). Amidephrine increased cardiac output distribution to the kidneys and brain by 32% and 55% respectively, relative to the distributions found in the saline-treated control rats.

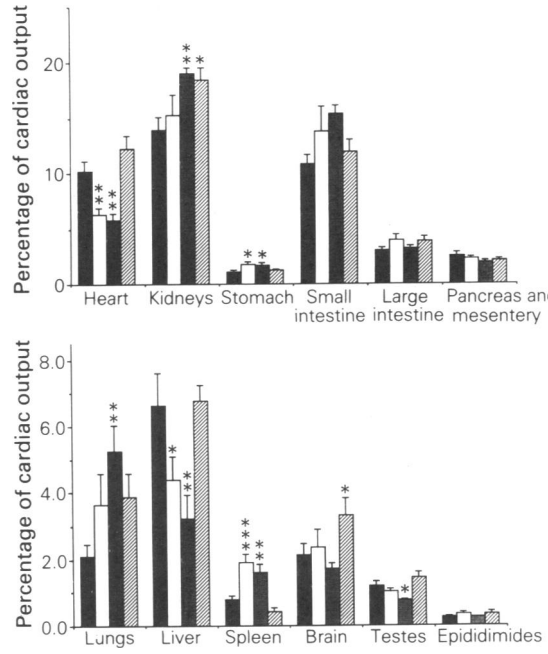


Figure 3 Effects of B-HT 933 and amidephrine on the distribution of cardiac output in anaesthetized rats. The first (solid) columns show the results from rats given saline alone; the open columns show data from rats give a single depressor dose ($1\,mg\,kg^{-1}$) of B-HT 933; the third (stippled) column in each group shows the results from rats during a second pressor dose of B-HT 933 ($1\,mg\,kg^{-1}$ bolus + $100\,\mu g\,min^{-1}$ infusion); and the hatched columns the data from rats given amidephrine ($6\,\mu g\,kg^{-1}$ bolus + $0.5\,\mu g\,min^{-1}$ infusion). $n = 8$ for each group. Statistical differences between the saline control group and the drug-treated groups were assessed by analysis of variance: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure 4 shows the effects of haemorrhage, before and after administration of B-HT 933 and amidephrine, on the distribution of cardiac output to the organs in the anaesthetized rat. Haemorrhage reduced the proportions of cardiac output received by the lungs, spleen, kidneys, testes and pancreas/mesentery, by 71%, 41%, 29%, 40% and 41% respectively, relative to the saline/saline controls. When the B-HT 933/haemorrhage rats are compared to the haemorrhagic, hypotensive controls, administration of the α_2-adrenoceptor agonist can be seen to have increased the fractions of cardiac output passing to the spleen (by 115%), kidneys (38%) and stomach (56%), but reduced that passing to the heart by 43%. After B-HT 933 administration to haemorrhagic, hypotensive animals, the percentages of cardiac output distributed to the spleen, kidneys and testes were no longer significantly different from

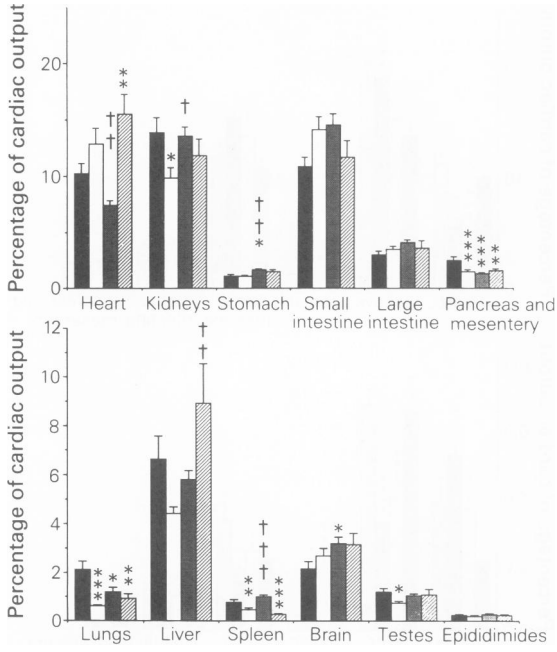


Figure 4 Effects of haemorrhage before and after B-HT 933 and amidephrine administration on the distribution of cardiac output in anaesthetized, haemorrhagic rats. The first (solid) columns show the results from non-haemorrhaged control rats given saline alone; the open columns give the data from haemorrhaged rats given saline alone; the third (stippled) column in each group shows results from haemorrhaged rats given B-HT 933 ($1\,mg\,kg^{-1}$ bolus + $100\,\mu g\,min^{-1}$ infusion); and the hatched columns the data from haemorrhaged rats given amidephrine ($6\,\mu g\,kg^{-1}$ bolus + $0.5\,\mu g\,min^{-1}$ infusion). $n = 8$ for each group. Statistical differences were assessed by analysis of variance: between the haemorrhagic groups and the non-haemorrhagic control group; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$: between the drug-treated haemorrhagic rats and the saline-treated haemorrhagic control group; $\dagger P < 0.05$, $\dagger\dagger P < 0.01$, $\dagger\dagger\dagger P < 0.001$.

those observed in the normotensive saline control group. Indeed, B-HT 933 actually increased the fractions of cardiac output passing to the brain (by 50%) and stomach (by 45%) compared to those in the normotensive control group. After amidephrine administration to the haemorrhagic, hypotensive rats, there was an increase in the percentage of cardiac output delivered to the liver (of 103%) relative to the haemorrhage/saline group and, when the amidephrine/haemorrhage rats are compared to the saline treated normotensive rats, it can be seen that there is a 52% greater fraction of cardiac output distributed to the heart, whilst there were decreases in the proportion of cardiac output distributed to the lungs (56%) and spleen (68%).

Table 2 shows the effects of B-HT 933 and amidephrine on organ blood flows in anaesthetized rats. A single dose of B-HT 933 induced reductions in cardiac and hepatic blood flows (of 45% and 48% respectively) due to the combined effects of the decreases in both cardiac output and its distribution to these vascular beds relative to the saline/saline group. The increased fractions of cardiac output received by the spleen and stomach were responsible for the respective 117% and 57% increases in blood flow through these organs. The second, pressor dose of B-HT 933 increased blood flow in the lungs (by 153%) and spleen (by 86%), as a direct result of increasing distribution of cardiac output to these organs, whilst blood flows through the liver and testes were reduced (by 54% and 34%, respectively) due to the reductions in the percentages of cardiac output passing to them. Administration of amidephrine increased blood flows through the heart (49%), lungs (124%), brain (89%), testes (54%), epididimides (82%), skin (41%) and large intestine (58%) relative to the saline/saline control group. Whilst the increased flow through the brain was due to both an increase in cardiac output and the fraction received, the other increases in blood flow can only be explained in part by the 16% increase in cardiac output; this indicates a degree of amidephrine-induced vasodilatation in these organs which may explain the absence of any overall change in total peripheral resistance by amidephrine.

Table 3 shows the effects of haemorrhage and the subsequent administration of either B-HT 933 or amidephrine on organ blood flows in anaesthetized rats. Haemorrhage alone caused, relative to the saline/saline group, decreased blood flows through the heart (34%), lungs (83%), liver (66%), spleen (74%), kidneys (66%), brain (35%), testes (70%), epididimides (57%), skin (83%), stomach (53%), small intestine (32%), large intestine (42%) and pancreas/mesentery (76%). In the lungs, spleen, kidneys, testes and pancreas/mesentery, the decreased blood flow was a result of the combined effects of the 52% reduction in cardiac output and its distribution to these organs. For the remainder of the organs, the reductions in blood flow may be explained by the decreased cardiac output alone. Administration of B-HT 933 to the haemorrhagic, hypotensive animals increased blood flow through the spleen, kidneys, brain, testes, epididimides, skin, stomach and large intestine (by 165%, 66%, 53%, 106%, 84%, 153%, 119% and 59% compared with the haemorrhagic controls). In the splenic, renal and gastric vascular beds this can be accounted for by the combined effects of the 31% increase in cardiac output and the restoration of its fractional distribution to these vascular beds. For the remainder, the increase in blood flow was largely due to the increase in cardiac

Table 2 The effects of B-HT 933 and amidephrine on organ blood flow (ml min^{-1} g^{-1} organ wt)

	Saline/ saline	*B-HT 933/ saline*	*B-HT 933/ B-HT 933*	*Amidephrine/ saline*
Heart	6.44 ± 0.74	3.52 ± 0.30*	4.43 ± 0.87	9.61 ± 1.09**
Lungs	1.01 ± 0.12	1.47 ± 0.36	2.56 ± 0.38**	2.26 ± 0.41*
Liver	0.375 ± 0.061	0.196 ± 0.031*	0.174 ± 0.046**	0.410 ± 0.049
Spleen	0.78 ± 0.08	1.69 ± 0.17**	1.45 ± 0.32*	0.45 ± 0.08
Kidneys	4.35 ± 0.64	4.09 ± 0.45	5.06 ± 0.28	5.92 ± 0.58
Brain	0.793 ± 0.088	0.709 ± 0.106	0.653 ± 0.061	1.495 ± 0.206***
Testes	0.263 ± 0.031	0.209 ± 0.015	0.174 ± 0.014*	0.404 ± 0.041**
Fat	0.026 ± 0.006	0.030 ± 0.002	0.030 ± 0.005	0.033 ± 0.006
Epididimides	0.148 ± 0.022	0.193 ± 0.029	0.150 ± 0.013	0.269 ± 0.036**
Skeletal muscle	0.203 ± 0.060	0.153 ± 0.027	0.154 ± 0.020	0.119 ± 0.012
Skin	0.098 ± 0.014	0.084 ± 0.011	0.079 ± 0.008	0.138 ± 0.011*
Stomach	0.476 ± 0.050	0.745 ± 0.109*	0.718 ± 0.124	0.730 ± 0.069
Small intestine	1.76 ± 0.22	1.99 ± 0.16	2.14 ± 0.25	2.14 ± 0.16
Large intestine	0.88 ± 0.07	1.10 ± 0.11	0.99 ± 0.09	1.39 ± 0.10***
Pancreas and mesentery	0.526 ± 0.066	0.488 ± 0.043	0.421 ± 0.043	0.595 ± 0.072

$n = 8$ for all groups. Data are for: rats given saline only (saline/saline); rats given a single, depressor dose of B-HT 933 (B-HT 933/saline); those given two doses of B-HT 933, the second having a pressor effect (B-HT 933/B-HT 933); and for rats treated with amidephrine (amidephrine/saline). Statistical differences between the drug-treated groups and the saline/saline control group were assessed by analysis of variance: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Table 3 The effects of haemorrhage followed by administration of saline, B-HT 933 or amidephrine on organ blood flow (ml min^{-1} g^{-1} organ wt)

	Saline/ saline	*Haemorrhage/ saline*	*B-HT 933/ haemorrhage*	*Amidephrine/ haemorrhage*
Heart	6.44 ± 0.74	4.23 ± 0.57*	3.10 ± 0.28***	7.13 ± 0.81††
Lungs	1.01 ± 0.12	0.17 ± 0.03***	0.41 ± 0.1***	0.40 ± 0.07***
Liver	0.375 ± 0.061	0.126 ± 0.010***	0.195 ± 0.015**	0.378 ± 0.049†††
Spleen	0.78 ± 0.08	0.20 ± 0.03***	0.53 ± 0.07**,†††	0.14 ± 0.03***
Kidneys	4.35 ± 0.64	1.50 ± 0.19***	2.49 ± 0.20***,†	2.67 ± 0.11**,†
Brain	0.793 ± 0.088	0.513 ± 0.065*	0.783 ± 0.085†	0.834 ± 0.029†
Testes	0.263 ± 0.031	0.079 ± 0.012***	0.163 ± 0.016**,†	0.186 ± 0.029*,††
Fat	0.026 ± 0.006	0.013 ± 0.003	0.026 ± 0.007	0.028 ± 0.008
Epididimides	0.148 ± 0.022	0.063 ± 0.013**	0.116 ± 0.019†	0.091 ± 0.012*
Skeletal muscle	0.203 ± 0.060	0.139 ± 0.019	0.139 ± 0.015	0.108 ± 0.02
Skin	0.098 ± 0.014	0.017 ± 0.003***	0.043 ± 0.008***,†	0.054 ± 0.006**,†
Stomach	0.476 ± 0.05	0.225 ± 0.026***	0.493 ± 0.040†††	0.439 ± 0.033†††
Small intestine	1.76 ± 0.22	1.19 ± 0.21*	1.31 ± 0.09	1.26 ± 0.17
Large intestine	0.88 ± 0.07	0.51 ± 0.05**	0.81 ± 0.05††	0.74 ± 0.11†
Pancreas and mesentery	0.526 ± 0.066	0.160 ± 0.017***	0.188 ± 0.018***	0.248 ± 0.021***

$n = 8$ for all groups. For clarity, the saline/saline group from Table 2 has been included here. Data shown are for the saline-treated control rats and haemorrhagic rats (saline/saline, haemorrhage/saline) and haemorrhagic rats treated with a single dose of B-HT 933 or amidephrine (B-HT 933/haemorrhage, amidephrine/haemorrhage). Statistical differences were assessed by analysis of variance: between the haemorrhagic, hypotensive groups and the normotensive saline control group, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$: between the drug-treated haemorrhagic groups and the haemorrhagic, hypotensive control group, † $P < 0.05$, †† $P < 0.01$, ††† $P < 0.001$.

output, although this cannot totally explain the increased blood flows, indicating possible B-HT 933-induced decreases in relative resistance in some vascular beds. It is of interest to note that, after B-HT 933 administration to the haemorrhagic, hypotensive animals, the blood flows through the brain, epididimides, stomach, small intestine and large intestine were no longer significantly different from those observed in the normotensive saline control group. In the haemorrhagic, hypotensive

rats, amidephrine increased blood flow through the heart (by 69%), liver (200%), kidneys (78%), brain (63%), testes (135%), skin (218%), stomach (95%) and large intestine (45%) relative to the haemorrhagic, hypotensive control group. In the liver the change was due to the combined effects of the 56% increase in cardiac output and restoration of its fractional distribution to the hepatic arterial vascular bed. Restoration of the blood flow in the other organs appears to be due solely to the increased cardiac output. After amidephrine administration, blood flows through the brain, stomach, small intestine and large intestine, in the haemorrhagic, hypotensive animals were no longer significantly different from those observed in the normotensive saline control group.

Discussion

The effect of B-HT 933 and amidephrine on the cardiovascular system of the anaesthetized rat

In intact, normotensive and hypertensive animals, α_2-adrenoceptor agonists, including B-HT 933, induce a fall in blood pressure by stimulating central α_2-adrenoceptors leading to central sympathoinhibition and vagal reflex activation (Kobinger & Pichler, 1977; 1978; Kobinger, 1978; Hammer *et al.*, 1980). Accordingly, this study shows that in the anaesthetized, intact rat, a single dose of B-HT 933 induced a fall in both mean arterial and diastolic blood pressures. This was caused by a decrease in cardiac output resulting from bradycardia which is consistent with the hypotension and bradycardia observed after central α_2-adrenoceptor activation (Kobinger, 1978). Total peripheral resistance was unchanged but cardiac and hepatic blood flows were reduced due to the combined effects of the lowered cardiac output and its redistribution away from the heart and liver. B-HT 933 also increased the fractions of the cardiac output passing to the spleen and stomach sufficiently to overcome the effects of the lowered cardiac output and produce greater blood flows through these organs.

After a first dose of B-HT 933 subsequent doses induced dose-dependent pressor responses. These pressor responses were mediated by α_2-adrenoceptors since they were inhibited by yohimbine but not affected by the α_1-adrenoceptor antagonist prazosin. These pressor responses were associated with a greater total peripheral resistance; cardiac output was not significantly affected. Blood flows through the hepatic artery and testes were reduced due to reductions in the fractional distribution of cardiac output to these vascular beds, whilst increases in the percentages of cardiac output passing to the lungs and spleen caused increases in blood flow through these organs.

It should be noted that the microspheres detected in the lungs include not only those passing through the bronchial arteries but also those passing through arteriovenous anastomoses which are subsequently trapped in the lungs after passage along the great veins. Thus, the effects of the α_1-adrenoceptor agonists on lung blood flow could be due to actions on either the anastomoses or the bronchial arteries. Another point of note is that the two vascular inputs into the liver (the hepatic artery and the hepatic portal vein) interact by apparently purely mechanical processes, such that increases or decreases in hepatic portal venous supply are at least in part compensated by changes in hepatic arterial inflow resistance (Sato *et al.*, 1977). Thus a fall in resistance to portal inflow results in an increase in hepatic arterial resistance and apparent changes in hepatic vascular resistance seen in this study may reflect opposing effects in gastrointestinal vascular resistance.

Amidephrine increased blood pressure in normotensive rats by increasing cardiac output; this resulted from a stroke volume increase sufficient to overcome the observed small decrease in heart rate. As the results demonstrate, amidephrine augmented blood flows through the heart, lungs, brain, testes, epididimides, skin and large intestine. The combined effects of increases in cardiac output and its distribution to the brain account for the increase in cerebral blood flow, whilst the increases in blood flow through the other organs were primarily due to the increased cardiac output and, probably, some degree of amidephrine-induced changes in relative resistances in these vascular beds.

The effects of B-HT 933 and amidephrine on the cardiovascular changes induced by haemorrhage in anaesthetized rats

In this study, the mean volume of blood removed from the rats to simulate haemorrhage ranged from 4.0 ml to 4.3 ml. The blood volume of the rat is 62 ml kg^{-1} body weight (Wang, 1959) and so, for rats in the weight range 250–300 g, this approximates to a 22–28% haemorrhage. This degree of haemorrhage lowered mean arterial blood pressure by 50–60% due to decreases both in total peripheral resistance and cardiac output; here it should be noted that the microsphere method has been shown to be as reliable as alternative techniques for measuring cardiac output in physiological conditions in which cardiac output is low (Lundberg & Schoenberg, 1985). The decreased cardiac output was caused by bradycardia as well as a decrease in stroke volume. Similar cardiovascular changes induced by an equivalent degree of haemorrhage have previously been observed in

both conscious (Darlington *et al.*, 1986) and anaesthetized rats (Chance *et al.*, 1985). Bradycardia following haemorrhage has been observed previously not only in the rat but also in the cat, dog and man (Oberg & White, 1970; Castenfors & Sjöstrand, 1972; Schrier *et al.*, 1979; Matsui *et al.*, 1983).

Baroreceptor activation and the subsequent increase in sympathetic drive after haemorrhage do not induce the otherwise expected increase in heart rate, as accompanying chemoreceptor stimulation (Daly & Scott, 1959) and the increased central and peripheral endogenous opioid peptide levels evoked by stress states (Madden *et al.*, 1977; Rossier *et al.*, 1977) favour bradycardia (Laubie *et al.*, 1977). Pendleton *et al.* (1980) demonstrated that vagotomy and atropine abolish the bradycardia following haemorrhage in the rat showing that it is mediated by increased vagal activity, apparently afferent as well as efferent (Castenfors & Sjöstrand, 1972).

The decreases in cardiac output and stroke volume observed here in the anaesthetized rat following haemorrhage are similar to changes occurring in other animal models of shock and in man. Thus, previously described consequences of haemorrhage include decreases in cardiac contractility (Spitzer *et al.*, 1985), venous return, cardiac output and blood pressure and increased levels of circulating catecholamines, angiotensin II and vasopressin (Feuerstein *et al.*, 1977; Cameron *et al.*, 1985; Corwin *et al.*, 1985). Together, these lead to severe impairment of tissue blood flow following haemorrhage (Carrière, 1969; Forsyth, 1970; Hollenberg & Nickerson, 1970; Hardaway, 1985).

Bereiter *et al.* (1986) showed that, in the anaesthetized cat, the threshold required for adrenal medullary adrenaline release was a haemorrhage of 20% of the measured blood volume. In rats, plasma adrenaline and noradrenaline levels increase significantly 5 min after haemorrhage and remain elevated for up to 1 h (Feuerstein *et al.*, 1981; 1984; Darlington *et al.*, 1986). Therefore, the circulating catecholamine levels are highly likely to be increased in the haemorrhagic, hypotensive rats used in this study. In these animals blood flows were found to be severely compromised through the lungs, spleen, kidneys, testes and the pancreas/mesentery owing to reductions in both the cardiac output and in its distribution to these organs. Blood flow was also reduced through the heart, liver, brain, epididimides, skin and gastrointestinal tract. Similar changes in organ blood flow following experimental haemorrhage have been observed in the rat, monkey and cat (Sapirstein *et al.*, 1960; Forsyth *et al.*, 1970; Chance *et al.*, 1985).

It is apparent from the present results that the physiological compensations to haemorrhage in the anaesthetized rat successfully maintain the fractions of cardiac output distributed to such vital organs as the heart and brain, at the expense of other less vital organs such as the pancreas/mesentery and the spleen. This is in accordance with the knowledge that the splanchnic blood reservoir plays an important role in cardiovascular homeostasis during haemorrhage (Greenway, 1983). However, the blood flow through these vital organs is still reduced because of the decrease in cardiac output.

Restoration of cerebral and peripheral blood flow, especially that through the heart and kidneys still remains paramount in the search for drugs for the treatment of haemorrhagic shock. Hence any agent which will restore renal blood flow has therapeutic potential for the treatment of haemorrhage. Restoration of blood flow through the splanchnic region may also be beneficial, as there seem to be cardiotoxic substances in the plasma of animals and man in shock (for review, see Goldfarb, 1982) which appear to be generated by the splanchnic circulation, especially the gastrointestinal tract and pancreas. Increasing splanchnic blood flow or stabilizing lysosomal membranes decrease the release and cardiac effects of these toxic agents (Lefer, 1978; 1982).

The present study shows that administration of B-HT 933 to haemorrhagic, hypotensive rats increases blood pressure by enhancing cardiac output through an increase in stroke volume. It also restored blood flow through the spleen, kidneys, brain, testes, epididimides, skin, stomach and large intestine. The increases in blood flow through the splenic, renal and gastric vascular beds were the result of both the increased cardiac output and its redistribution in their favour. The enhanced blood flow through the other organs can be partially explained by the increased cardiac output, but there is evidence of some B-HT 933-mediated vasodilatation relative to the haemorrhage/saline control group. Stimulation of prejunctional α_2-adrenoceptors inhibits noradrenaline release from presynaptic nerve terminals (Langer, 1981) and would therefore reduce the effects of the increase in sympathetic tone in haemorrhage. This may account for the vasodilatation in some vascular beds (relative to the haemorrhage/saline group) seen in the haemorrhagic, hypotensive animals given B-HT 933. The results indicate that B-HT 933 has favourable effects in the anaesthetized, haemorrhagic, hypotensive rat, in that it increases cardiac output, without greatly increasing heart rate, as well as restoring blood flow through vital organs such as the brain and kidneys, and through the splachnic vascular beds. However, coronary blood flow is not restored by B-HT 933 to the level found in normotensive control rats.

Amidephrine also increased the blood pressure of the haemorrhagic, hypotensive rats through an increase in cardiac output mediated by an enhanced

stroke volume. It also restored blood flow through the heart, liver, kidneys, brain, testes, skin, stomach and large intestine. In the hepatic artery, this increase was due to the increases in both cardiac output and its distribution to this vessel, but the change in cardiac output alone caused the increased blood flow through the other organs. These increases in cardiac output and blood flows through the cardiac, renal, cerebral and splenic vascular beds in the haemorrhagic animals could also be favourable therapeutically. That amidephrine successfully restored coronary blood flow and B-HT 933 did not suggest that α_1-adrenoceptor rather than α_2-adrenoceptor agonists may have greater therapeutic potential.

In the anaesthetized animal, it is thought that α_2-adrenoceptors on arteriolar smooth muscle are extrajunctional and respond primarily to circulating catecholamines, whilst the α_1-adrenoceptors are activated by noradrenaline released from nerve endings during sympathetic neural activation (Langer, 1981; McGrath, 1982). Hence, after haemorrhage, the initial compensatory response of the peripheral vasculature involves the increased activation of both smooth muscle α_1-adrenoceptors, through the increase in sympathetic nerve activity, and α_2-adrenoceptors, by the catecholamines released from the adrenal medulla. It has been suggested previously that α_2- rather than α_1-adrenoceptor activation is more effective in maintaining the compensatory vasoconstriction to haemorrhage over long periods of time (Bond & Johnson, 1985). This theory is in accord with the observation that, in pithed rats, the pressor responses to infusions of α_2-adrenoceptor agonists are maintained for longer than those to infusions of α_1-adrenoceptor agonists (McGrath & O'Brien, 1987). It is interesting to note, however, that in the present study, haemorrhage potentiated the pressor response to the amidephrine infusion, whilst it depressed the response to the infusion of B-HT 933. In other experiments (MacLean *et al.*, 1989) we have observed that, in the anaesthetized rat, haemorrhage not only potentiates the pressor response to an infusion of amidephrine, but also to two other α_1-adrenoceptor agonists, phenylephrine and cirazoline. This suggests that haemorrhage may increase the beneficial effects of α_1-adrenoceptor agonists and therefore further increase their therapeutic potential.

The above observations also suggest that there may be a redistribution of the relative importance of responses to α_1- and α_2-adrenoceptor activation in shock, as hypothesized by McGrath (1982). For example, in the spleen, before haemorrhage, B-HT 933 affected both the distribution of cardiac output to this organ and its blood flow whilst amidephrine was without effect. In the haemorrhagic, hypotensive animals, however, amidephrine had a greater influence than B-HT 933 on these parameters and, in fact, B-HT 933 no longer had any significant effect on the distribution of cardiac output to the spleen. Alternative explanations for the potentiation of the amidephrine pressor response and the reduction of the pressor response to B-HT 933 infusion after haemorrhage require consideration of the complex interactions of α-adrenoceptors with the other physiological changes induced by haemorrhage. These include the changes in blood gases and pH, basal vascular tone, circulating angiotensin II, catecholamines and other vasomodulators, e.g. vasopressin (Cameron *et al.*, 1985) and neuropeptide Y (Morris *et al.*, 1987), released in response to haemorrhage.

In this study, the haemorrhagic, hypotensive rats showed arterial acidosis, hypocapnia and hyperoxia. This may be explained by very slow capillary blood flow, caused by hypotension, arteriolar vasoconstriction and open capillaries (Hardaway, 1985), leading to reduced oxygen delivery and an increase in anaerobic metabolism; the resulting lactic acidaemia causing hypocapnia as CO_2 is blown off. Pressor responses to phenylephrine and xylazine, respectively selective agonists at α_1- and α_2-adrenoceptors, are not affected by changes in blood gases and pH within the range observed after haemorrhage here (MacLean & Hiley, 1988a). Therefore it is unlikely that such changes caused the altered pressor responses to amidephrine and B-HT 933. Indeed, Lew & Angus (1985) showed that, in ganglion blocked, anaesthetized rats, lowering basal blood pressure by haemorrhage, nitroprusside or pentobarbitone preferentially inhibited the pressor response to the α_2-adrenoceptor agonist B-HT 920, whilst only haemorrhage potentiated the pressor response to the α_1-adrenoceptor agonist methoxamine. This suggests that reduced vascular tone is not the cause of the potentiation of α_1-adrenoceptor-mediated pressor responses after haemorrhage, but might be the cause of the inhibition of the pressor response to infusions of B-HT 933.

After haemorrhage, circulating angiotensin II is increased and we have demonstrated previously that, in pithed rats, endogenous angiotensin II facilitates pressor responses to both phenylephrine and xylazine (MacLean & Hiley, 1988b). Angiotensin II may therefore contribute to the facilitatory effect of haemorrhage on the pressor responses to infusions of amidephrine.

In conclusion, this study indicates that in the anaesthetized, haemorrhagic, hypotensive rat, administration of either amidephrine or B-HT 933 had some beneficial effects on cardiac output and on blood flow through the vital organs, although amidephrine might seem the more useful at present due

to the differential effects of these drugs on coronary blood flow. This suggests that careful use of selective α-adrenoceptor agonists may be of therapeutic use in the treatment of haemorrhagic shock.

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Limited interference of specific Paf antagonists with hyper-responsiveness to Paf itself of lungs from actively sensitized guinea-pigs

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1 The interference of various platelet-activating factor (Paf) antagonists with Paf- and antigen-induced effects in isolated lungs from actively sensitized guinea-pigs was investigated.

2 WEB 2086 and BN 52021, two antagonists structurally unrelated to Paf, at concentrations 10–100 fold above those exerting a potent and selective inhibition of the effects of Paf in lungs from non-immunized guinea-pigs, failed to inhibit bronchoconstriction and mediator release evoked by the phospholipid when administered into lungs from actively sensitized animals.

3 In contrast to WEB 2086 and BN 52021, antagonists such as Ro 19-3704 and Ro 19-1400, structurally related to the Paf molecule, at concentrations which abrogate the effects of Paf in lungs from non-immunized guinea-pigs, inhibited bronchoconstriction and release of histamine and leukotriene-like material evoked by the intra-arterial administration of Paf into lungs from actively sensitized animals.

4 Ro 19-3704 and Ro 19-1400 also inhibited markedly the release of leukotriene C_4 (LTC_4)-like material and, to a smaller extent, the histamine secretion induced by 10 μg arachidonic acid.

5 CV 6209, another Paf antagonist structurally related to the phospholipid, failed to antagonize its bronchopulmonary and secretory effects in sensitized lungs.

6 All the antagonists used, irrespective of their ability to interfere or not with bronchoconstriction and mediator release triggered by Paf, suppressed oedema formation as measured by the increase in lung wet weight induced by either Paf or ovalbumin.

7 Our results indicate that: (i) the increase in vascular permeability and the subsequent oedema formation on one hand and the bronchopulmonary effects of Paf on the other hand are mediated by different mechanisms; and (ii) active sensitization provokes a marked modification of the lung reactivity to Paf.

Introduction

It has been recently established that active sensitization of guinea-pigs leads to *in vitro* bronchopulmonary hyper-responsiveness to different agonists, including platelet-activating factor (Paf) (Pretolani *et al.*, 1988). Indeed, lungs from these animals exhibit an enhanced bronchoconstrictor response, release more cyclo-oxygenase and lipoxygenase metabolites and secrete histamine in response to Paf when compared to lungs from non-immunized guinea-pigs. This phenomenon appears to be independent of the ability of the lung to undergo anaphylaxis, since lungs from passively sensitized animals show bronchopulmonary responses and mediator release following Paf administration similar to those observed in lungs from non-immunized guinea-pigs, even though they respond to antigen as lungs from actively sensitized animals (Pretolani *et al.*, 1988).

The differences between lungs from actively sensitized and non-sensitized guinea-pigs were further emphasized by studying the pharmacological modulation of the bronchoconstrictor and secretory effects of Paf. Indeed, bronchoconstriction induced by Paf, which is blocked by cyclo-oxygenase inhibitors when lungs isolated from non-immunized guinea-pigs are used (Lefort *et al.*, 1984), becomes refractory to aspirin or indomethacin when lungs are obtained from actively sensitized animals (Pretolani *et al.*, 1988). Furthermore, the increased bronchoconstrictor response to Paf observed in sensitized

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lungs is markedly reduced by BW 755C, a mixed cyclo-oxygenase and lipoxygenase inhibitor (Higgs et al., 1979), in the presence of mepyramine, indicating that leukotrienes participate in the guinea-pig lung hyper-responsiveness to Paf.

Recently, potent and selective Paf antagonists have been described which, besides their activity against Paf in various experimental models, are claimed to inhibit allergic reactions (reviewed by Braquet et al., 1987). Among these compounds, Ro 19-3704, Ro 19-1400 (Burri et al., 1985) or CV 3988 (Terashita et al., 1983), represent structural analogues of the Paf molecule, whereas others, such as WEB 2086 (Casals-Stenzel, 1987; 1988; Pretolani et al., 1987) or BN 52021 (Braquet et al., 1985), are structurally unrelated. Since active sensitization modifies the guinea-pig lung reactivity to Paf (Pretolani et al., 1988), the interference of its antagonists with the bronchopulmonary and mediator-releasing effect of the phospholipid in lungs from actively sensitized guinea-pigs was now investigated. In addition, since peptido-leukotrienes are claimed to be essential for allergy (Barnes & Costello, 1986), to participate in acute lung anaphylaxis and to be released by Paf from sensitized lungs (Pretolani et al., 1989), the interference of Paf antagonists with their formation was also investigated.

Methods

Sensitization procedure

Hartley guinea-pigs of either sex (400–600 g) were actively sensitized by two s.c. injections separated by two weeks of 0.5 ml of 0.9% NaCl (saline) containing 10 μg ovalbumin dispersed in 1 mg $Al(OH)_3$. The animals were used 7 days after the second injection. This procedure promotes the production of high titres of specific homocytotropic antibodies (Ovary, 1964). The titre, measured in the serum obtained from animals killed one week after the booster injection of the antigen, was approximately of 1/800 (Pretolani et al., 1988). In some experiments guinea-pigs were used at defined time-intervals during the sensitization period; in others the booster injection was omitted and the animals were used 14 or 21 days after the first injection of the antigen. Since lungs from non-immunized or $Al(OH)_3$-injected guinea-pigs exhibited similar responses to Paf (Pretolani et al., 1988), the former were used as controls.

Tracheal strip preparation

Non-immunized guinea-pigs were anaesthetized with sodium pentobarbitone (30 mg kg^{-1}, i.p.) and tracheae were removed. To increase the sensitivity of the strips to contractile agents (Hay et al., 1986), the epithelium was removed by gentle rubbing of the mucosal surfaces with a cotton wool-coated probe and the tissues were cut in spirals. Each preparation was connected to a Gould Statham isometric force transducer under 2 g tension in an organ bath and superfused with a Krebs solution at 37°C containing 2.5 g l^{-1} bovine serum albumin (Krebs-BSA) and gassed continuously with 95% O_2:5% CO_2. The Krebs-BSA solution was supplemented with phenoxybenzamine (3 μM), mepyramine (2 μM), methysergide (0.5 μM), atropine (10 μM) and indomethacin (3 μM), to avoid contractile responses by mechanisms other than those evoked by the release of leukotriene-like material from the lung. The tissues were allowed to equilibrate for 2 h and, after this period, the stable endoperoxide analogue, U 46619 was administered at doses of 1 or 2 nmol to assess their sensitivity.

Lung perfusion experiments

Actively sensitized guinea-pigs were anaesthetized as above, tracheae were cannulated and the animals ventilated (60 strokes min^{-1}, 10 ml kg^{-1} body weight) with a Palmer miniature respiratory pump. A thoracotomy was performed and the lungs were removed as described (Lefort et al., 1984). Each organ was placed in a plastic chamber and immediately ventilated (60 strokes min^{-1}, 10 ml kg^{-1} body weight) and perfused (10 ml min^{-1}, 37°C) with a gassed (95% O_2:5% CO_2) Krebs-BSA solution containing 3 μM indomethacin and 2 μM mepyramine. Indomethacin was used to prevent the formation of any cyclo-oxygenase metabolites which contract the tracheal strip and might interfere with the responses to leukotriene-like material from the lung. Mepyramine abolishes the histamine component in the bronchoconstrictor action of Paf in lungs from actively sensitized guinea-pigs (Pretolani et al., 1988). After passage through the lung, the perfusion solution was directed to the tracheal strip and the superfusion system for the latter organ was stopped. Thus, the solution eluting from the lung was used to perfuse the tracheal strip. After a 10 min period for equilibration, increasing doses (1 and 100 ng in 100 μl of saline containing 0.1% BSA) of Paf were injected into the pulmonary artery at 10 min intervals. Bronchoconstrictor responses were recorded by a pressure transducer located between the trachea and the respiratory pump and expressed as percentages of increase, calculated over the basal values measured prior to the administration of the agonists. Before the end of each experiment, 10 μg ovalbumin was administered and the lung response was recorded. Fractions of lung effluent were collected before,

during the first 3 min and between the 3rd and 6th min after the successive injections of the two doses of Paf and ovalbumin for the determination of immunoreactive leukotriene C_4 (LTC_4)-like material and histamine, as described below. In a series of experiments, arachidonic acid was injected at the dose of 10 μg instead of Paf and lung response was investigated, as described above.

When the effect of the various Paf antagonists was studied, the compounds were dissolved up to the indicated concentrations in the Krebs-BSA solution used for the lung perfusion and Paf and ovalbumin were administered, as described above.

Determination of immunoreactive LTC_4-like material and histamine

Immunoreactive LTC_4-like material in fractions of lung perfusate was quantified by radioimmunoassay, as previously described (Aehringhaus *et al.*, 1982). Dextran-coated charcoal was used to separate unbound ligand by centrifugation at 1200 *g* for 10 min at 4°C. The monoclonal anti-LTC_4 antibody employed (kindly provided by Dr U. Zor, Weizmann Institut, Rehovot, Israel) was 10% crossreactive with LTD_4 and less than 0.1% with LTA_4 and LTB_4. The sensitivity of the assay was approximately 15 pg of immunoreactive LTC_4 per 0.1 ml sample.

For the determination of histamine , 1 ml-aliquots of the collected fractions were mixed with 1 ml of a 0.8 N perchloric acid solution. After centrifugation for 10 min at 1000 *g* and 4°C, the supernatants were stored at 4°C. An automatic spectrofluorometric assay for histamine was performed according to previously described methods (Shore *et al.*, 1959; Lebel, 1983).

Measurement of lung oedema formation

The increase in lung wet weight following the intra-arterial injections of Paf and ovalbumin into lungs from actively sensitized guinea-pigs was used to evaluate the oedema formation. Lungs were weighed before, after the administration of 1 and 100 ng Paf and at the end of the experiments, after the injection of 10 μg ovalbumin in the absence and in the presence of the various Paf antagonists. The increases in lung weights were compared to those of lungs perfused for 40 min and injected at 10 min intervals with 0.1 ml of the Paf vehicle, i.e. saline containing 0.1% BSA.

Materials

The composition of the Krebs solution was (in mM): NaCl 118, KCl 4.7, $CaCl_2 \cdot 2H_2O$ 2.5, $MgSO_4 \cdot 7H_2O$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 5.6. The drugs used were as follows: Paf (1-O-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine), indomethacin, phenoxybenzamine, atropine sulphate, BSA fraction V (Sigma, St. Louis, MO, U.S.A.); $Al(OH)_3$, perchloric acid (Merck, Darmstadt, GFR); mepyramine maleate (Rhône-Poulenc, Vitry/Seine, France); methysergide hydrogen maleinate (Sandoz, Basel, Switzerland); pentobarbitone (sodium pentobarbital, Clin-Midy, Montpellier, France); chicken ovalbumin (Miles, Naperville, IL, U.S.A.); U 46619 (15 (S)-hydroxy-11,9 (epoxymethano)prosta-5Z, 13E-dienoic acid) was kindly provided by Dr M. Bach, Upjohn Co, Kalamazoo, U.S.A.; LTC_4(PIRMED-CNRS, Paris, France); WEB 2086 (3-[4-(2-chlorophenyl)-9-methyl-6H-thieno[3,2-f][1,2,4]-triazolo-[4,3-a]-[1,4]-diazepin-2-yl]-1-(4-morpholinyl)-1-propanone, from Boerhinger-Ingelheim, F.R.G.), was initially dissolved in HCl 50 mN in saline and further diluted to the appropriate concentrations in saline; BN 52021 (9H-1,7a-(epoxymethano)-1H,6aH-cyclopenta[c]furo - [2,3 - b]furo - [3',2',:3,4]cyclopenta - [1,2-d]-furan-5,9,12-(4H)-trione, 3-tert-butylhexahydro-4,4b-11-tri-hydroxy-8-methyl) from Institut Henri Beaufour, Le Plessis Robinson, France), was dissolved in NaOH 0.1 N and the pH was adjusted to 7.4 with HCl 0.1 N; Ro 19-3704 (3-[4-[(**R**)-2 - [(methoxycarbonyl)oxy] - 3 - [(octadecylcarba - moyl)oxy]propoxy]butyl]thiazolium iodide and Ro 19-1400 (3-[3[[(**R**)-2-(methoxycarbonyl)-3 - [(octadecylcarba - moyl) oxy] propoxy] carbonyl]-propyl]thiazolium hydroxide), from Roche, Basel, Switzerland) were dissolved in ethanol and further diluted in saline; CV-6209 (2-[N-acetyl - N - (2 - methoxy - 3 - octadecyl - carbamoyloxy - propoxycarbonyl) - aminomethyl] - 1 - ethylpy - ridinium chloride) from Takeda Chemical Ind., Osaka, Japan, was dissolved in saline.

Statistical analysis

Results are expressed as mean ± s.e.mean of the indicated number of experiments and significance was assessed by Student's *t* test for unpaired values.

Results

Interference of the antagonists with Paf- and antigen-induced bronchoconstriction and tracheal strip contraction of lungs from actively sensitized guinea-pigs

The administration of 1 ng Paf into the pulmonary artery of lungs from actively sensitized guinea-pigs

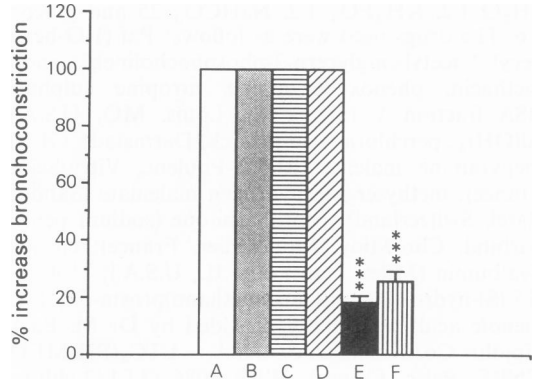


Figure 1 Bronchoconstriction induced by intra-arterial injections of 1 ng Paf into isolated perfused lungs from actively sensitized guinea-pigs in the absence (A) and in the presence of WEB 2086 (B), BN 52021 (C), CV 6209 (D), Ro 19-3704 (E) and Ro 19-1400 (F). All drugs were dissolved in the Krebs-BSA solution at the final concentration of 10 μM, except BN 52021, which was used at 0.1 mM. Bronchoconstriction is expressed as % increase of the bronchial resistance to inflation measured prior to the administration of 1 ng Paf. Results represent the mean of 5 to 8 experiments; vertical bars show s.e.mean. *** $P < 0.001$.

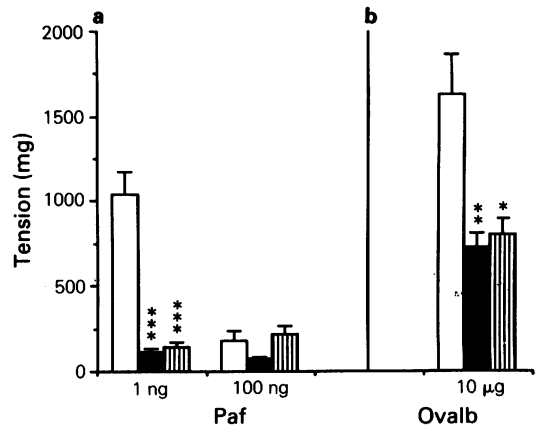


Figure 3 Tracheal strip response (in mg tension) induced by the intra-arterial administrations of Paf (1 and 100 ng, a) and ovalbumin (Ovalb, 10 μg, b) into isolated perfused lungs from actively sensitized guinea-pigs, in the absence (open columns) and in the presence of Ro 19-3704 (solid columns) and Ro 19-1400 (lined columns), used at the concentration of 10 μM. Data are expressed as mean of 5 to 8 experiments; vertical bars show s.e.mean. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

induced a maximal and irreversible bronchoconstriction (Figure 1), which prevented further responses to 100 ng of the phospholipid to be recorded. Following the triggering of bronchoconstriction, a marked contraction of the superfused tracheal strip was noted (Figure 2). When Paf was then injected into the pulmonary artery at a dose of 100 ng, only a minimal contraction of the tracheal strip was observed (Figure 2). At the end of the experiment, 10 μg ovalbumin was injected into the lung but, due to the maximal and irreversible bronchoconstriction evoked by the previous administrations of Paf, no further increase in bronchial resistance was recorded. By contrast, the superfused tracheal strip contracted largely following the antigen challenge (Figure 2), demonstrating the presence of a spasmogenic substance in the effluent from antigen-challenged lungs, in spite of desensitization to Paf.

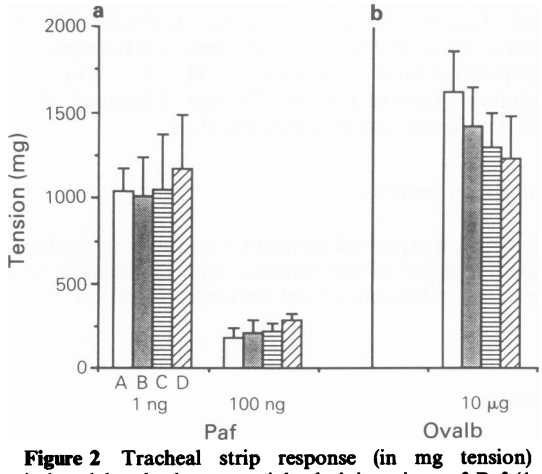


Figure 2 Tracheal strip response (in mg tension) induced by the intra-arterial administrations of Paf (1 and 100 ng, a) and ovalbumin (Ovalb, 10 μg, b) into isolated perfused lungs from actively sensitized guinea-pigs in the absence (A) and in the presence of 10 μM WEB 2086 (B), 0.1 mM BN 52021 (C) and 10 μM CV 6209 (D). Data are expressed as mean of 5 to 8 experiments; vertical bars show s.e.mean.

In the presence of Paf antagonists WEB 2086 or CV 6209 (10 μM) or BN 52021 (0.1 mM), bronchoconstriction induced by 1 ng Paf was equivalent to controls, i.e., reached a maximal value which precluded the determination of statistical variations (Figure 1). In a separate series of experiments the effect of BN 52021, WEB 2086 and CV 6209 on bronchoconstriction and tracheal strip contraction induced by lower doses (0.01 or 0.1 ng) of Paf was investigated and, as in the case of higher doses of the phospholipid, these antagonists were ineffective against bronchoconstriction as well as tracheal strip contraction (not shown).

The amplitude of the tracheal contraction in response to the injection of 10 μg ovalbumin into the lung was also unaffected by WEB 2086, BN 52021 or

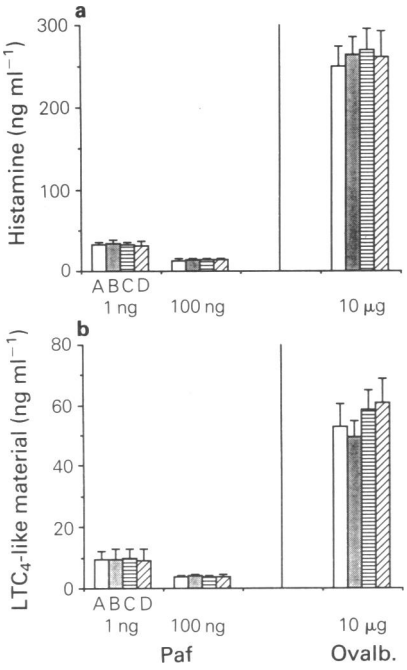


Figure 4 Release of histamine (a) and immunoreactive leukotriene C_4 (LTC_4)-like material (b) induced by the intra-arterial injection of 1 and 100 ng Paf (left) and 10 μg ovalbumin (Ovalb, right) to isolate perfused lungs from actively sensitized guinea-pigs in the absence (A) and in the presence of 10 μM WEB 2086 (B), 0.1 mM BN 52021 (C) and 10 μM CV 6209 (D). Fractions of lung effluent were collected before, during the first 3 min and between the 3rd and 6th min after successive injections of Paf and ovalbumin, as described in the Methods section. Amounts of immunoreactive LTC_4-like material and histamine are expressed as ng ml^{-1} of the values obtained at the peak release, i.e. between 3 and 6 min for LTC_4-like material and between 1 and 3 min for histamine. Results are expressed as mean of 5 to 8 experiments; s.e.mean shown by vertical bars.

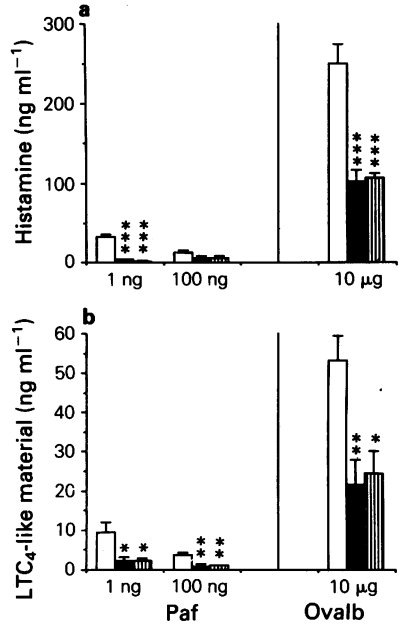


Figure 5 Release of histamine (a) and immunoreactive leukotriene C_4 (LTC_4)-like material (b) induced by the intra-arterial injection of 1 and 100 ng Paf (left) and 10 μg ovalbumin (Ovalb, right) to isolated perfused lungs from actively sensitized guinea-pigs in the absence (open columns) and in the presence of Ro 19-3704 (solid columns) and Ro 19-1400 (lined columns), at the concentration of 10 μM. The experimental protocol was the same as the one described in the legend of Figure 4. Results are expressed as mean of 5 to 8 experiments; vertical bars show s.e.mean. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

CV 6209 (Figure 2). By contrast, when the lungs from actively sensitized guinea-pigs were perfused with the Krebs-BSA solution supplemented with Ro 19-3704 or Ro 19-1400 (10 μM), a marked inhibition of bronchoconstriction induced by 1 ng Paf was observed (Figure 1). Ro 19-3704 and Ro 19-1400 also strongly antagonized tracheal strip contractions following the administration of 1 ng Paf and significantly reduced that evoked by antigen challenge (Figure 3).

The possibility that Ro 19-3704 and Ro 19-1400 might affect the reactivity of the tracheal tissue to exogenous stimuli was ruled out, since 940 ± 110 and 965 ± 155 mg contractions to 2 pmol U 46619 were measured in the presence and in the absence of the two Paf antagonists.

Interference of the antagonists with Paf- and antigen-induced immunoreactive LTC_4-like material and histamine release from perfused guinea-pig lungs

Intra-arterial administration of 1 ng Paf into lungs from actively sensitized guinea-pigs evoked the release of much less histamine and immunoreactive LTC_4-like material in the effluent than did antigen (Figure 4). Histamine release was maximal during the first 3 min after the injection of Paf, whereas the peak of immunoreactive LTC_4-like material production was reached between the 3rd and 6th min. Upon injection of 100 ng of the phospholipid, lower amounts of both mediators were detected in the effluent (Figure 4). When ovalbumin was injected last, the amounts of immunoreactive LTC_4-like

material and histamine measured in the effluent were approximately 10 fold above those released upon administration of 1 ng Paf (Figure 4). In the presence of 10 μM WEB 2086 or CV 6209 or 0.1 mM BN 52021 the release of histamine and of LTC_4-like material following Paf or ovalbumin administration was unaffected (Figure 4).

When the experiments were performed in the presence of Ro 19-3704 and Ro 19-1400 at 10 μM, release of LTC_4-like material and histamine secretion evoked by the intra-arterial administration of 1 and 100 ng Paf into lungs from actively sensitized guinea-pigs was markedly reduced (Figure 5). Following the administration of ovalbumin the release of LTC_4-like material and histamine was also inhibited by both antagonists (Figure 5).

In these experiments and in those illustrated in Figure 6, the background values for mediator release measured prior to challenge were: histamine, 1.20 ± 0.30 ng ml^{-1} and LTC_4 0.68 ± 0.20 ng ml^{-1}.

Interference of Ro 19-3704 and Ro 19-1400 with arachidonic acid-induced LTC_4-like material and histamine release from lungs from actively sensitized guinea-pigs

LTC_4-like material and histamine were released following the injection of 10 μg arachidonic acid into lungs from actively sensitized guinea-pigs (Figure 6). In the presence of 10 μM Ro 19-3704 or 19-1400, a marked inhibition of LTC_4-like material production was observed, whereas the release of histamine was only partially reduced (Figure 6).

Kinetics of bronchopulmonary response and mediator release: effect of WEB 2086

To determine the possible relationship between the development of lung hyperresponsiveness and the lack of effect of Paf antagonists, the interference of WEB 2086 with the bronchopulmonary and secretory response to Paf of lungs from guinea-pigs killed at various time intervals during the sensitization period was investigated. As shown in Figure 7, at days 0 and 14, bronchoconstriction induced by 1 ng Paf was minimal as compared to the situation prevailing later and was inhibited by WEB 2086. At this time, neither histamine was released nor was the trachea contracted. By contrast, 4 days after the booster injection of the antigen performed at day 14, lung hyper-responsiveness to Paf developed, reached its maximum at day 21 and persisted for at least 3 months. Concomitantly, in lungs obtained 7 days after the booster injection and up to day 98, bronchoconstriction and mediator release in response to 1 ng Paf were unaffected by WEB 2086 (Figure 7).

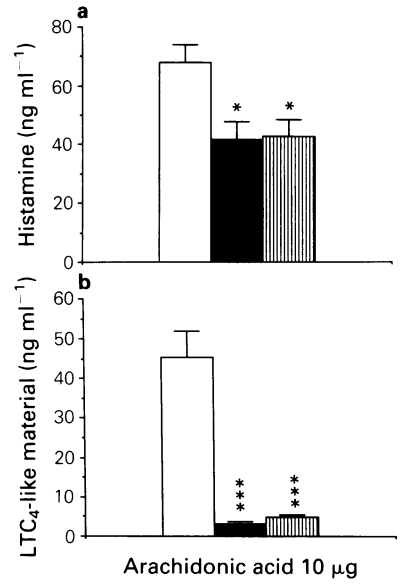


Figure 6 Release of histamine (a) (upper panel) and immunoreactive leukotriene C_4 (LTC_4)-like material (b) induced by the intra-arterial injection of 10 μg arachidonic acid to isolated perfused lungs from actively sensitized guinea-pigs in the absence (open columns) and in the presence of Ro 19-3704 (solid columns) and Ro 19-1400 (lined columns), both used at the concentration of 10 μM. The experimental protocol was the same as the one described in the legend of Figure 4. Results are expressed as mean of 4 experiments; s.e.mean shown by vertical bars. * $P < 0.05$; *** $P < 0.001$.

Similar results were obtained upon injection of lower doses (0.1 and 0.01 ng) of Paf (Table 1). Interestingly, when the booster injection was omitted and the experiments were performed at day 21, the lung response to Paf was similar to that observed at day 0 and 14 and bronchoconstriction was suppressed by WEB 2086 (Figure 7).

Table 1 Effect of WEB 2086 (10 μM) on Paf (0.1 and 0.01 ng)-induced bronchoconstriction (BC) and mediator release from isolated perfused lungs from actively sensitized guinea-pigs

Paf (ng)	*BC* (%)	*Histamine* (ng ml^{-1})	*Trachea tension* (mg)
0.1	100	19.0 ± 4.9	1120.0 ± 381.6
0.1 (+ WEB 2086)	100	11.8 ± 0.7	720.0 ± 69.3
0.01	100	26.8 ± 6.0	693.3 ± 70.6
0.01 (+ WEB 2086)	100	32.3 ± 6.4	740.0 ± 207.3

Results are expressed as mean $\pm$ s.e.mean of 3–6 experiments.

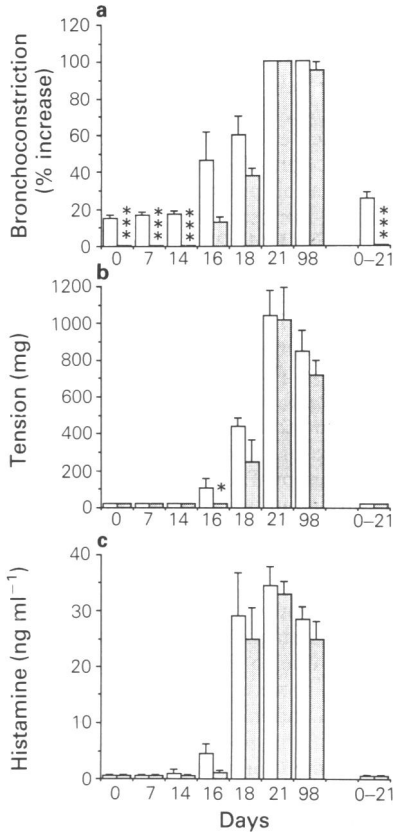


Figure 7 Bronchoconstriction (a), tracheal contraction (b) and histamine release (c) induced by the intra-arterial administration of 1 ng Paf into lungs from actively sensitized guinea-pigs in the absence (open columns) and in the presence of 10 μM WEB 2086 (stippled columns). The animals were killed at defined time intervals after the first sensitizing injection at day 0. The booster injection of the antigen was performed at day 14. Bronchoconstriction is expressed as % increase of the bronchial resistance to inflation measured prior to the administration of Paf. Tracheal strip response is expressed in mg tension. Results are expressed as mean of 5 to 7 experiments; s.e.mean shown by vertical bars. * $P < 0.05$; *** $P < 0.001$.

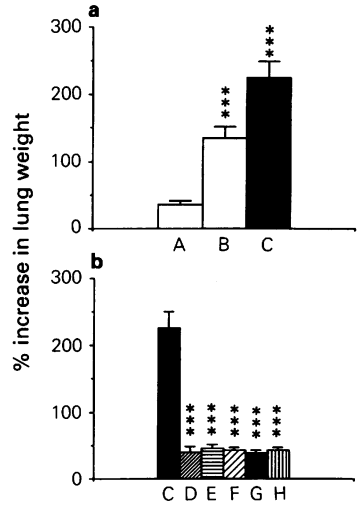


Figure 8 Interference of the various Paf antagonists with lung oedema formation, expressed by the % increase in lung wet weight. In (a) are shown the increments in lung weight measured in lungs perfused for 40 min and injected at 10 min intervals with 0.1 ml saline containing 0.1% BSA (column A); after the injection of 1 and 100 ng Paf (column B) and at the end of the experiments, after ovalbumin administration (column C). In (b), the increase of the lung wet weight measured at the end of the experiments (column C) is compared to that obtained in the presence of 10 μM WEB 2086 (column D), 0.1 mM BN 52021 (column E), 10 μM CV 6209 (column F), 10 μM Ro 19-3704 (column G) and 10 μM Ro 19-1400 (column H). Data are expressed as mean of 5 to 8 experiments; s.e.mean shown by vertical bars. *** $P < 0.001$.

Effect of Paf antagonists on Paf- and antigen-induced lung oedema formation

When lungs from actively sensitized guinea-pigs were injected at 10 min intervals with 0.1 ml saline containing 0.1% BSA, only a slight increase ($35.5 \pm 6.6\%$, $n = 5$) of the wet weight was observed after 40 min (Figure 8). By contrast, following the injection of 1 and 100 ng Paf, the lung weight was markedly increased (Figure 8). A further increase was measured at the end of the experiment, after ovalbumin administration (Figure 8). In the presence of the Paf antagonists, the increase of lung wet weight due to either Paf or ovalbumin was suppressed (Figure 8).

Discussion

The present results confirm our claim that the responsiveness to Paf of lungs of actively sensitized guinea-pigs is markedly increased when compared to that of lungs of non-immunized animals and that the pharmacological modulation of the broncho-pulmonary and secretory effects of Paf in sensitized lungs is different from that of control preparations (Pretolani *et al.*, 1988). Indeed, bronchochonstriction to Paf in lungs from actively sensitized animals is not mediated by products from the cyclo-oxygenase

pathway (Pretolani *et al.*, 1988), whereas cyclo-oxygenase inhibitors are effective against Paf-induced bronchoconstriction in non-sensitized lungs (Lefort *et al.*, 1984). This observation was further strengthened by the fact that lipoxygenase metabolites of arachidonic acid play a major role in Paf-induced bronchoconstriction in lungs from actively sensitized guinea-pigs (Pretolani *et al.*, 1989).

In the present work we demonstrate that the potent Paf antagonists WEB 2086, BN 52021 and CV 6209 become ineffective against Paf-induced bronchoconstriction and release of leukotriene-like materials and histamine when the mediator is administered to lungs from actively sensitized guinea-pigs. This phenomenon does not result simply from a shift in the potency of the drugs since the experiments were performed with concentrations of Paf antagonists 10 to 100 fold above those effective in control preparations. Furthermore, in the time-course experiments, we observed that failure of WEB 2086 was strictly related to the development of the lung hyper-responsiveness which follows the booster injection of the antigen. In confirmation, the response to Paf of lungs from animals which did not receive the booster injection at day 14 was similar to that of control preparations and was suppressed by WEB 2086. This supports our previous contention (Pretolani *et al.*, 1988) that the hyper-responsiveness to Paf of lungs from actively sensitized guinea-pigs is triggered by the booster injection of the antigen which leads to a marked modification of the lung reactivity to the phospholipid mediator.

Even though WEB 2086, BN 52021 and CV 6209 were ineffective against the bronchopulmonary effects of Paf in sensitized lungs, the other two antagonists Ro 19-3704 and Ro 19-1400 retained their activity against Paf. However, inhibition by Ro 19-3704 and Ro 19-1400 of the release of immunoreactive LTC_4-like material induced by arachidonic acid indicates that these compounds are not specific Paf antagonists and that they may interfere with the activation of the lipoxygenase pathway. This concept is reinforced by the ability of Ro 19-3074 and Ro 19-1400 to inhibit the release of LTC_4-like material induced by the antigen administration. The blockade of the generation of lipoxygenase metabolites by both compounds strongly supports a role of leukotrienes in the bronchopulmonary manifestations induced by Paf in sensitized lungs and agrees with our demonstration of the ability of the mixed cyclo-oxygenase and lipoxygenase inhibitor BW 755C (but not of pure cyclo-oxygenase inhibitors) to counteract bronchoconstriction by Paf (Pretolani *et al.*, 1989). The interference by Ro 19-3704 and Ro 19-1400 with the release of peptido-leukotrienes evoked by agonists other than Paf also agrees with data of Lagente *et al.* (1988) showing that Ro 19-3704 inhibits the production of superoxide anions from guinea-pig alveolar macrophages stimulated with various agonists, including the secretagogue tripeptide FMLP. Furthermore, recent results from our laboratory (Desquand *et al.*, 1988) show that Ro 19-3704 impairs the release of thromboxane B_2 and LTB_4 and the production of superoxide anions following stimulation of rabbit polymorphonuclear neutrophils with opsonized-zymosan and of guinea-pig alveolar macrophages with FMLP.

Platelet activating factor has the ability to increase pulmonary vascular permeability *in vivo* and *in vitro* (Bureau *et al.*, 1987; Evans *et al.*, 1987a; Imai *et al.*, 1988) and, accordingly, its specific antagonists inhibit this phenomenon (Evans *et al.*, 1987a; Imai *et al.*, 1988). In our present experiments, the Paf antagonists which failed to inhibit bronchoconstriction and release of mediators from lungs from actively sensitized guinea-pigs by Paf itself suppressed altogether Paf- and antigen-induced oedema formation. This indicates that the recognized Paf antagonists continue to block the effects of the agonist on resident endothelial cells, which account for permeability changes. We recently showed that BW 755C is as effective as Paf antagonists in inhibiting the increase in lung weight due to Paf and antigen (Pretolani *et al.*, 1989). Since those experiments were performed in the presence of indomethacin, it is likely that only the products of the lipoxygenase pathway account for the development of oedema induced by Paf in the perfused guinea-pig lung. Indeed, leukotrienes have been also demonstrated to induce alterations in lung vascular permeability (Evans *et al.*, 1987b). Thus, it can be hypothesized that following the immunological stimulations, several mediators are released including Paf, which then triggers the production of leukotrienes creating a mediator network, as already suggested by Murphy & Henson (1985).

It is possible that the effects of Paf which become refractory to the antagonists that were used are mediated through another receptor, either brought in by hypothesized inflammatory cells that it is suggested migrate to the lungs in response to the booster injection of the antigen or modified upon the resident cells, particularly mast cells responsible for histamine release. This receptor might not be recognized by the antagonists employed. Nevertheless, the efficacy of Ro 19-3704 and Ro 19-1400, under conditions where CV 6209 was inactive, does not support this possibility, since the three agents are Paf structural analogues. Alternatively, the effects of Paf may become non-receptor mediated, even though this concept is unclear presently.

In conclusion, our results demonstrate that: (i) WEB 2086, BN 52021 and CV 6209 fail to inhibit bronchoconstriction and mediator release induced by Paf administered to lungs from sensitized guinea-

pigs; (ii) the production of Paf by the lungs during anaphylaxis (Fitzgerald *et al.*, 1986) is important for the development of oedema, since the antagonists tested prevented the increase in lung wet weight following ovalbumin administration; (iii) the activation of the lipoxygenase pathway is involved in the increased reactivity of lungs from boosted animals.

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Interaction of palmitoyl carnitine with calcium antagonists in myocytes

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1 Beating of aggregates of embryonic chick myocytes, in primary culture, was quantified by use of a motion-detector and video-recorder technique. Interactions of palmitoyl carnitine, a putative endogenous ligand at Ca^{2+} channels, with calcium antagonists were investigated.

2 Bay K 8644 (1–100 nM) and palmitoyl carnitine (0.2–30 μM) increased edge movement of the aggregates; beats fused so that there was an increase in baseline 'tone'. The concentrations required to produce a 50% increase in edge movement were 2.5 nM for Bay K 8644 and 2 μM for palmitoyl carnitine. Higher concentrations (20–30 μM) of palmitoyl carnitine caused tachycardia of abrupt onset but resulted in cessation of beating. The effects of palmitoyl carnitine were not stereo-selective in that the (+)- and (−)-isomers were equieffective. Lysophosphatidyl choline (LPC) had no effect in concentrations up to 10 μM but higher concentrations caused tachycardia followed by cessation of beating. High concentrations of both palmitoyl carnitine and LPC (100 μM) caused break-up of the aggregates, presumably as a result of detergent effects.

3 Palmitoyl carnitine (1–100 μM) reversed the inhibitory effects of nisoldipine (0.3 μM), diltiazem (10 μM) and verapamil (1 μM). Ouabain was ineffective in reversing the effects of nisoldipine, differentiating the effects of palmitoyl carnitine from those of Na^+/K^+ATPase inhibition. In contrast, palmitoyl carnitine did not reverse the inhibitory effects of pimozide (2 μM) or lidoflazine (7 μM); palmitoyl carnitine showed a similar profile to Bay K 8644 in this respect.

4 These findings indicate that the effects of palmitoyl carnitine closely resemble those of Bay K 8644 and can be differentiated from those of lysophospholipids. As palmitoyl carnitine accumulates in the sarcolemma during myocardial ischaemia, the mode of action in the Ca^{2+} channel may have clinical relevance for the use of calcium antagonists in ischaemia.

Introduction

Although there are several high affinity binding sites for exogenous ligands (dihydropyridines, verapamil, diltiazem) on voltage-dependent Ca^{2+} channels (VOCs), there are no endogenous ligands which have high affinity for these sites and modulation of VOC activity appears to be indirect, as a result of changes in membrane potential (Reuter, 1967), channel phosphorylation (Tsien *et al.*, 1972; Speralakis & Schnieder, 1976) or interactions with G proteins (Holz *et al.*, 1986).

However, palmitoyl carnitine, a lipid metabolite which accumulates in the cytosol and sarcolemma during ischaemia (Idell-Wenger *et al.*, 1978; Liedtke *et al.*, 1978; Neely & Feuvray, 1981; Corr *et al.*, 1984), has been shown to resemble Bay K 8644, a dihydropyridine Ca^{2+} channel activator (Schramm *et al.*, 1983), in K^+-depolarized smooth muscle (Mir & Spedding, 1986; Spedding & Mir, 1987). As palmitoyl carnitine has selective interactions with tritiated calcium-antagonists ([^{3}H]-nitrendipine, [^{3}H]-verapamil and [^{3}H]-diltiazem) in rat cerebral cortex membranes at concentrations which may be achieved during ischaemia, it has been suggested that acylcarnitines may be endogenous modulators of Ca^{2+} channel function, at least during ischaemic conditions (Mir & Spedding, 1986; Spedding & Mir, 1987).

We have therefore investigated whether the effects of palmitoyl carnitine mimic those of Bay K 8644 in beating aggregates of chick myocytes, maintained in primary cell culture. Beating was quantified with a motion detector video system which was developed to investigate the effects of Ca^{2+} channel activators on myocardial cells (Patmore & Whiting 1985;

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Patmore & Duncan 1988). Calcium-antagonists may act at various sites in VOCs to block the channels (Spedding, 1985b) and very distinct interactions have been shown to occur between the various subgroups of antagonists and dihydropyridine Ca^{2+} channel activators both in smooth muscle (Schramm & Towart, 1984; Su *et al.*, 1984; Spedding & Berg, 1984; Spedding, 1985a) and in heart (Boddeke *et al.*, 1988; Patmore & Duncan, 1988).

Thus, Bay K 8644 has been shown to reverse competitively the effects of the dihydropyridine calcium-antagonists, to reverse non-competitively the effects of verapamil and diltiazem, but not to reverse the inhibitory effects of lipophilic 'class III' (Spedding, 1985b) calcium antagonists, such as flunarizine, pimozide and lidoflazine. These interactions have also been defined in chick myocytes (Patmore & Duncan, 1988). The aims of the present study were to assess whether the interactions between Bay K 8644 and the different groups of calcium-antagonists in myocardium were similar to those between palmitoyl carnitine and the calcium antagonists. Such an approach would indicate whether palmitoyl carnitine had a site of action in the myocardial Ca^{2+} channel and could be considered as an endogenous activator. As palmitoyl carnitine has detergent effects in high concentrations, we have also compared its effects with those of the lysophospholipids which also accumulate during ischaemia; much of this accumulation will be consequent to inhibition of lysophospholipase and lysophospholipase transacylase by acylcarnitines (Gross *et al.*, 1982; Gross & Sobel, 1983). Some of this work has been presented to the British Pharmacological Society (Duncan *et al.*, 1986; 1987).

Methods

Cell cultures

Eleven day old Leghorn K chicken embryos were killed by decapitation and the hearts removed under aseptic conditions. Ventricles were chopped into fragments and were washed in Hanks Ca^{2+}- and Mg^{2+}-free balanced salt solution (Gibco) and then agitated at 37°C in the presence of 0.05% trypsin as described by Clusin (1981). Trypsinisation was then repeated 3–4 times and the dispersed cells were pooled, centrifuged and resuspended in culture media containing 20% nutrient media M-199 (Gibco), 6% heat-inactivated foetal calf serum, 1% penicillin/streptomycin solution and 73% low potassium salt solution the composition of which was (mM): NaCl 116, $NaHCO_3$ 26.2, $MgSO_4$ 0.8, NaH_2PO_4 0.9, $CaCl_2$ 1.8 and dextrose 5.5, adjusted to pH 7.3. The cells were seeded at a density of 3–4 $\times 10^5$ cells ml^{-1} into 96 well trays. The bottom of each well was coated with a layer of Sylgard 184 (Dow Corning) silicon elastomer resin. This process inhibits the formation of monolayers and over a period of 3 days in culture the cells reaggregate to form roughly spherical clusters of 100–200 μm diameter.

Assessment of beating and experimental procedures

Aggregates normally display automaticity and the edge movement associated with contractions could be detected in each aggregate on observation under low power (40 ×) microscopy. Around 15 aggregates formed in each culture well and contractility was assessed by scoring the % of aggregates in each well which were beating. This was done on an all-or-nothing basis and 10 randomly identified aggregates were scored. Control beating was normally 100%. The salt solution was designed to resemble the ionic composition of the culture media and contained (mM): NaCl 110, KCl 1.07, $CaCl_2$ 1.7, $MgSO_4$ 0.73, HEPES 19.3, $NaHCO_3$ 0.83 and dextrose 5.5. Following drug application cultures were incubated for 30 min at 37°C and then identified wells scored again for % of aggregates beating. Only 40 of the 96 wells were used per tray and different treatments (drugs or concentrations) were applied to each of the 40 wells. Replicate tests for each concentration of each drug was made on 3 further trays. On each tray a series of wells were treated as 'sham' controls where salt solution containing no drug was added. This normally caused no inhibition of beating. Concentration-response curves were calculated for drugs by assessing the % of aggregates in which beating was completely inhibited.

In experiments where the interaction between palmitoyl carnitine and antagonists was investigated, concentrations of antagonists which caused approximately 85% inhibition of beating were added simulataneously with varying concentrations of palmitoyl carnitine. The scoring protocol was the same as that described for antagonist experiments and the data were again expressed as % of the sham-treated control aggregate beating. Data were fitted to a sigmoidal function between 15 and 100% of control and EC_{50} values (actually taken at 57.5% of control) calculated to express the potency of the palmitoyl carnitine reversal of antagonist-induced inhibition of beating.

Measurements of contractility

Contractility of individual spontaneously beating aggregates was estimated by measuring edge movement of individual aggregates. This technique has

been described briefly elsewhere (Patmore & Whiting, 1985). Aggregates grown on sylgard elastomer treated dishes were transfered to individual wells from Lux select-a-well culture plates. Over a period of 15–20 min very firm contacts were usually made with the substrate and the well was transfered to an inverted microscope stage and aggregates viewed at 400 × magnification. An aggregate was selected which displayed substantial edge movement, the area occupied by the aggregate changing on contraction. A video camera was attached to the microscope and on the video image a detection window was superimposed on the area of aggregate showing most edge movement. The window was composed of 20–30 raster lines and the point at which the image changed from light to dark (background to aggregate) along each line was compared with the length of the line. This was repeated for each line, the data integrated to calculate the area of aggregate occupying the window and this value was updated every video scan (20 ms). On contraction the area of aggregate occupying the detection window changes and an analogue output of area describing the waveform of contraction was obtained. This is essentially a measure of edge displacement and would typically be of the order of 5 μm. Edge movement has been previously shown to be a function of the force of contraction (Clusin, 1981) and in this study edge movement has been used synonymously with contraction. Aggregates were continually superfused with culture media substitute salt solution as described earlier prewarmed to 37°C. Drugs were added to the superfusate in the presence of half the normal level of calcium to allow observation of positive inotropism.

Drugs

The following susbtances were used: palmitoyl (−)- and (±)-carnitine, lysophosphatidyl choline (grade 1) (Sigma); (±)-verapamil (Sigma); pimozide, lidoflazine (Janssen); Bay K 8644 (methyl 1,4-dihydro-2,6-methyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine - 5-carboxylate) and nisoldipine (Bayer). Palmitoyl (+)-carnitine was synthesized by Dr G. Ramirez, Institute of Organic Chemistry, Palo Alto, Ca, U.S.A. Throughout the text, the racemic form is referred to as palmitoyl carnitine.

Results

Aggregates of myocytes beat spontaneously with a rate of 107 ± 4 beats min^{-1} ($n = 20$) at 37°C. The extent of edge movement of each aggregate, which was taken as being equivalent to an inotropic effect

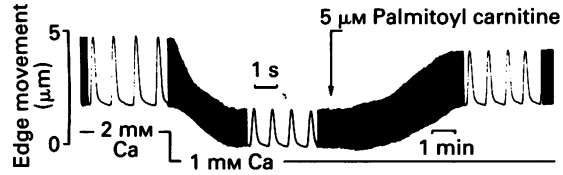


Figure 1 Effects of palmitoyl carnitine (5 μM) on edge movement of aggregates of myocytes from chick embryos.

was dependent on the Ca^{2+} concentration of the medium; 1 mM Ca^{2+} resulted in half maximal contractility with a near maximal effect occurring with 2 mM Ca^{2+} (Figures 1 and 2; Patmore & Duncan, 1988). Increasing the Ca^{2+} concentration caused the beats to fuse partially so that the base-line increased; presumably relaxant processes were insufficient to account for Ca^{2+} entry so that cytosolic Ca^{2+} increased during diastole. Baseline changes reflected edge movement in all the experiments.

The protocol for investigation of the effects of palmitoyl carnitine is depicted in Figure 1. Preparations were set up in high Ca^{2+} (2 mM), allowing an estimate of near-maximal inotropic effects followed by a 10 min incubation in 1 mM Ca^{2+}; edge movement after 10 min was taken as 100%. Palmitoyl carnitine (0.5–30 μM) caused a concentration-dependent increase in contractility, with a maximum effect similar to doubling the Ca^{2+} concentration. The concentration which caused a 50% increase in edge movement was 2 μM (Figure 3). However, prolonged incubation (> 10 min at 10 μM) or shorter incubation with high concentrations (> 5 min with 20–30 μM) resulted in a short period (5–360 s) of tachycardia followed by cessation of beating (Figures 2a and 4a). The inotropic effects of palmitoyl carnitine were not stereo-selective, because concentration-response curves to the racemate, (−)-isomer (concentration for 50% increase in edge movement: 3.5 μM) and (+)-isomer (concentration for 50% increase in edge movement: 2.5 μM) were superimposable in > 4 experiments.

Bay K 8644 (1–100 nM) augmented edge movement in a concentration-dependent manner and also increased the baseline (Figures 2b and 3); rate of beating was not affected. The augmented contractility was maintained for > 30 min.

Lysophosphatidyl choline (LPC; 1–10 μM) did not increase edge movement (Figure 3), although higher concentrations (30–50 μM; Figure 4b) increased edge movement and rate of beating transiently; these concentrations were associated with break-up of the edge so that tracking was no longer possible. The aggregates broke up at higher concentrations and in four experiments at 100 μM break-up occurred within 2 min without marked inotropic effects. Palmitoyl

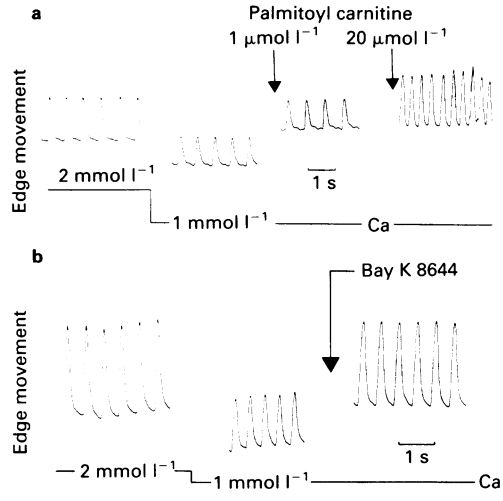


Figure 2 Effects of lowering the Ca^{2+} concentration from 2 mM to 1 mM on edge movement in chick myocytes and of perfusing for 10 min with palmitoyl carnitine (a, 1 μM then 10 min at 20 μM) or with Bay K 8644 (b, 10 nM).

carnitine caused edge break-up at high concentrations (> 100 μM).

Acyl carnitines inhibit the degradation of LPC in cell membranes, so the effects of a threshold concentration of LPC (10 μM) in the presence of palmitoyl carnitine (1 μM) were determined. In four experiments, LPC (10 μM) caused a similar threshold effect (14.5 ± 22% augmentation, $P > 0.1$) to that obtained previously (Figure 3). However, after incubation with palmitoyl carnitine, LPC caused a rapid tachycardia

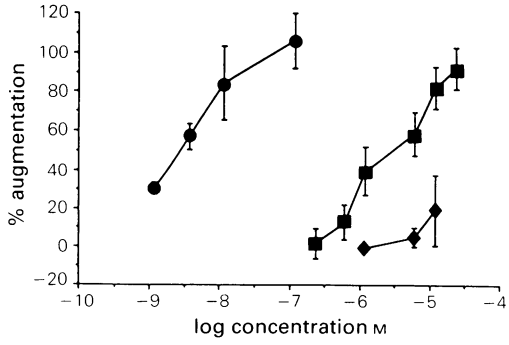


Figure 3 Concentration-response curves for the increase in edge movement caused by Bay K 8644 (●, $n = 5$), palmitoyl carnitine (■, $n = 5$–18) or lysophosphatidyl choline (◆, $n = 5$–6). Edge movement in the presence of 1 mM Ca^{2+} was taken as 100%. Edge movement in the presence of 2 mM Ca^{2+} was augmented to 190% ($n = 9$). Vertical bars represent s.e.mean.

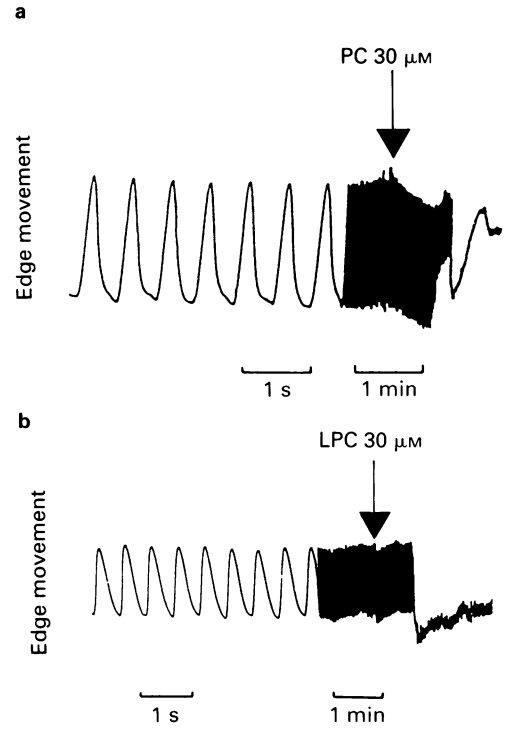


Figure 4 Effects of palmitoyl carnitine (PC) (a, 30 μM) and lysophosphatidyl choline (LPC) (b, 30 μM) on edge movement in chick myocytes.

in all 17 preparations tested and within 10 min beating ceased in 9 of the preparations; the maximal increase in edge movement observed was 19.0 ± 17% above that caused by palmitoyl carnitine (11% augmentation, $n = 17$). Thus it would appear that although palmitoyl carnitine may increase the susceptibility of tissues to the well-known arrhythmogenic properties of LPC, pre-exposure to acyl carnitines did not reveal marked inotropic effects.

Calcium-antagonists

The inhibitory effects of calcium-antagonists on cell automaticity were quantified by an all or none observational technique whereby groups of 10 aggregates are scored for evidence of edge movement; incubation with increasing concentrations of calcium antagonists results in a concentration-dependent inhibition of the number of aggregates beating. Preliminary experiments established concentrations of the antagonists (nisoldipine 0.3 μM, diltiazem 10 μM, verapamil 1 μM, pimozide 2 μM, lidoflazine 7 μM) which were equieffective in causing approximately 85% inhibition of beating. Increasing the concentration of Bay K 8644 completely counteracted the

Table 1 Comparison of the potencies of Bay K 8644 and palmitoyl carnitine in augmenting edge movement and reversing the effects of calcium-antagonists in chick myocytes and in augmenting contractility in K^+-depolarized smooth muscle preparations (taenia caeci from the guinea-pig) or in displacing tritiated ligands from the Ca^{2+} channels in rat cerebral cortex

	Palmitoyl carnitine (A)	*Bay K 8644* (B)	*A/B*
Myocytes:			
Inotropy	5.7	8.6	954
Reversal:			
Nisoldipine	4.1	7.1′	1000
Verapamil	3.9	7.1′	3010
Diltiazem	4.4	6.9′	700
Pimozide	NE	NE	—
Lidoflazine	NE	NE	—
*Smooth muscle:**			
Augmentation	4.1	7.9	6310
*Cerebral cortex:**			
[^{3}H]-nitrendipine	3.9	7.7	6310
[^{3}H]-verapamil	4.0	8.1	12590
[^{3}H]-diltiazem	4.0	7.9	7940

Data are expressed as pEC_{50}s or pIC_{50}s, except for the ratio.
* Data from Spedding & Mir, 1987; Patmore & Duncan, 1988.
NE – not effective.

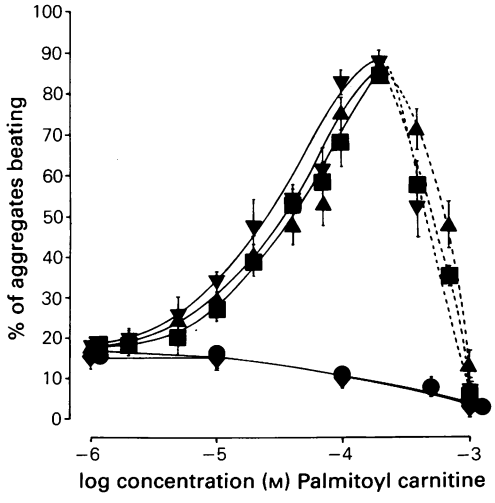


Figure 5 Reversal of the effects of nisoldipine (0.3 μM, ▲), verapamil (1 μM, ■), diltiazem (10 μM, ▼) but not lidoflazine (7 μM, ●) or pimozide (2 μM, ◆) by palmitoyl carnitine. The number of aggregates beating in a culture plate was quantified, using 10 plates per observation. Vertical bars represent s.e.mean.

inhibitory effects of nisoldipine (0.3 μM) with a pEC_{50} of 7.1 (Table 1).

Palmitoyl carnitine (1–200 μM) reversed the inhibitory effects of nisoldipine (0.3 μM) with a pEC_{50} of 4.1 (Figure 5; Table 1). However the reversal was not complete and at higher concentrations of palmitoyl carnitine (0.5 and 1 mM) inhibitory effects were observed, presumably due to antagonist effects or to the detergent properties of the substance. Palmitoyl carnitine reversed the inhibitory effects of verapamil and diltiazem with pEC_{50}s of 3.9 and 4.4 respectively (Figure 5; Table 1). As was the case with Bay K 8644, palmitoyl carnitine did not reverse the inhibitory effects of pimozide and lidoflazine (Figure 5).

Unlike palmitoyl carnitine, ouabain (10 μM) did not reverse the inhibitory effects of nisoldipine in this model ($n = 4$), indicating that the reversal of the effects of nisoldipine by palmitoyl carnitine were not mediated via inhibition of Na^+/K^+ ATPase.

Discussion

This study shows that palmitoyl carnitine (0.5–30 μM) increased edge movement in myocytes, and the effects are similar to those of Bay K 8644 and of increasing the Ca^{2+} concentration. This is consistent with the findings of Inoue & Pappano (1983), who reported that higher concentrations (30–300 μM) increased Ca^{2+}-dependent action potentials in avian ventricular muscle, and of Spedding & Mir (1987) who found that palmitoyl carnitine (10–100 μM) resembled Bay K 8644 in K^+-depolarized smooth muscle. However, the concentrations required to increase edge movement in the myocytes were much lower than in the previous studies, although the concentration in the sarcolemma is not known. Thus the myocytes appeared particularly susceptible, due either to a greater accumulation of acyl carnitines than in the other preparations, which would be facilitated by easier access to these small preparations, or perhaps because of a greater susceptibility of the Ca^{2+} channels in these cells. The low concentrations are relevant because Knabb *et al.* (1986) have shown, in cultured rat myocytes, that sarcolemmal acylcarnitines increase 70 fold during hypoxia and after 60 min of hypoxia constituted approximately 3.5% of total sarcolemmal phospholipid. It is therefore likely that such an accumulation would interact with Ca^{2+} channels, as seen in our study. Piper *et al.* (1984) found that the concentrations of acyl carnitines present during ischaemia were insufficient to cause mitochondrial damage directly, to affect Na^+/Ca^{2+} exchange or Ca^{2+} pumping activity in the sarcoplasmic reticulum. However, these workers did not consider the possi-

bility of effects on Ca^{2+} mobilisation. Increased Ca^{2+} mobilisation during hypoxia or ischaemia (see Allen & Orchard, 1987; Barry et al., 1987) via Ca^{2+} channels would be deleterious to cellular function and would be a major contributor to cell death following ischaemia and is discussed later.

What evidence is there that palmitoyl carnitine interacts with Ca^{2+} channels or tightly associated proteins? The effects of palmitoyl carnitine mimic those of the selective Ca^{2+} channel activator Bay K 8644 and although palmitoyl carnitine is approximately 1000 fold less potent than the dihydropyridine there is a good parallelism between the concentrations required to augment contractility and to counteract the effects of the calcium-antagonists (Table 1). Bay K 8644 is a powerful tool for defining the site of action of agents at the Ca^{2+} channel (Su et al., 1984; Spedding & Berg, 1984; Spedding, 1985a,b). Using the present experimental protocol, Patmore & Duncan (1988) have shown that Bay K 8644 counteracts the effects of nisoldipine at nonomolar concentrations (Table 1), which is compatible with the competitive interaction between dihydropyridines observed in smooth muscle. Bay K 8644 also counteracted the effects of verapamil and diltiazem; the binding sites for these drugs are linked by an allosteric mechanism within the Ca^{2+} channel (Galizzi et al., 1985; Glossmann et al., 1985; Mir & Spedding, 1987). However, Bay K 8644 did not reverse the inhibitory effects of pimozide or lidoflazine, lipophilic class III calcium antagonists (Spedding, 1985b). Bay K 8644 does not reverse the effects of these agents in smooth muscle (Spedding & Berg, 1984) nor in heart (Boddeke et al., 1988; Patmore & Duncan, 1988) implying that these drugs either do not act at the Ca^{2+} channel (Spedding, 1985b; Grima et al., 1986) or act at a distinct site in the channel, perhaps trapping it in a state which has low affinity for dihydropyridines (Spedding, 1985b).

Thus, palmitoyl carnitine shows the same selective interactions between the different classes of calcium-antagonists as does Bay K 8644. Furthermore, the pIC_{50}s for palmitoyl carnitine to reverse nisoldipine, diltiazem and verapamil in the heart (Table 1) are very similar to the pIC_{50}s for inhibition of [^{3}H]-nitrendipine, [^{3}H]-diltiazem and [^{3}H]-verapamil binding (Table 1) pointing to a direct interaction with the Ca^{2+} channel. However, the lack of stereoselectivity indicates that the interaction may be consequent to some form of selective accumulation and increased Ca^{2+} availability, perhaps consequent to a surface charge effect (Inoue & Papanno, 1983; Spedding, 1984). Electrophysiological studies are underway to define the mode of action.

Acyl carnitines and lysophospholipids have marked detergent properties and Adams et al. (1979) considered these substances to be 'naturally occurring detergents', although Spedding & Mir (1987) found that a wide range of surface-active molecules did not mimic the effects of palmitoyl carnitine in smooth muscle. Nevertheless the detergent effects of lysophospholipids have been implicated as causative factors of arrhythmogenesis during ischaemia (Corr et al., 1981; 1982; 1984; 1987) by affecting a wide variety of membrane-linked processes, particularly by inhibiting the conductance of the inward rectifier K^+ channel (Clarkson & Ten Eick, 1983; Kiyosue & Arita, 1986). The threshold concentration for affecting K^+ conductance in guinea-pig myocytes was found to be 10 μM (Kiyosue & Arita, 1986), which was the threshold concentration for the effects of LPC in the present study. Tachycardia followed by asystole would be expected from an agent affecting K^+ conductance and this parallels the excitability changes seen in ischaemic tissue (Corr et al., 1987). A full electrophysiological analysis in myocytes will be required to ascertain whether the tachycardia followed by asystole caused by LPC and palmitoyl carnitine, at high concentrations (20 μM) is due to this phenomenon.

Nevertheless although high concentrations of LPC and palmitoyl carnitine have similar detergent properties (see Spedding & Mir, 1987), LPC tends to inhibit rather than augment Ca^2 current (Clarkson & Ten Eick, 1983; Corr et al., 1987) and in the present study was effectively devoid of positive inotropic effects. The positive inotropic effects of palmitoyl carnitine can therefore be clearly differentiated from those of LPC. Will an effect of palmitoyl carnitine on Ca^{2+} availability be of relevance during ischaemia?

Knabb et al. (1986) have shown a 70 fold accumulation of acylcarnitines in the sarcolemma of rat myocytes during hypoxia and that inhibition of this accumulation with an acylcarnitine transferase inhibitor prevented the electrophysiological changes and depolarization which accompanied hypoxia, indicating that acylcarnitines are major determinants of ischaemic processes. Acyl carnitines would also be pivotal in controlling the lysophospholipid content of the sarcolemma. It has previously been assumed by many workers that Ca^{2+} overload following reperfusion which leads to cell death was the result of non-specific membrane disruption. However, Crake & Poole-Wilson (1986) have shown that the massive Ca^{2+} influx following ischaemia and reoxygenation in rabbit isolated heart is not accompanied by non-specific membrane disruption and we consider that it may be via modified calcium channels, especially as the number of channels available to tritiated calcium antagonists is modified in myocardial ischaemia (Dillon & Nayler, 1987; Matucci et al., 1987); such data are consistent with a pool of channels being affected by acyl carnitines.

In conclusion, acyl carnitines have inotropic effects in myocytes and selective interactions with calcium-antagonists which closely resemble those of Ca^{2+} channel activators. Such findings support further work investigating the hypothesis that Ca^{2+} overload following ischaemia and reperfusion may be via channels modified by acyl carnitines.

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Prevention of physostigmine-, DFP-, and diazinon-induced acute toxicity by monoethylcholine and N-aminodeanol

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1 Choline, and the choline analogues monoethylcholine (MEC) and N-aminodeanol (NAD) were examined for prophylactic activity in acute acetylcholinesterase inhibitor toxicity in mice. The rank order of potency of the compounds was MEC > NAD > choline.

2 Simultaneous administration of MEC (60 mg kg^{-1}) or NAD (200 mg kg^{-1}) with physostigmine reduced lethality to 17 and 13% respectively. MEC (60 mg kg^{-1}) completely protected against diisopropylfluorophosphate (DFP) and diazinon toxicity, and NAD reduced lethality to 17% for both agents. Choline (200 mg kg^{-1}) exhibited only negligible antidotal activity against the inhibitors.

3 *In vitro* concentrations of choline, MEC, and NAD, similar to the estimated concentration obtained *in vivo* in the acute toxicity study, produced mixed inhibition of mouse brain acetylcholinesterase. The inhibition was dose-related and was additive to the inhibition produced by the cholinesterase inhibitors.

4 All three analogues reduced ligand binding at the nicotinic, M_1, and M_2 receptors. The rank order of potencies for the analogues at each receptor was nicotinic: (choline > MEC > NAD), M_1: (MEC > choline > NAD), and M_2: (MEC > choline > NAD).

5 It is proposed that the analogues prevent acetylcholinesterase inhibitor toxicity peripherally by interacting with acetylcholinesterase, and/or by competing with acetylcholine for binding to cholinoceptors.

Introduction

Cholinesterase inhibitors primarily inhibit acetylcholinesterase (AChE) and secondarily inhibit other esterases that hydrolyze choline esters (Wills, 1970; Chemnitius *et al.*, 1988). After inhibition of these enzymes acetylcholine accumulates at all cholinergic nerve endings and cholinoceptors (Holmes, 1953) and in human plasma (Okonek & Kilbinger, 1974). Toxic doses of these agents may produce cardiovascular and respiratory depression mediated by excessive central and peripheral acetylcholine receptor stimulation. Although the probable cause of death is anoxia (Clement & Lee, 1980) it is unclear whether central respiratory depression (Meeter & Wolthuis, 1968) or peripheral respiratory paralysis (Clement & Lockwood, 1982) is responsible. Central or peripheral toxicity also appears to vary with the nature of the acetylcholinesterase inhibitor (van Helden *et al.*, 1983).

A standard clinical antidote for AChE inhibitor toxicity is available which consists of atropine to block the muscarinic effects of acetylcholine and pralidoxime to reactivate acetylcholinesterase. However, the nicotinic effects of acetylcholine (ganglionic and diaphragmatic stimulation) remain untreated. The effectiveness of the antidote also depends on the type of inhibitor (i.e. organophosphate or carbamate)

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responsible for the toxicity as well as the time lag between exposure and treatment (Fleisher & Harris, 1965). The combination of an oxime and anticholinoceptor agent is effective for organophosphate toxicity (Leadbeater *et al.*, 1985). However, in carbamate intoxication, atropine, but not pralidoxime, is recommended since the latter is ineffective against some carbamates and may actually increase toxicity in the case of carbaryl (Natoff & Reiff, 1973). Experimental antidotal approaches have included the use of soman simulator 'Som-Sim' (Wolthuis *et al.*, 1981), choline acetyltransferase inhibitors (Schoene *et al.*, 1977), reversible cholinesterase inhibitors (Harris *et al.*, 1978), and hemicholinium-3 (HC-3) (Lin *et al.*, 1987).

Since the available treatment of AChE inhibitor toxicity is less than satisfactory for carbamates, it was the purpose of the present study to examine analogues of choline as possible antidotes. The rationale for this approach was based on the premise that the analogues will compete with acetylcholine for binding to acetylcholine receptors. A reduction in acetylcholine-mediated toxicity should result since the analogues possess both reduced affinity and intrinsic activity for these receptors. Mechanisms of action of the analogues with respect to their effect on the kinetics of inhibition of AChE and their affinity for M_1, M_2, and nicotinic receptors are also presented.

Methods

Virus free male ICR mice (15–20 g) and Sprague Dawley rats (175–200 g) were obtained from Harland Industries (Prattville, AL and Indianapolis, IN, U.S.A., respectively). All animals were housed in a temperature-controlled environment (25°C) with a 12 h light/dark cycle. Mouse brain or rat heart tissue was obtained following decapitation with a guillotine.

Prevention of acetylcholinesterase inhibitor toxicity

The ability of choline, monoethylcholine (MEC), or N-aminodeanol (NAD) to prevent acetylcholinesterase inhibitor toxicity was determined by simultaneous administration (i.p.) with acetylcholinesterase inhibitor in mice. The observation period for the acute toxicity of physostigmine was 30 min, and 6 h for diisopropylfluorophosphate (DFP) or diazinon. Physostigmine (0.8 mg kg^{-1}) and diazinon (60 mg kg^{-1}) were administered intraperitoneally and DFP (6 mg kg^{-1}) was administered subcutaneously.

Estimation of in vivo *concentration*

Initial *in vivo* concentrations of the analogues after intraperitoneal administration were estimated on the following assumptions: (1) 80% of the weight of the animal constituted total body water, (2) the dose was distributed through total body water. The initial concentration was then estimated to be the dose, divided by the total body water.

Acetylcholinesterase inhibition

A modification of the spectrophotometric method of Ellman *et al.* (1961) was used to determine acetylcholinesterase activity. A 25% homogenate of whole mouse brain (free of cerebellum and lower brain stem) in 0.1 M sodium potassium phosphate buffer (pH 7.9) containing 1% Triton-X-100 was prepared using a Polytron (setting = 5). The homogenate was centrifuged at 40,000 *g* for 30 min, and the supernatant used for determining cholinesterase activity. Acetylcholinesterase inhibitor or choline analogue was pre-incubated with enzyme for 2 min before addition of substrate. All enzyme velocities were determined at 20°C during the initial 1–2 min of reaction.

Binding assays

The affinity of choline, MEC and NAD for three types of acetylcholine receptors was examined by variations of a filtration assay (Aronstam *et al.*, 1978). Affinity was determined from the ability of the analogue to inhibit the binding of ligand to the receptor. The ratio of the concentration of labelled ligand to its K_d was 0.5 for [^{3}H]-pirenzepine, 1.0 for [^{3}H]-N-methylscopolamine, and 230 for [^{125}I]-α-bungarotoxin. Data analysis was performed using the programme LIGAND.

The affinity of the analogues for the M_1 receptor was examined in mouse brain cerebral cortex tissue by use of [^{3}H]-pirenzepine. Cerebral cortex tissue was homogenized in 10 volumes of sodium potassium phosphate buffer (pH 7.4, 0.05 M) in an Elvehjem apparatus with a teflon pestle. The homogenate was then centrifuged at 1000 *g* for 10 min, the supernatant recentrifuged at 40,000 *g* for 1 h and the pellet resuspended in buffer. The reaction volume was 1 ml and contained 200 μg protein, 100 μM DFP, 3 nM [^{3}H]-pirenzepine, choline analogue, and sodium potassium phosphate buffer. Choline analogue and ligand were added 20 min after the addition of DFP, and incubated an additional 40 min at 20°C. The assay was terminated by filtration through Whatman GF/B filters. Non-specific binding was determined with 1 μM atropine.

Table 1 Reversal of acetylcholinesterase inhibitor toxicity by monoethylcholine (MEC), N-aminodeanol (NAD) and choline (Ch)

Treatment	n	*Observation period* (min)	*Time to death* (min)	% *deaths*
Physostigmine ($0.8\,mg\,kg^{-1}$)	21	30	6.0 ± 3.0	87.7
Physostigmine + MEC ($60\,mg\,kg^{-1}$)	6	30	27.0*	16.7
Physostigmine + NAD ($200\,mg\,kg^{-1}$)	8	30	7.4*	12.5
Physostigmine + Ch ($200\,mg\,kg^{-1}$)	6	30	5.9	83.3
DFP ($6\,mg\,kg^{-1}$)	26	360	12.1 ± 0.8	96.2
DFP + MEC ($60\,mg\,kg^{-1}$)	8	360	0	0
DFP + NAD ($200\,mg\,kg^{-1}$)	9	360	20.2 ± 3.4	100
DFP + NAD ($600\,mg\,kg^{-1}$)	6	360	22.5*	16.7
DFP + Ch ($200\,mg\,kg^{-1}$)	9	360	16.7 ± 2.8	88.9
Diazinon ($60\,mg\,kg^{-1}$)	29	360	33.1 ± 8.8	93.1
Diazinon + MEC ($60\,mg\,kg^{-1}$)	6	360	0	0
Diazinon + NAD ($400\,mg\,kg^{-1}$)	6	360	35.0*	16.7
Diazinon + NAD ($600\,mg\,kg^{-1}$)	6	360	7.0 ± 2.2	50.0
Diazinon + Ch ($200\,mg\,kg^{-1}$)	9	360	22.1 ± 3.1	77.8

Male ICR mice (18–25 g) were administered inhibitor alone or concurrently with choline analogue. All agents were administered i.p. except for DFP (administered s.c.). Solvents used were: water (physostigmine, MEC, NAD, Ch); 0.15 M NaCl (DFP); and propyleneglycol (diazinon). Average time to death does not include survivors.
n = Number of animals; $\pm$ = standard error of mean
* $n = 1$

Rat heart was the tissue source for M_2 muscarinic receptor material. After rinsing in buffer, the tissue was minced with scissors, and homogenized in 10 volumes of sodium potassium phosphate buffer (pH 7.4, 0.05 M) in a Polytron (2 × 30 s, speed 6), and further homogenized in an Elvehjem apparatus with a teflon pestle. The homogenate was then centrifuged at 1000 *g* for 10 min, the pellet rehomogenized with fresh buffer as above and centrifuged at 1000 *g*. The 1000 *g* supernatants were pooled and centrifuged at 40,000 *g* for 35 min, and the pellet resuspended in buffer. The reaction volume used was 2 ml and contained 500 μg protein, 100 μM DFP, 0.3 nM [^{3}H]-scopolamine methyl chloride, choline analogue, and sodium potassium phosphate buffer. The remaining assay procedure was the same as for the M_1 receptor assay.

The source of nicotinic receptor material was the electric organ of *Torpedo californica* (Pacific Biomarine, California). Frozen tissue was homogenized in 13 volumes of NaCl-Tris buffer (0.4 M NaCl:0.01 M Tris, pH 7.2) in a Waring blendor for 1 min (high speed). The homogenate was centrifuged at 2000 *g* for 10 min, the supernatant recentrifuged at 30,000 *g* for 30 min, and the pellet resuspended in buffer. The reaction volume used was 1 ml and contained 25 μg of protein, 100 μM DFP, 1 nM [^{125}I]-α-bungarotoxin, choline analogue, and NaCl-Tris buffer. Incubation conditions were similar to the M_1 assay above. Methylated bovine serum albumin (BSA) was added at the end of the incubation period (final concentration $3\,mg\,ml^{-1}$) to reduce non-specific filter binding to Whatman GF/C filters. Non-specific binding was defined with unlabelled α-bungarotoxin (1 μM).

Reagents and isotopes

Physostigmine sulphate (Sigma) and choline chloride (Sigma) were prepared in distilled water and diisopropylfluorophosphate (DFP, Sigma) was prepared in 0.15 M sodium chloride. Diazinon (Ciba-Geigy) was dissolved in propylene glycol and diazoxon (Ciba-Geigy) was dissolved in water. All cholinesterase inhibitor solutions were prepared fresh immediately before use. [N-methyl-^{3}H]-scopolamine methyl chloride ($72\,Ci\,mmol^{-1}$) was purchased from Amersham, U.K. [N-methyl-^{3}H]-pirenzepine ($85\,Ci\,mmol^{-1}$) and [^{125}I]-α-bungarotoxin ($17.3\,\mu Ci\,\mu g^{-1}$) were purchased from New England Nuclear.

Monoethylcholine iodide (MEC) was prepared by reacting N,N-dimethyl-aminoethanol and ethyl iodide. N-aminodeanol perchlorate (N-amino-N,N-dimethylaminoethanol) was prepared according to the method of Newton *et al.* (1983). All synthesized compounds were recrystallized and their structures confirmed using IR, n.m.r., and melting points.

Results

Prevention of acetylcholinesterase inhibitor toxicity

Table 1 summarizes the ability of several choline analogues to prevent the acute toxicity of reversible and irreversible acetylcholinesterase inhibitors. Physostigmine produced fatalities in ~88% of mice tested with an average time to death of 6 min. Simultaneous administration of monoethylcholine (MEC, 60 mg kg^{-1}) or N-aminodeanol (NAD, 200 mg kg^{-1}) reduced the deaths to ~17% and ~13% respectively. Simultaneous administration of choline did not appear to alter toxicity or the time to death. DFP was lethal in ~96% of mice tested. Simultaneous administration of MEC (60 mg kg^{-1}) afforded complete protection against DFP. NAD, inactive at 200 mg kg^{-1}, reduced lethality to ~17% when administered at 600 mg kg^{-1}. Choline (200 mg kg^{-1}) reduced lethality to 89%. The acute toxicity of diazinon was completely blocked by simultaneous administration of 60 mg kg^{-1} MEC. NAD (400 mg kg^{-1}) reduced deaths to ~17% but increased toxicity when administered at 600 mg kg^{-1}. Choline at 200 mg kg^{-1} reduced deaths to ~78%.

Acetylcholinesterase inhibition

The choline analogues were also examined for their ability to affect the kinetics of acetylcholinesterase (AChE) in the presence and absence of AChE inhibitors. The cuvette concentration of the choline analogues was selected to match the estimated *in vivo* concentration achieved in the acute toxicity studies. In Figure 1, MEC60 corresponds to a cuvette concentration of 0.33 mM monoethylcholine (MEC) which was approximated *in vivo* with a dose of 60 mg kg^{-1} (i.p.). MEC alone produced a mixed inhibition of AChE. In the presence of the reversible inhibitor physostigmine, MEC60 produced a mixed inhibition which was additive to the effect of physostigmine. Further inhibition was observed with a two fold higher concentration of MEC (0.66 mM, MEC120). In the presence of the irreversible inhibitor DFP, MEC also produced a dose-related mixed inhibition which was additive to the effect of DFP. The *in vitro* AChE inhibitor activity of the irreversible agent diazinon was determined with its biologically active metabolite, diazoxon (Kreuger *et al.*, 1960). In the presence of diazoxon, MEC also produced a dose-related inhibition which was additive to the effect of diazoxon. In Figure 2, NAD200 corresponds to a cuvette concentration of N-aminodeanol (NAD) of 1.8 mM which was approximated *in vivo* with a dose of 200 mg kg^{-1} (i.p.). NAD alone produced a mixed inhibition of AChE. In the presence of physostigmine, DFP, or diazoxon, NAD also produced a dose-dependent

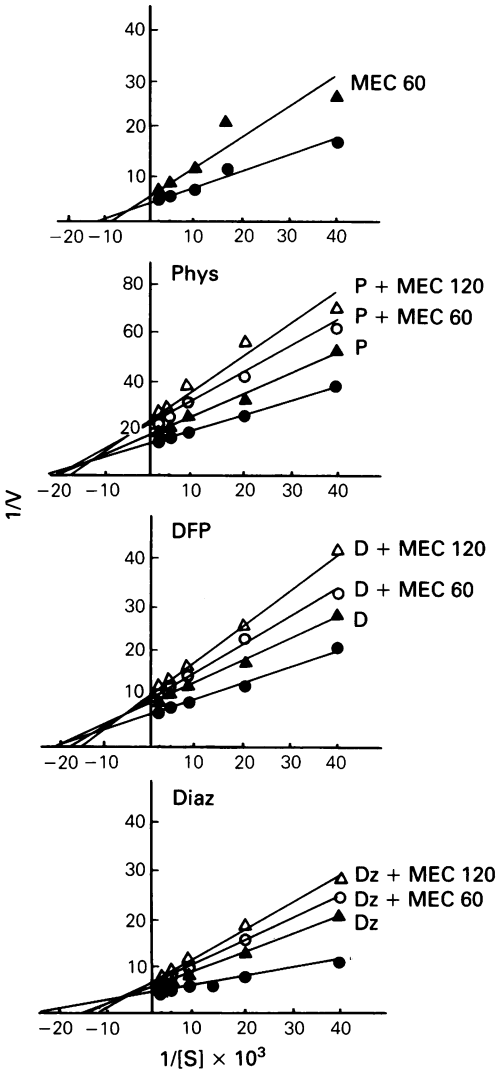


Figure 1 Effect of monoethylcholine (MEC) and inhibitor on acetylcholinesterase (AChE). AChE activity was determined with 40,000 *g* mouse brain supernatant at 20°C by the Ellman method. Inhibitor and analogue were preincubated for 2 min before addition of substrate. Velocity (V) is expressed as μM min^{-1} mg^{-1} protein, and (S) as M l^{-1}. Phys and P refer to physostigmine; DFP and D refer to diisopropylfluorophosphate; Diaz and Dz refer to diazinon (diazoxon). Additional experimental details appear in the legend to Table 2 and in the methods section.

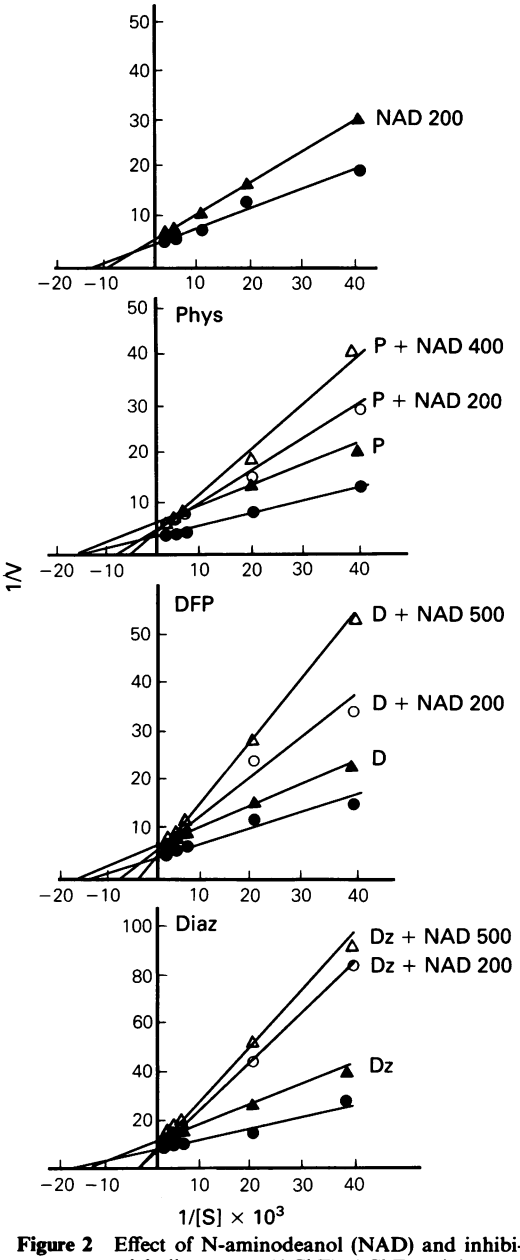


Figure 2 Effect of N-aminodeanol (NAD) and inhibitor on acetylcholinesterase (AChE). AChE activity was determined with 40,000*g* mouse brain supernatant at 20°C by the Ellman method. Inhibitor and analogue were preincubated for 2 min before addition of substrate. Velocity (V) is expressed as μM min^{-1} mg^{-1} protein, and (S) as M l^{-1}. Phys and P refer to physostigmine; DFP and D refer to diisopropylfluorophosphate; Diaz and Dz refer to diazinon (diazoxon). Additional experimental details appear in the legend to Table 2 and in the methods section.

mixed inhibition of AChE which was additive. Mixed inhibition of AChE was also observed for choline at 1.8 mM (CH200) in the presence or absence of all three AChE inhibitors (Figure 3).

Table 2 contains the kinetic constants obtained in the AChE studies. Choline, MEC, and NAD all decreased the velocity of AChE, as shown by increased slopes, alone or in the presence of the AChE inhibitors. In the absence of inhibitor or in the presence of physostigmine, choline, MEC, and NAD decreased V_{max}. However in the presence of DFP, MEC decreased V_{max}, whereas NAD and choline increased V_{max}. In the presence of diazoxon, choline, MEC, and NAD all increased V_{max}. With the exception of the effect of MEC in the presence of physostigmine, the choline analogues appeared to increase K_i for each AChE inhibitor.

Affinity of choline analogues for cholinoceptors

The affinity of the choline analogues for cholinoceptors was determined by competition binding assays. MEC appeared to be the most potent at the M_1 receptor displacing [^{3}H]-pirenzepine with an IC_{50} of 1.2 mM (Figure 4, Table 3). A similar pattern was observed at the M_2 receptor for MEC, displacing [^{3}H]-methylscopolamine with an IC_{50} of 0.51 mM. At the nicotinic receptor, the effect of the analogues was less clear due to intersection of the binding curves near their IC_{50} values. Choline appeared to be the most potent in displacing [^{125}I]-α-bungarotoxin with an IC_{50} value of 26 mM. For both the M_1 and M_2 receptor there is fairly close agreement (see Table 3) between the IC_{50} value of the analogue and its binding affinity (K_i). The IC_{50} and K_i values are less similar for the nicotinic receptor and may reflect covalent attachment of α-bungarotoxin to the receptor (Bennet, 1978), and the higher ligand/K_d ratio used in the assay. As shown by the B_{max} values, there is approximately 50 times more receptor material/mg protein in nicotinic than in M_1 and M_2 tissue sources.

Discussion

Choline, and the choline analogues monoethylcholine (MEC) and N-aminodeanol (NAD) were examined for their ability to prevent acute acetylcholinesterase (AChE) inhibitor toxicity in mice. The AChE inhibitors used included a reversible agent (physostigmine) and two irreversible organophosphates (DFP and diazinon). Diazinon is a thionophosphate and was included as an example

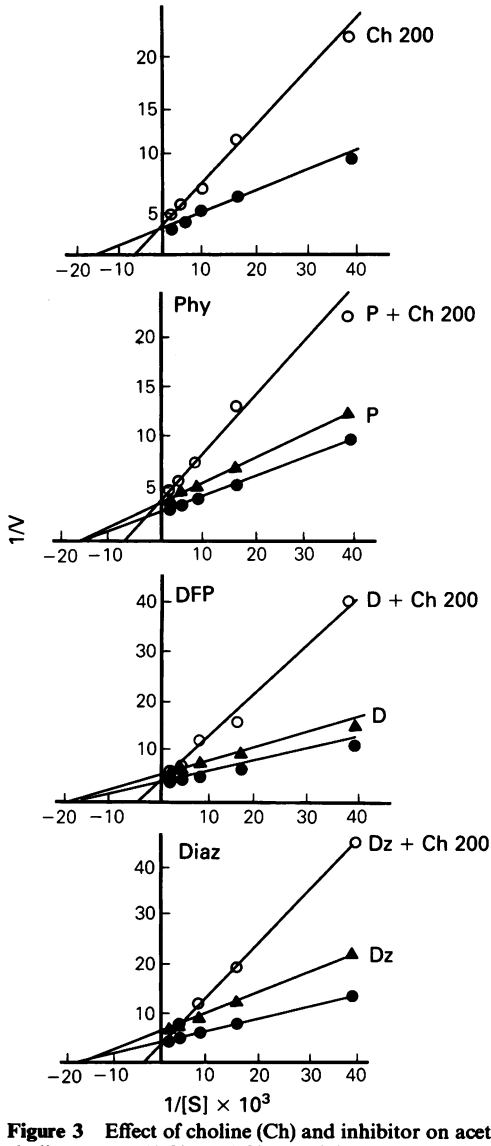


Figure 3 Effect of choline (Ch) and inhibitor on acetylcholinesterase (AChE). AChE activity was determined with 40,000 *g* mouse brain supernatant at 20°C by the Ellman method. Inhibitor and analogue were preincubated for 2 min before addition of substrate. Velocity (V) is expressed as $\mu M\,min^{-1}\,mg^{-1}$ protein, and (S) as $M\,l^{-1}$. Phy and P refer to physostigmine; DFP and D refer to diisopropylfluorophosphate; Diaz and Dz refer to diazinon (diazoxon). Additional experimental details appear in the legend to Table 2 and in the methods section.

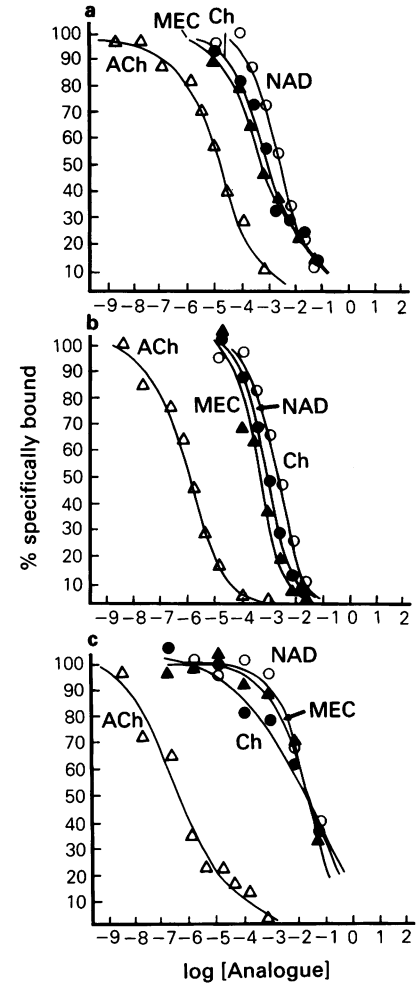


Figure 4 Binding affinity of choline (Ch), monoethylcholine (MEC) and N-aminodeanol (NAD) at M_1 (a), M_2 (b) and nicotinic (c) cholinoceptors. Assay parameters (tissue source, ligand, incubation volume) for each procedure were: nicotinic (torpedo electric organ, [^{125}I]-α-bungarotoxin, 1 ml); M_1 (mouse brain cortex, [^{3}H]-pirenzepine, 1 ml); and M_2 (rat heart, [^{3}H]-methylscopolamine, 2 ml). After 20 min preincubation with DFP (100 μM), ligand was added and incubated an additional 40 min at 20°C. Additional experimental details appear in the Methods section. Data were analysed using the programme LIGAND.

of an agent that undergoes metabolic activation by the host to the more active phosphate analogue diazoxon (Kanazawa, 1983). Accordingly a slower onset in toxicity would be expected compared to physostigmine or DFP. The average time to death for diazinon was found to be approximately 3 times

Table 2 Effect of monoethylcholine (MEC), N-aminodeanol (NAD), or choline (Ch) on the kinetics of acetylcholinesterase inhibitors

Inhibitor[a]	*Analogues*[b]	*Relative slope*[c]	V_{max} (% *of control*)	K_i (M)
Physostigmine	—	1.7	81	5.0×10^{-5}
	MEC (60)	2.0	64	4.9×10^{-5}
	MEC (120)	2.3	55	5.1×10^{-5}
	NAD (200)	2.7	78	1.3×10^{-4}
	NAD (400)	3.9	88	2.3×10^{-4}
	Ch (200)	2.7	74	1.3×10^{-4}
DFP	—	1.3	68	5.1×10^{-5}
	MEC (60)	1.5	59	5.7×10^{-5}
	MEC (120)	2.3	62	8.6×10^{-5}
	NAD (200)	2.3	76	1.3×10^{-4}
	NAD (500)	3.9	87	2.1×10^{-4}
	Ch (200)	4.5	100	2.8×10^{-4}
Diazoxon[d]	—	1.5	61	6.6×10^{-5}
	MEC (60)	3.1	73	7.3×10^{-5}
	MEC (120)	3.8	67	8.0×10^{-5}
	NAD (200)	4.0	100	3.1×10^{-4}
	NAD (500)	4.3	72	2.2×10^{-4}
	Ch (200)	4.3	96	2.5×10^{-4}
—	MEC (60)	1.7	57	9.7×10^{-5}
—	NAD (200)	1.7	88	1.1×10^{-4}
—	Ch (200)	2.7	94	1.6×10^{-4}

Acetylcholinesterase activity was determined with 40,000 *g* mouse brain supernatant at 20°C by the Ellman method. Inhibitor and analogue were preincubated for 2 min before addition of substrate.

[a] Concentrations used for physostigmine, diisopropylfluorophosphate (DFP), and diazoxon were 25 μM, 3 μM, and 1 μM, respectively.

[b] The cuvette concentration of the analogue approximated the *in vivo* concentration achievable following parenteral administration. The number in parentheses corresponds to the dose administered in $mg\,kg^{-1}$. The cuvette concentration for MEC (60) was 0.33 mM, and was 1.8 mM for NAD (200) and Ch (200), respectively.

[c] Compared to the uninhibited enzyme rate.

[d] Biologically active metabolite of diazinon.

Table 3 Cholinoceptor binding constants for monoethylcholine (MEC), N-aminodeanol (NAD) and choline (Ch)

Receptor	*Agonist*	η_H	B_{max} (fmol mg^{-1})	IC_{50} (M)	K_i (M)
Nicotinic	ACh	0.40	2,530	2.5×10^{-7}	1.1×10^{-9}
	MEC	0.67	2,430	3.3×10^{-2}	1.5×10^{-4}
	NAD	0.63	2,580	4.8×10^{-2}	2.1×10^{-4}
	Ch	0.40	2,250	2.6×10^{-2}	1.1×10^{-4}
Muscarinic M_1	ACh	0.56	37	1.6×10^{-5}	7.9×10^{-6}
	MEC	0.44	38	1.2×10^{-3}	5.8×10^{-4}
	NAD	0.71	41	4.3×10^{-3}	2.2×10^{-3}
	Ch	0.48	40	1.5×10^{-3}	7.7×10^{-4}
Muscarinic M_1	ACh	0.67	53	7.0×10^{-7}	3.4×10^{-7}
	MEC	0.89	58	5.1×10^{-4}	2.5×10^{-4}
	NAD	0.79	58	2.6×10^{-3}	1.3×10^{-3}
	Ch	0.83	62	9.2×10^{-4}	4.5×10^{-4}

Assay parameters (tissue source, ligand, incubation volume) for each procedure were: nicotinic (torpedo electric organ, [^{125}I]-α-bungarotoxin, 1 ml); M_1 (mouse brain cortex, [^{3}H]-pirenzepine, 1 ml); and M_2 (rat heart, [^{3}H]-methylscopolamine, 2 ml). After 20 min preincubation with DFP (100 μM), ligand was added and incubated an additional 40 min at 20°C. Additional experimental details appear in the legend to Figure 4 and in the Methods section.

that of DFP (Table 1), and correlates with the time of maximal diazoxon formation in the mouse (Kreuger *et al.*, 1960).

The rank order of effectiveness for the analogues against AChE inhibitor toxicity appears to be MEC $\gg$ NAD > choline. MEC was approximately three times more active than NAD in physostigmine toxicity, and 6 and 10 times more active in diazinon and DFP toxicity respectively. The higher dose of NAD used ($600\,mg\,kg^{-1}$) exacerbated diazinon toxicity but was maximally effective in DFP toxicity. This may reflect an interaction with diazinon rather than toxicity of the analogue itself since doses of NAD alone up to $800\,mg\,kg^{-1}$ were non-toxic. The slight activity observed with choline agrees with data reported by Stovner (1956). In the present study, the maximal antidotal dose of choline was limited to $200\,mg\,kg^{-1}$. Administration of choline at 400 or $600\,mg\,kg^{-1}$ ($n = 3$, data not shown), produced a dose-related synergistic toxicity with DFP or diazinon. The toxicity data suggest that the prevention of toxicity observed reflects a structure-activity relation of the analogues and is not based solely on a non-specific quaternary function modality. Since choline, MEC, and NAD are quaternary agents with limited distribution to the CNS, it is probable that the antidotal action observed against the AChE inhibitors occurs primarily on the periphery.

The *in vitro* data presented demonstrate that choline, MEC, and NAD interfere with the kinetics of acetylcholinesterase (AChE). A similar inhibitory action for choline on AChE and serum cholinesterase has been observed (Adams & Whittaker, 1950). The analogues produced a dose-related mixed inhibition of AChE in the presence or absence of each of the three AChE inhibitors (Figures 1, 2, 3). Based on the cuvette concentrations used, MEC appeared to be the most active with NAD and choline approximately equal to each other in potency. In general, the presence of the analogue and inhibitor increased the K_i values compared to the inhibitor alone. However, the effect of the analogues was more complex with respect to the effect on V_{max} (Table 2). Depending on the AChE inhibitor, V_{max} values were observed to increase and decrease. The data suggest that the analogues temporarily bind to the enzyme, causing AChE velocity to slow. It is proposed, therefore, that the rate of carbamylation or phosphorylation of the enzyme would also slow. A similar effect has been observed for the effect of tetra-N-alkylammonium ions on the rate of phosphonylation of AChE by the organophosphate O-ethyl S-n-propyl methyl phosphonothiolate (Sellers *et al.*, 1983). The *in vivo* reduction in toxicity observed could then be explained in terms of the additional time that would then be allowed for inactivation of the AChE inhibitor by mixed-function oxidases (Smith *et al.*, 1974; Cremlyn, 1978). A similar protective effect has been reported for the reduction in toxicity of organophosphates by reversible AChE inhibitors (Harris *et al.*, 1978) and clonidine (Aronstam *et al.*, 1986). It is also possible that the observed reduction in physostigmine toxicity is due to an increase in the rate of decarbamoylation of AChE. A parallel effect has been noted for the ability of tetra-N-alkyl ammonium ions to alter the rate of deacylation of AChE (Sellers *et al.*, 1983) and for excess free carbamate to increase decarbamoylation of AChE (Watts & Wilkinson, 1977). This hypothesis is supported by the observation that the amount of AChE inhibition produced by the combination of inhibitor and analogue was not additive when compared to the sum of the effects of either agent alone.

Choline, MEC, and NAD exhibited binding affinities for M_1, M_2 and nicotinic receptors (Figure 4). At M_1 and M_2 receptors, the rank order of potency was MEC > choline > NAD, whereas at the nicotinic receptor, the order of potency was choline > MEC $\cong$ NAD. Although different ligands were used in the present study, similar K_i values and rank order potencies were obtained for choline and MEC on M_1 and nicotinic receptors (Buccafusco & Aronstam, 1988). Based on the *in vivo* concentrations achieved and the IC_{50} values obtained in the binding experiments, the analogues should compete *in vivo* to a greater extent with acetylcholine for binding to M_1 and M_2 receptors than to nicotinic receptors. Although less competition with acetylcholine would occur at the nicotinic receptor with the *in vivo* doses used, the extent of acetylcholine displacement at the diaphragm during AChE inhibitor toxicity may still be significant.

The present study demonstrates that choline analogues prevent acetylcholinesterase inhibitor toxicity if administered at the time of exposure to the inhibitor. A more practical treatment would be one that could be instituted after exposure to the inhibitor. Preliminary data suggests that post treatment with MEC may be an effective method of reducing DFP toxicity. Administration of MEC ($60\,mg\,kg^{-1}$) 2 min after DFP administration increased the time to death from 12.1 ± 1.2 ($\pm$s.e.) to 19.3 ± 3.8 ($\pm$s.e.) min and reduced fatalities from 96% to 85% ($n = 7$, data not shown). It should be noted that the pharmacokinetics of MEC are unknown at present and further studies with respect to the dose of MEC and the interval of post-treatment are needed to evaluate fully post-treatment efficacy. Data are not available for the post-treatment use of NAD or choline.

It is unclear why choline was the least effective antidote for the three cholinesterase inhibitors examined, yet exhibited receptor binding and AChE interactions with a potency similar to NAD. Also,

differences in activity and potency were observed for the analogues, suggesting that a structural-activity relationship exists for the pharmacological parameters investigated. From the data obtained, it is proposed that the mechanism of antidotal activity for MEC and NAD in acute cholinesterase inhibitor toxicity is peripheral rather than central. It is further proposed that the analogues bind reversibly to AChE to prevent covalent attachment of the inhibitor and/or compete with acetylcholine for binding to muscarinic and nicotinic receptors.

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Arachidonic acid-induced mobilization of calcium in human neutrophils: evidence for a multicomponent mechanism of action

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1 The mechanism(s) involved in the mobilization of calcium induced by arachidonic acid in human neutrophils was investigated.

2 The addition of arachidonic acid to a suspension of human neutrophils led to a time- and concentration-dependent mobilization of calcium which was the result of two separate and experimentally differentiable processes. The latter consisted of a rapid and transient phase followed by a slower and more sustained response.

3 The initial phase of calcium mobilization elicited by arachidonic acid was decreased in the presence of EGTA, inhibited by pertussis toxin as well as by nordihydroguaiaretic acid (NDGA), and diminished following a pre-incubation with leukotriene B_4, but not platelet-activating factor.

4 The characteristics of the first phase of the mobilization of calcium were consistent with an interaction of the fatty acid with the leukotriene B_4 receptors, either directly or indirectly following the synthesis of leukotriene B_4, as well as with a release of internal calcium.

5 The second, slower and more sustained phase of calcium mobilization was more apparent at high concentrations ($\geqslant 8$–$16\,\mu M$) of arachidonic acid, and was relatively insensitive to pertussis toxin, EGTA or NDGA.

6 The characteristics of the 'slow' phase of calcium mobilization by arachidonic acid are consistent with its being associated primarily with a release of calcium from internal storage pools.

7 The data presented indicate that the mechanism of mobilization of calcium by arachidonic acid in human neutrophils is complex and involves specific activation pathways employed, in part at least, by other neutrophil agonists. These findings may have relevance to various inflammatory situations in which the elevated levels of extracellular arachidonic acid known to be present could modulate the functional responsiveness of the neutrophils to other stimuli.

Introduction

Arachidonic acid is a multifunctional agonist for neutrophilic polymorphonuclear leukocytes (neutrophils). The addition of the fatty acid stimulates several of the characteristic functions of the cells including: the mobilization of calcium (Volpi *et al.*, 1980; Sha'afi *et al.*, 1980; Naccache *et al.*, 1983), the polymerization of actin (Yassin *et al.*, 1985), degranulation (Naccache *et al.*, 1979), aggregation (Naccache *et al.*, 1983; O'Flaherty *et al.*, 1979), and the stimulation of superoxide production (Badwey *et al.*, 1981; 1987). Furthermore, arachidonic acid has been found to augment the activity of purified protein kinase C (McPhail *et al.*, 1984; Murakami *et al.*, 1986; Morimoto *et al.*, 1988), an enzyme potentially involved in the initiation and/or regulation of neutrophil responsiveness (White *et al.*, 1984; Naccache *et al.*, 1985; Rossi, 1986; Tauber, 1987), as well as that of partially purified NADPH-oxidase preparations from these cells (Rossi, 1986; Tauber, 1987; Curnutte *et al.*, 1987; Clark *et al.*, 1987). These various biological activities of arachidonic acid are generally assumed to support the concept that the fatty acid is an important modulator of the pathophysiological functions of the neutrophils.

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However, whereas the mode of action of 'classical' neutrophil stimuli such as the small molecular weight fragment of the fifth component of complement, C5a, and of the formylated chemotactic peptides such as fMet-Leu-Phe are relatively well established (for reviews, see Snyderman & Verghese, 1987; Sha'afi & Molski, 1988), little attention has been paid to the mechanism of activation of the neutrophils by arachidonic acid. This problem is compounded by the fact that arachidonic acid is metabolized by the 5-lipoxygenase system of the neutrophils (Borgeat & Samuelsson, 1979; Borgeat *et al.*, 1985) resulting in the generation of neutrophil active molecules such as leukotriene B_4 (Ford-Hutchinson *et al.*, 1980; Palmer *et al.*, 1980; Naccache *et al.*, 1988).

We have recently observed that the synthesis of lipoxygenase products in response to the addition of arachidonic acid is the result of more than the simple provision of substrate to the 5-lipoxygenase enzyme system. This conclusion was supported in part by the finding that the synthesis of lipoxygenase products observed following the addition of arachidonic acid was sensitive to inhibition by pertussis toxin, even though the latter did not affect directly the activity of the 5-lipoxygenase (McColl *et al.*, 1989). Since the neutrophil functional responses stimulated by arachidonic acid (e.g., degranulation, superoxide production, protein kinase C translocation and leukotriene synthesis) are calcium-dependent, we have attempted to determine the mechanism(s) by which arachidonic acid mobilizes calcium in these cells. The results are supportive of a model of the mechanism of action of arachidonic acid involving at least three separate sites of action, two of which are closely associated with the activation of receptor-dependent events and which collectively contribute to the mobilization of calcium induced by the fatty acid.

Methods

The human neutrophils used in this study were isolated following dextran sedimentation on Ficoll-Paque cushions. The remaining erythrocytes were lysed by hypotonic shock (20 s at room temperature). Neutrophils represented at least 97% of the cell suspensions. Cell viability, as estimated by trypan blue exclusion, was better than 98%. The cells were isolated and resuspended in Hanks' balanced salt solution (HBSS) (Gibco, Burlington, Ontario, Canada), pH 7.4, containing 1.6 mM $CaCl_2$ but no added $MgCl_2$.

Intracellular free calcium was monitored with the fluorescent probe fura-2 as described in Grynkievicz *et al.* (1985) and Faucher & Naccache (1987). Briefly, neutrophil suspensions (1×10^7 cells ml^{-1}) were incubated with 1 μM fura-2/AM for 30 min at 37°C. The cells were then washed free of the extracellular probe, resuspended at 5×10^6 cell ml^{-1} and allowed to re-equilibrate for 10 min at 37°C. They were then transferred to the thermostatted cuvette compartment (37°C) of the fluorimeter and the fluorescence monitored (excitation and emission wavelengths, 340 and 510 nm respectively). The internal calcium concentrations were calculated as described in Tsien *et al.* (1982) by individually calibrating each run.

Formyl-methionyl-leucyl-phenylalanine (fMet-Leu-Phe), platelet-activating factor (Paf), nordihydroguaiaretic acid (NDGA) and cholera toxin were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Ficoll-Paque was from Pharmacia (Dorval, Québec, Canada), fura-2/AM was purchased from Molecular Probes (Junction City, OR, U.S.A.), and pertussis toxin was from List Biochemical Laboratories (Campbell, CA, U.S.A.). Leukotriene B_4 was generously provided by Dr J. Rokach (Merck-Frosst Canada, Pointe-Claire, Dorval, Canada). Arachidonic acid was purified by silicic acid chromatography or by r.p.-h.p.l.c. prior to use. The purity of the leukotriene B_4 was determined by r.p.-h.p.l.c., and the concentration of its stock solutions by u.v. spectrophotometry (270 nm). All the other reagents were analytical grade.

Results

Concentration-dependence of the mobilization of calcium induced by arachidonic acid and its sensitivity to NDGA

The addition of micromolar concentrations of arachidonic acid to human neutrophils elicited a rapid and concentration-dependent increase in the concentration of cytoplasmic free calcium (as monitored by the fluorescent calcium probe fura-2) (Figure 1). The threshold concentration of arachidonic acid required consistently to elicit a calcium response from the neutrophils was found to be about 1 μM. The magnitude of the calcium signal increased with the concentration of arachidonic acid added and showed signs of saturation at or above 33 μM. The fura-2 response to relatively low concentrations of arachidonic acid ($\leqslant 8\ \mu$M) characteristically comprised a rapid and transient peak that decayed to resting or higher than resting level of cytoplasmic free calcium.

The potential involvement of 5-lipoxygenase products in the mediation of the calcium mobilizing properties of arachidonic acid was tested with the help of the 5-lipoxygenase inhibitor NDGA (Tappel

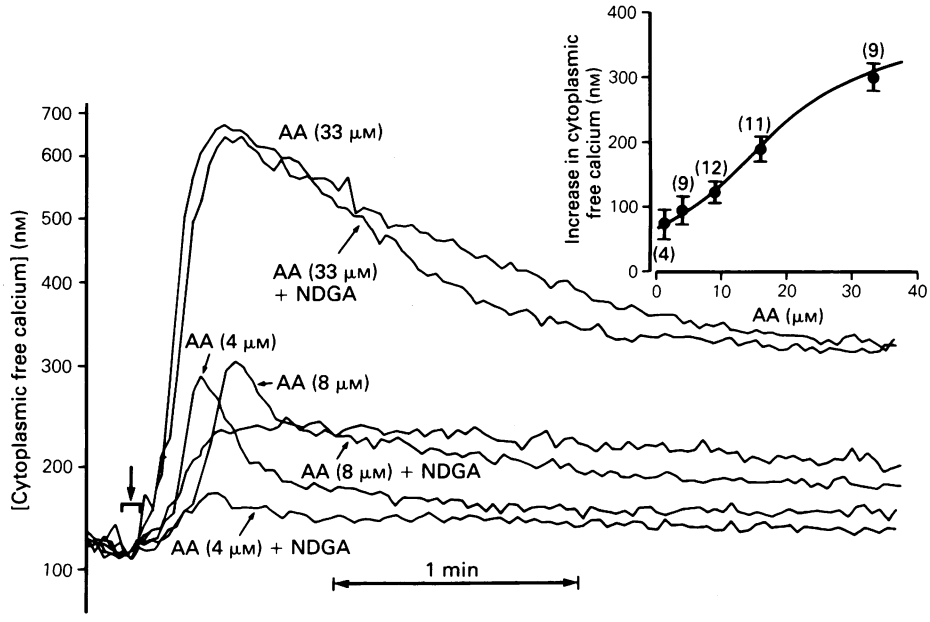


Figure 1 Arachidonic acid-mobilization of calcium in human neutrophils. The levels of calcium were measured as described in Methods. Arachidonic acid (AA), at the indicated concentrations, was added at the arrow and where indicated, cells were pre-incubated with NDGA (10 μM) for 3 min before the addition of the fatty acid. The results shown are representative of at least three such experiments. The concentration–response curve for increases in cytoplasmic free calcium induced by arachidonic acid is inset. The values represent the mean of the number of experiments indicated in parentheses with s.e.mean shown by vertical bars.

et al., 1953; Hamberg, 1976; Walenga *et al.*, 1980; Salari *et al.*, 1984). The pre-incubation of the cells with NDGA was found to have differential effects depending on the concentration of arachidonic acid used (Figure 1). The calcium responses to low concentrations of arachidonic acid ($\leqslant 4\,\mu$M) were found to be significantly reduced in the presence of the inhibitor, while those of high concentrations of ($\geqslant 33\,\mu$M) were essentially unaffected. At intermediate concentrations of arachidonic acid (about 8 μM), NDGA preferentially inhibited the initial spike in calcium but had little effect on the raised level of cytoplasmic free calcium that was maintained during the course of these experiments.

Dependence on extracellular calcium

The contribution of extracellular calcium to the increase in cytoplasmic free calcium was evaluated by adding the calcium chelator EGTA. Under these conditions, little, if any, extracellular calcium is available to diffuse into the cells, and the changes in the fluorescence of fura-2 that are recorded can be assumed to be due to calcium released from internal stores (Molski *et al.*, 1983). The results of one such experiment are shown in Figure 2. EGTA reduced by similar amounts (about 50 nM) the increase in calcium induced by 2 and 33 μM arachidonic acid. However, the calcium chelator had, on a relative

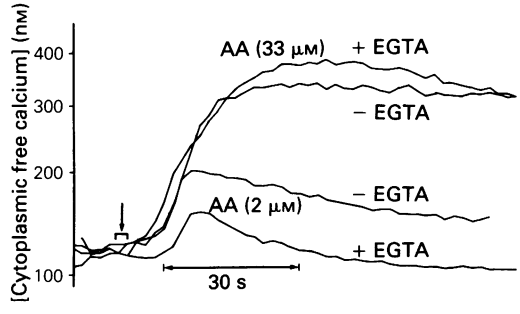


Figure 2 Effect of EGTA on the increase in cytoplasmic free calcium elicited by arachidonic acid (AA). The experimental conditions were as described in Methods. The arrow represents the time of addition of arachidonic acid (at the indicated concentrations) and, where indicated, EGTA (3 μM) was added 1 min before arachidonic acid. The concentration of free calcium in the buffer in the presence of the calcium chelator was calculated to be 4.1×10^{-8} M. The result is representative of at least three such experiments.

basis, a much larger effect on the response to 2 μM arachidonic acid than on 33 μM.

Sensitivity to pertussis and cholera toxin

Pre-incubation of human neutrophils with pertussis toxin (0.5 μg ml^{-1}, 2 h at 37°C) decreased the magnitude of the calcium response to low concentrations of arachidonic acid principally by affecting the initial calcium spike (Figure 3a). In the presence of this toxin, arachidonic acid (8 μM) caused a slow increase in cytoplasmic calcium that levelled off at a value close to the sustained plateau level achieved in control, untreated cells. The calcium response to 33 μM arachidonic acid was, on the other hand, unaffected by pertussis toxin. Pre-incubation with cholera toxin (0.5 μg ml^{-1}, 2 h at 37°C) did not affect significantly, either the magnitude or the shape of calcium responses to arachidonic acid 2 μM (Figure 3b) or 33 μM (results not shown).

Cross-desensitization of the calcium responses to arachidonic acid, leukotriene B_4 or platelet-activating factor

Potential interrelationships among the mechanism(s) of mobilization of calcium by arachidonic acid and either leukotriene B_4 or platelet-activating factor were investigated by examining the effects of pre-exposure to the cells to the latter two lipid mediators before stimulating with arachidonic acid. The results of these experiments are depicted in Figure 4. Note that the ordinate axis of the three panels in this Figure are slightly different from each other due to the individual calibrations that were performed on each run. In these experiments, the response to arachidonic acid in control cells (Figure 4c) is compared to that observed in cells pre-treated with either leukotriene B_4 or platelet-activating factor (Figure 4a and b). In each case the calcium response to the first stimulus was allowed to decay as close as possible to its resting level before adding arachidonic acid. The data shown are taken from successive runs in the same experiment. Figure 4a and c demonstrates that pre-incubation of the cells with leukotriene B_4 essentially abolished the rapid calcium spike induced by 8 μM arachidonic acid. However, a slow increase in calcium was still observed in response to arachidonic acid in the cells pretreated with leukotriene B_4. In contrast, the response to arachidonic acid was essentially unchanged by previous exposure to platelet-activating factor at concentrations that increased intracellular calcium to the same extent as leukotriene B_4 (Figure 4b and c).

The pre-incubation of the cells with arachidonic acid, on the other hand, inhibited in a concentration-dependent manner, the calcium response to platelet-activating factor as well as to leukotriene B_4 (results not shown). These results are reminiscent of the previously documented inhibition by arachidonic acid of the mobilization of calcium induced by fMet-Leu-Phe (Naccache *et al.*, 1983). The study of the biochemical mechanism(s) underlying this heterologous desensitization is beyond the aims of the present investigation.

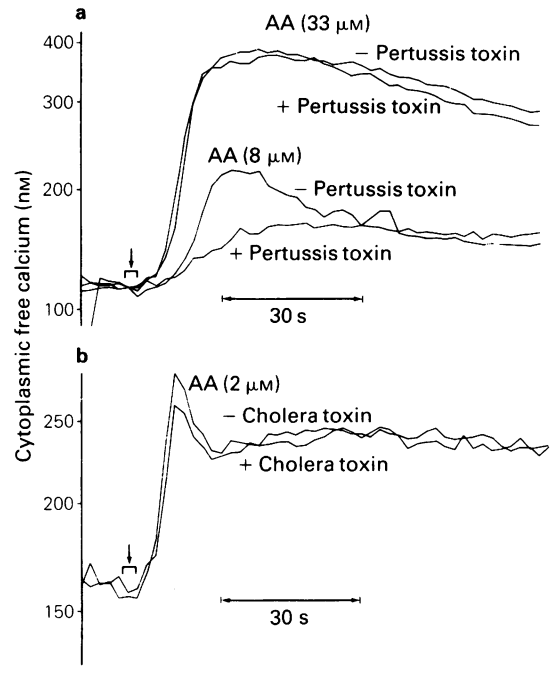


Figure 3 Effect of pertussis toxin and cholera toxin on the mobilization of calcium induced by arachidonic acid (AA) in human neutrophils. The experimental conditions are as described in Methods. Where indicated, the cells were incubated with 0.5 μg ml^{-1} of the toxins for 2 h at 37°C. The arrow indicates the time of addition of arachidonic acid. Note that the graphs in (a) and (b) were drawn to different scales in order to optimize the display of the data. The result is representative of at least three experiments.

Discussion

Arachidonic acid is one of the most commonly used stimuli for neutrophils, having been shown to stimulate several of these cells' characteristic functions and biochemical correlates (see references in Introduction). In the present paper, we have examined the

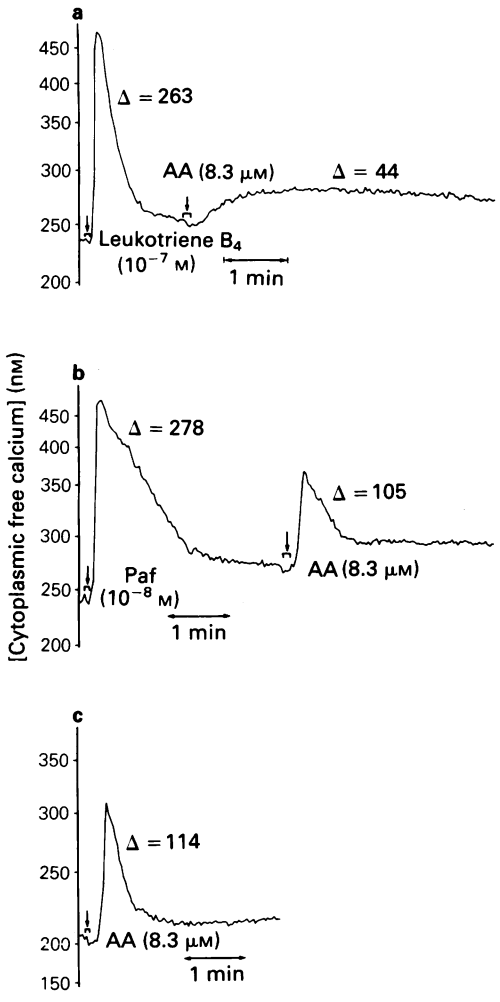


Figure 4 Cross-densitization of the mobilization of calcium induced by pre-stimulation of human neutrophils with leukotriene B_4 or arachidonic acid (AA). The experimental conditions are as described in Methods. Leukotriene B_4 (10^{-7} M), platelet-activating factor (Paf 10^{-8} M), or arachidonic acid (8 μM) were added at the arrows as indicated. The 'deltas' represent the increases in the concentrations of cytoplasmic free calcium calculated from the differences between the prestimulation levels of calcium and the peaks attained after the addition of the respective stimuli. The result is representative of at least three experiments.

calcium mobilizing ability of arachidonic acid in order to characterize the mechanism of action of the fatty acid. The results indicate that the changes in intracellular calcium of human neutrophils in response to arachidonic acid are complex and result from interactions at several distinct sites of action that are likely to include: (1) internal calcium storage sites, (2) direct or indirect receptor-activation mediated, in part at least, through the formation of biologically active metabolites such as leukotriene B_4.

As previously observed in rabbit peritoneal neutrophils (Volpi *et al.*, 1980; Sha'afi *et al.*, 1980; Naccache *et al.*, 1983), and more recently, in the presence of cytochalasin B, in human neutrophils (Smith *et al.*, 1987), the addition of arachidonic acid elicits a rapid mobilization of calcium. As described in the current paper, both the magnitude and the shape of the fura-2 signals induced by arachidonic acid were concentration-dependent. Low concentrations of the fatty acid induced a transient increase in the fluorescence signal, high concentrations a much more lasting response, while at intermediate concentrations the effect was composed of the sum of these two effects. This summation resulted in an initial spike of calcium followed by a plateau at a level significantly higher than the resulting levels of calcium. It should be noted that, while this general pattern was quite reproducible, the relative magnitude of the two phases of calcium mobilization was found to vary among different cell preparations. It is unknown at present whether this variability represents inherent differences in the neutrophils or was induced by uncontrolled influences affecting the cells during the isolation or incubation procedures, e.g., endogenous liberation and metabolism of arachidonic acid (Gorman *et al.*, 1985).

Several lines of evidence indicate that the biphasic shape of the calcium response to arachidonic acid is a reflection of at least two independent sites of action. Indeed, these two phases were differentially sensitive to a variety of pharmacological and experimental manipulations.

The calcium response to arachidonic acid was decreased in the presence of the calcium chelator EGTA, thus indicating its partial dependence on extracellular calcium. The sensitivity of the initial calcium transient to inhibition by pertussis toxin implies that a GTP binding protein (and by implication, a specific receptor site) is involved in the mediation of this response. This interpretation was supported by the finding that the pre-exposure of the cells to leukotriene B_4 led to a significant decrease in the magnitude of the initial phase of the calcium response to arachidonic acid, even though concentrations of the latter close to 100 fold larger than those of leukotriene B_4 were used. The lack of effect of platelet-activating factor on the ability of arachidonic acid to mobilize calcium underlines the specificity of this desensitization. These results make it likely that part of the effects of arachidonic acid are due to direct or indirect interactions with the leukotriene B_4 receptors (see Figure 5). It is possible that the amount of leukotriene B_4 generated as a result of

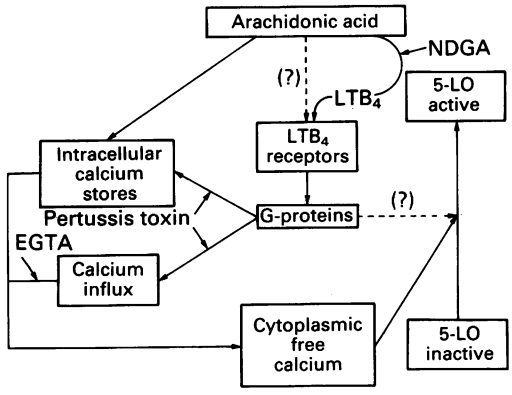


Figure 5 Schematic representation of the events initiated by arachidonic acid in human neutrophils. The complete lines represent pathways, the existence of which is relatively well-established; the dotted lines indicate potential regulatory mechanisms that remain to be firmly demonstrated. The primary site of action of the inhibitors NDGA, pertussis toxin and EGTA are also indicated: LTB_4 = leukotriene B_4;5-LO = 5-lipoxygenase.

the presentation of substrate (arachidonic acid) to the 5-lipoxygenase (8.5, 3.5 and 1.0 ng of leukotriene B_4 and its Ω-oxidation products per 10^6 neutrophils after a 15 min incubation with 33, 10 and 3 μM arachidonic acid, respectively (McColl *et al.*, 1989) would account for the initial phase of the mobilization of calcium by arachidonic acid. The sensitivity of the arachidonic acid-induced initial calcium spike to inhibition by NDGA, as well as that of the arachidonic acid stimulated influx of ^{45}Ca in rabbit neutrophils (Volpi *et al.*, 1980) support this conclusion.

The second phase of calcium mobilization that is observed upon the addition of arachidonic acid is likely to be due to direct effects of the fatty acid on intracellular pools of calcium (see Figure 5). It is most apparent at high concentrations of arachidonic acid, under which conditions the direct release of internal calcium overwhelmed the contribution of the receptor-activated mechanisms that are more prominent at lower concentrations of the fatty acid. Arachidonic acid has previously been shown to elicit a release of calcium from non-mitochondrial calcium storage pools in detergent-permeabilized neutrophils (Beaumier *et al.*, 1987), as well as in isolated sarcoplasmic reticulum of striated myocytes (Cheah, 1981), pancreatic islets (Wolf *et al.*, 1986) and rat liver microsomes (Chan & Turk, 1987). The ability of arachidonic acid to induce a release of calcium from intracellular stores in permeabilized cells has been found to be independent of its metabolism by either 5-lipoxygenase or the cyclo-oxygenase (Beaumier *et al.*, 1987; Chan & Turk, 1987). The relative intensitivity of the slow phase of the arachidonic acid-induced mobilization of calcium in human neutrophils to the presence of extracellular calcium chelators as well as to pertussis toxin, NDGA and to preincubation with leukotriene B_4 are all consistent with this interpretation. The present results thus demonstrate that the mobilization of internal calcium by arachidonic acid, an effect previously only described in permeabilized cell preparations, can also be observed in intact cells. A corollary of this interpretation is that the effect of arachidonic acid on the internal calcium pools is independent of the pertussis toxin-sensitive G protein, and is not shared by leukotriene B_4.

The question remains as to whether the mobilization of calcium by arachidonic acid represents a causal event in the initiation of the physiological responses of the neutrophils to the fatty acid. Arachidonic acid, as previously mentioned, has been shown to stimulate neutrophil aggregation, degranulation, superoxide production and the synthesis of 5-lipoxygenase products. It is noteworthy that all of these functional responses of the cells have been shown to be calcium-dependent. Moreover, the activation of 5-lipoxygenase by arachidonic acid has recently been found to be pertussis toxin-sensitive (McColl *et al.*, 1989). Taken together with the results presented above, these data imply that receptor occupation and activation play a pivotal, and yet to be described, role in the activation of the 5-lipoxygenase enzymatic pathway.

In summary, the present results demonstrate that arachidonic acid, in the low micromolar range, elicits a significant mobilization of calcium that is the result of its direct and indirect interactions with specific receptor sites, as well as with the internal calcium storage pools of the neutrophils. A schematic representation of the events initiated by arachidonic acid that are supported by the present data is shown in Figure 5. These observations are relevant to the patho-physiological behaviour of the neutrophils at inflammatory sites in which levels of free arachidonic acid similar to those used in this study have been observed (Hammarstrom *et al.*, 1975; Greaves, 1986). Indeed, under those conditions, arachidonic acid may modulate, positively as well as negatively, the responses of the neutrophils to other agonists such as C5a, platelet-activating factor, leukotriene B_4 or fMet-Leu-Phe. According to this view, arachidonic acid would be considered as an autocoid in its own right, and not simply as a substrate for a series of metabolizing enzymes.

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Interference of WEB 2086 and BN 52021 with Paf-induced effects on guinea-pig trachea

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1 The thienotriazolodiazepine WEB 2086 and the gingkolide BN52021 have been evaluated as antagonists of Paf-acether (Paf) by studying their effects on Paf-induced relaxation and Paf-induced prostaglandin E_2 (PGE_2) production in histamine-contracted guinea-pig tracheal preparations.

2 Relaxation induced by Paf 4 μM in histamine-contracted guinea-pig tracheal preparations was $39.67 \pm 3.5\%$ ($n = 30$). At the same concentration, Paf significantly increased PGE_2 production from histamine-contracted guinea-pig tracheal preparations.

3 WEB 2086 inhibited in a dose-related manner ($IC_{50} = 21.2$ nM) the relaxant effect induced by Paf and, at 1 μM, suppressed Paf-induced release of PGE_2.

4 BN 52021 100 μM inhibited to about 60% Paf-induced relaxation of histamine-contracted guinea-pig tracheal preparations, but completely abolished Paf-induced increase in PGE_2.

5 Both antagonists had no effects on relaxations induced by arachidonic acid 10 μM or PGE_2 0.1–1 μM in histamine-contracted guinea-pig tracheal preparations.

6 The results are consistent with the presence of specific Paf receptors in guinea-pig trachea and indicate that a relaxant prostanoid, namely PGE_2, at least partially mediates Paf-induced relaxation in this experimental model.

Introduction

Paf-acether (Paf, formerly platelet-activating factor; Benveniste *et al.*, 1972), identified as being 1-O-hexa/octadecyl-2-acetyl-sn-3- glycero-phosphocholine, is a potent mediator of inflammation, which induces bronchoconstriction and airway hyperreactivity in several animal species including man (Chung *et al.*, 1986; Cuss *et al.*, 1986; Anderson *et al.*, 1988). However, *in vitro*, this phosphoetherlipid does not contract bronchial and tracheal preparations from different species (Cerrina *et al.*, 1983; Stimler & O'Flaherty, 1983) and paradoxically relaxes resting guinea-pig tracheal preparations (Prancan *et al.*, 1982). We previously demonstrated that Paf relaxed, in a dose-dependent manner, guinea-pig tracheal preparations in which tone was induced by maximal effective concentrations of histamine. This effect was observed only in the presence of an intact epithelium and was largely dependent on the production of immunoreactive relaxant prostanoids, such as prostaglandin E_2 (PGE_2, Brunelleschi *et al.*, 1987).

The presence of specific Paf receptors was previously documented in rat trachea and in human and guinea-pig lung tissue homogenates, but, as the lungs contain more than 40 different cell types, these receptors remain to be localized precisely (Hwang *et al.*, 1983; 1985). The development of specific Paf receptor antagonists has largely contributed to a better definition of the role of this mediator in several pathophysiological conditions. Among the compounds available, BN 52021 (ginkgolide B; a component purified from *Gingko biloba* leaves) and the thienotriazolodiazepine WEB 2086, are two chemically unrelated antagonists, whose ability to inhibit competitively Paf-induced effects is well documented in different experimental models (Casals-Stenzel *et al.*, 1987; Braquet *et al.*, 1987). We

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have therefore evaluated the effects of BN 52021 and WEB 2086 on Paf-induced relaxation of guinea-pig tracheal preparations precontracted with histamine.

A preliminary account of this work was presented at the 1st Joint Meeting of the Hungarian and Italian Pharmacological Societies at Verona, Italy (Brunelleschi *et al.*, 1988).

Methods

Preparation of tissues

Adult male guinea-pigs (300–500 g) were killed by a blow to the head and exsanguinated. The trachea was removed, carefully cleaned of adherent fat and connective tissue, cut as a spiral and divided into two paired portions. Each tracheal segment was placed in a 10 ml water jacketed organ bath at 37°C containing Tyrode solution (composition in mM: NaCl 139.2, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 0.49, NaH_2PO_4 0.4 and glucose 5.5; pH 7.4) gassed with 95% O_2 and 5% CO_2.

Tissues were allowed to equilibrate for 90 min in Tyrode solution under a load of 2.5–3 g. This high initial load ensured optimal length of preparations and maximal and reproducible contractile responses. The length of the spiral was measured at the end of the equilibration period; the wet weight of each preparation was recorded at the end of the experiments.

Experimental protocols

After a 90 min equilibration period, tracheal preparations were contracted twice with a maximal effective concentration of histamine (50 μM) and extensively washed every 10–15 min with fresh Tyrode solution. When the basal tone was re-established, tracheal tissues were stimulated once more with histamine (50 μM). When the contraction reached a plateau, a fixed concentration of Paf (4 μM) was added to the organ bath in the presence or absence of specific Paf antagonists.

A concentration-effect curve for Paf was obtained in preliminary experiments, yielding the ED_{50} value of 0.4 μM. At this concentration, the amount of relaxation was less than 25% (data not shown); therefore, to evaluate better the effects of Paf antagonists and to avoid mistakes attributable to the small relaxant effect observed, we decided to use a higher concentration of Paf, 4 μM, which ensured about 40% relaxation (see Results).

BN 52021 and WEB 2086 were administered at the peak of histamine contraction (7 min), 3 min before the addition of the phosphoetherlipid. Preliminary experiments, in which antagonists were put into the organ bath 3–15 min before the addition of Paf, showed no differences in the amount of relaxation observed. Hence, a 3 min contact time with antagonists was used.

Because tissues were highly tachyphylactic to Paf, each tracheal segment was treated only once with this agent, that is, one tracheal segment was challenged with Paf and its pair with the antagonist + Paf. BN 52021 and WEB 2086 did not affect by themselves the amplitude of histamine-induced contraction. The specificity of these antagonists was also evaluated in guinea-pig tracheal preparations contracted with histamine and then relaxed by arachidonic acid or PGE_2.

Determination of PGE_2 in tracheal effluents

The measurement of PGE_2 was performed on samples of bath fluid derived from preparations at resting tone and after challenge with histamine, histamine + Paf or histamine + antagonist + Paf. The following protocol was used. A bath fluid sample was collected at the end of 20 min at basal tone. Preparations were re-equilibrated for 5 min and contracted with histamine (50 μM). A second sample was obtained 15 min after the addition of histamine. Thereafter, tissues were washed until they returned to resting tone and a third sample was collected after another 20 min period. The tissues were then re-equilibrated for 5 min, challenged with histamine (7 min), Paf antagonist or vehicle (3 more min) and Paf (5 min more) and the fourth sample was obtained. The samples were immediately frozen and kept at −20°C until use; aliquots (100 μl) of the sample were assayed without extraction.

PGE_2 content in guinea-pig tracheal effluents was determined by an enzymo-immunological assay according to Pradelles *et al.* (1985) and expressed as pg mg^{-1} tissue. The minimal detectable concentration of PGE_2 was 10 pg ml^{-1}. Basal release of PGE_2 was subtracted from the values obtained after agonist challenge.

Calculation of results

Changes in force were measured from isometric recordings, by using isometric force transducers (MARB, mod 83F) and Hewlett Packard (HP66) physiographs. The relaxation induced by Paf, in the presence or absence of a specific antagonist, was expressed as a % reversal of histamine-induced contractile response. IC_{50} value for WEB 2086 was calculated using the Cheng-Prusoff equation: $IC_{50} = K_B(1 + A)/ED_{50}$, where A is Paf 4 μM and the K_B was estimated from Paf concentration-effect curves according to the method of Arunlakshana & Schild (1959).

Statistical analysis of the results obtained was performed by use of Student's t test for paired variables. A probability value of less than 0.05 was considered to be statistically significant. All results are expressed as means ± s.e.mean.

Materials

The compounds used and their sources were: histamine dihydrochloride, PGE_2, fatty acid-free bovine serum albumin (BSA), arachidonic acid (Sigma, St. Louis, MO, USA); Paf (1-octadecyl-2-O-acetyl-sn-glycero-3-phosphocholine; Bachem, Bubendorf, Switzerland).

Kits for performing enzymo-immunological assay of PGE_2 were purchased from Laboratoires des Stallergenes (Fresnes, France). BN 52021 (3-[1,1-dimethylethyl]hexahydro-1,4,7b-trihydroxy-8-methyl-9H-1,7a(epoxymethanol)-1H,6aH-cyclopenta[c]furo-[2,3-b]furo[3',2':3,4]cyclopenta[1,2-d]furan-5,9,12-[4H]-trione) and WEB 2086 (3-(4-(2-chlorophenyl)-9-methyl-6H-thieno-(3,2-f)(1,2,4)-triazolo-(4,3-a)(1,4)-diazepine-2-yl)-1-(4-morpholinyl)-1-propanone) were kindly supplied by Institut Henri Beaufour (Le Plessis-Robinson, France) and by Boehringer Ingelheim Ltd. (Ingelheim am Rhein, West Germany), respectively. Paf was dissolved in Tyrode solution containing 0.25% BSA, stored as a stock solution (10 mM) at −20°C and added to the organ bath as aliquots of 100 µl, so that the BSA final concentration was 25 µg ml^{-1}. WEB 2086 was dissolved in water, BN 52021 was dissolved in dimethylsulphoxide (DMSO) 50%. Final concentration of DMSO was not more than 0.5%.

Results

Physical and mechanical characteristics of guinea-pig tracheal preparations

The length and wet weight of the guinea-pig tracheal preparations used were 20.5 ± 0.15 mm and 47.18 ± 1.5 mg, respectively (n = 70). Histamine (50 µM)-induced contraction was 0.93 ± 0.03 g (n = 70); the relaxation induced by Paf (4 µM) was 39.67 ± 3.5% reversal of histamine-induced contraction (n = 30).

Effects of antagonists on Paf-induced relaxation in guinea-pig tracheal preparations

WEB 2086 (1 nM–1 µM) dose-dependently inhibited the relaxation induced by Paf 4 µM (40 ± 3.2%; n = 20) and, at 1 µM, completely blocked it (Figure 1a). By taking as 100% the Paf-induced relaxation, a concentration-effect curve of antagonism could be drawn in the presence of increasing concentrations of

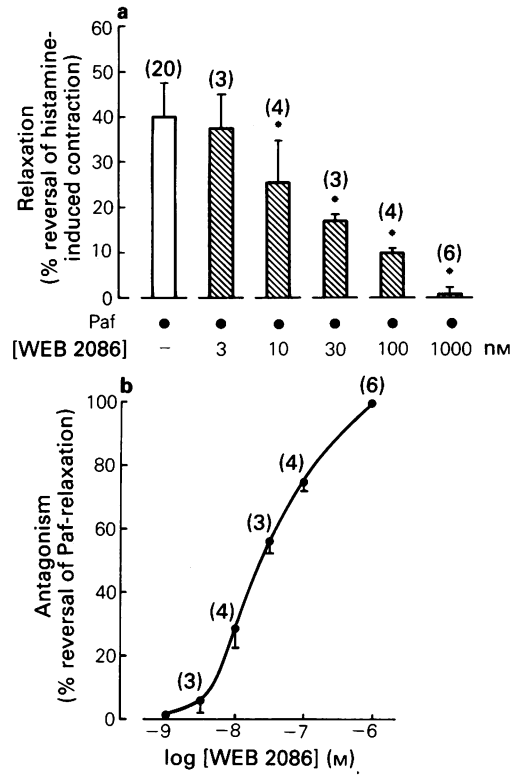


Figure 1 Effect of WEB 2086 on Paf-induced relaxation of histamine (50 µM)-contracted guinea-pig tracheal preparations. (a) Paf-induced relaxation in the absence or presence of WEB 2086. (b) Reversal by WEB 2086 of Paf-induced relaxation. Relaxation induced by Paf was taken as 100%. Each tracheal strip was divided into two paired segments, one was treated with Paf and the other with WEB 2086 + Paf. The number of experiments is indicated in parentheses. Means are shown with vertical lines indicating s.e.mean. * $0.05 > P > 0.01$.

WEB 2086 (1 nM–1 µM) (Figure 1b). In these conditions, the IC_{50} value for WEB 2086 was 21.2 nM.

As shown in Figure 2, BN 52021 100 µM blocked by 58% the mechanical effect induced by Paf. In this set of experiments, the relaxation evoked by Paf 4 µM was 38.5 ± 4.5% in the absence and 16.4 ± 4.7% (n = 6) in the presence of BN 52021 100 µM. At 10 µM BN 52021 did not significantly antagonize the effect of Paf (Figure 2).

Effects of Paf antagonists on arachidonic acid- and PGE_2-induced relaxation in guinea-pig tracheal preparations

As previously demonstrated, arachidonic acid or PGE_2 were able to relax histamine-contracted

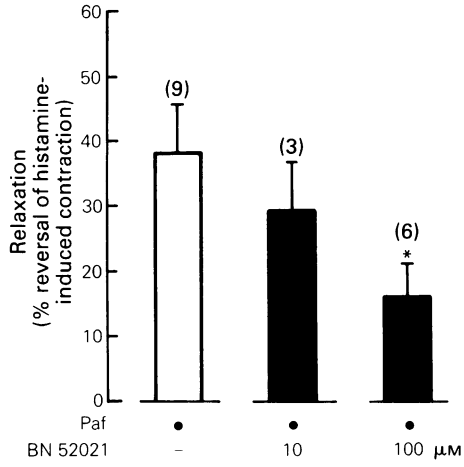


Figure 2 Effect of BN 52021 on Paf-induced relaxation of histamine-contracted guinea-pig tracheal preparations. Each tracheal strip was divided into two paired segments, one of which was treated with Paf and the other with BN 52021 + Paf. The number of experiments is indicated in parentheses. Means are shown with vertical lines indicating s.e.mean. * $P < 0.05$.

guinea-pig tracheal spirals (Braunstein *et al.*, 1988). Therefore, we decided to verify the specificity of WEB 2086 and BN 52021 as Paf receptor antagonists, by evaluating their effects on relaxation induced by fixed concentrations of arachidonic acid or PGE_2.

Arachidonic acid 10 μM and PGE_2 1 μM relaxed to 59.6 $\pm$ 3.5% ($n = 8$) and 76.2% ($n = 6$), respectively, histamine-contracted guinea-pig tracheal preparations (data not shown). In the presence of WEB 2086 1 μM the relaxations induced by the two agents were 55.7 $\pm$ 5.8% ($n = 4$) for arachidonic acid, and 79 $\pm$ 3% ($n = 3$) for PGE_2. Similar results were obtained by evaluating the effects of BN 52021. In the presence of this antagonist, arachidonic acid-induced relaxation was 60.2 $\pm$ 4% ($n = 4$), while PGE_2-induced relaxation was 80 $\pm$ 5.2% ($n = 3$). Moreover, WEB 2086 and BN 52021 were devoid of any antagonistic effect on relaxation evoked by lower concentrations (0.1 and 0.3 μM) of PGE_2, which induced 21.98 $\pm$ 3% ($n = 5$) and 44.75 $\pm$ 3.1% ($n = 4$), respectively (data not shown).

Effects of Paf antagonists on Paf-induced PGE_2 production in guinea-pig tracheal spirals

As previously found, Paf 4 μM caused a significant increase in detectable levels of PGE_2 in effluents from histamine-contracted guinea-pig tracheal preparations (Brunelleschi *et al.*, 1987). In the presence of WEB 2086 1 μM or BN 52021 100 μM, the increase in PGE_2 levels observed when Paf was added to histamine-contracted guinea-pig tracheal preparations was completely abolished and the actual levels of PGE_2 were similar to those measured in the presence of histamine alone (Figure 3a and b).

Discussion

These results confirm our previous observations that Paf relaxes histamine-contracted guinea-pig tracheal preparations and indicate that this effect can be significantly reduced, not only by cyclo-oxygenase

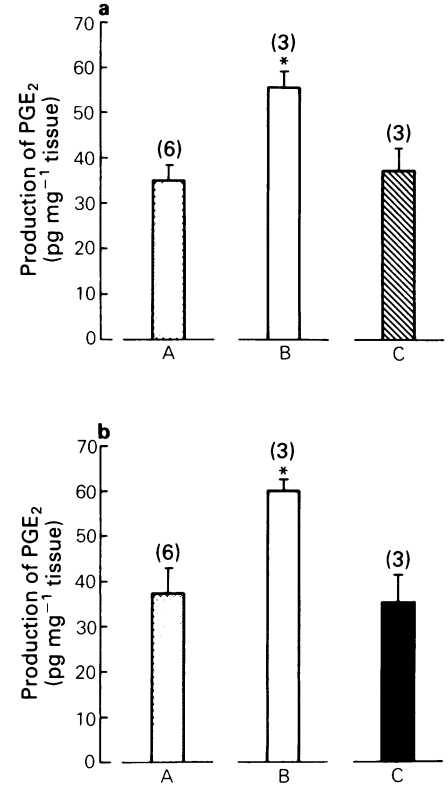


Figure 3 Effects of Paf antagonists on Paf-induced production of prostaglandin E_2 (PGE_2) in histamine-contracted guinea-pig tracheal preparations. (a) Effects of WEB 2086 on Paf-induced PGE_2 production. (b) Effects of BN 52021 on Paf-induced PGE_2 production. Each tracheal strip was divided into two segments, one of which was treated with Paf and the other by antagonist + Paf. Column A: PGE_2 production evoked in the presence of histamine 50 μM; column B: histamine 50 μM + Paf 4 μM; Column C: histamine 50 μM + WEB 2086 1 μM + Paf 4 μM (a) or histamine 50 μM + BN 52021 100 μM + Paf 4 μM (b). Means are shown with vertical lines indicating s.e.mean. The number of experiments is indicated in parentheses. * $P < 0.05$. See Methods for further details.

inhibitors (Brunelleschi *et al.*, 1987), but also by Paf receptor antagonists. In fact, BN 52021 and WEB 2086 inhibited, with different potencies, Paf-induced relaxation of guinea-pig trachea and this antagonism was specific, since relaxation induced by either arachidonic acid or PGE_2 was unchanged. WEB 2086 was the more active compound: at 1 μM it completely blocked the relaxant effect induced by Paf, IC_{50} value being 21.2 nM, while BN 52021 was less potent (about 60% inhibition at 100 μM). The greater potency of WEB 2086 has been previously documented in different experimental models; for instance, O'Donnell & Barnett (1988) obtained pA_2 values of 7.31 and 6.38 for WEB 2086 and BN 52021, respectively, on Paf-induced aggregation in rabbit platelets. According to Evans *et al.* (1988) the dose of WEB 2086 required to abolish completely Paf-induced microvascular leakage of guinea-pig airways *in vivo* was 500 fold lower than that of BN 52063, a standardized mixture of ginkgolides A, B and C.

A role for Paf as an important regulator of eicosanoid generation in pulmonary tissues may be claimed, based on a variety of experimental evidence (Stimler & O'Flaherty, 1983; Lefort *et al.*, 1984; Hamasaki *et al.*, 1984; Desquand *et al.*, 1986; Chung *et al.*, 1986; Brunelleschi *et al.*, 1987; this study). For example, intra-arterial injection of Paf to the perfused guinea-pig lungs elicits a dose-related liberation of thromboxane A_2 (Lefort *et al.*, 1984). Moreover, Desquand *et al.* (1986) showed that the contractile activity of Paf in guinea-pig lung parenchymal strips is accompanied by thromboxane A_2 (TXA_2) release: BN 52021 100 μM inhibited to about 60% the phosphoetherlipid-induced contraction, but totally suppressed the concomitant thromboxane A_2 release (Desquand *et al.*, 1986). The fact that the activation of the cyclo-oxygenase pathway preferentially leads to production of TXA_2 in guinea-pig parenchyma and PGE_2 in guinea-pig trachea (Grodzinska *et al.*, 1975; Lefort *et al.*, 1984) may help to explain the opposite functional effects (contraction or relaxation) elicited by Paf. However, the ability of BN 52021 to reduce Paf-induced mechanical events and Paf-induced eicosanoid generation was observed to the same extent in both tissues (Desquand *et al.*, 1986; this study).

In conclusion, our results indicate that WEB 2086 and BN 52021 are able to reduce Paf-induced effects (relaxation and PGE_2 generation) in histamine-contracted guinea-pig trachea and are consistent with the presence of specific Paf receptors in this tissue.

A role for PGE_2 as mediator of Paf-induced relaxation is strongly indicated, since both antagonists, besides reducing the relaxation, also prevent the phosphoetherlipid-induced increase of PGE_2 in tracheal effluents.

The observation that BN 52021 only partially reduced the mechanical event, while suppressing Paf-induced PGE_2 production, warrants two possible explanations. The first, and more obvious one, is that the concentration of BN 52021 we used was not high enough to block Paf-induced relaxation. However, 100 μM BN 52021 was the maximal concentration we could use in our experimental model, due to the concentration of DMSO in which the compound was dissolved. The second explanation which could be drawn from our results with BN 52021 is that PGE_2 is not the sole mediator of the relaxation elicited by Paf.

Participation of other mediators and/or a direct effect of Paf could be envisaged; as a matter of fact, we previously showed that pretreatment of guinea-pig tracheal spirals with indomethacin strongly reduced, but did not completely abolish, Paf-induced relaxation (Brunelleschi *et al.*, 1987). However it is noteworthy that a potent Paf receptor antagonist, namely WEB 2086, is able to block totally Paf-induced tracheal effects.

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Effect of changing extracellular levels of magnesium on spontaneous activity and glutamate release in the mouse neocortical slice

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1 The mouse neocortical slice preparation, maintained in a two compartment, grease gap bath, exhibits spontaneous depolarizing activity (with or without rhythmic after potentials) after perfusion with magnesium-free artificial cerebrospinal fluid.

2 If the magnesium concentration is decrementally lowered over an extended time period, then incrementally raised following a similar time course, the spontaneous depolarizing shift activity shows a hysteresis (with regard to both frequency and amplitude), the depolarizing shifts being more resistant to magnesium during the the incremental period.

3 The amino acid content of the perfusing fluid was analysed by high performance liquid chromatography (h.p.l.c.). Although a basal efflux of 6 amino acids was quantifiable, only glutamate levels increased following superfusion of the preparation with magnesium-free, artificial cerebrospinal fluid.

4 Glutamate release increased to 266% of the resting release in the presence of magnesium within the first 12 min of the change into magnesium-free artificial cerebrospinal fluid. This increase in release preceded the onset of spontaneous depolarising activity. The release of glutamate remained elevated at 182% of control up to 60 min after perfusion with magnesium-free buffer, when depolarizing activity was well established.

5 A model is presented and discussed for the genesis and maintenance of the spontaneous depolarizing shifts. It is suggested that the maintenance of this spontaneous activity reflects a long term enhancement of neocortical neurone excitability which may be related to long term potentiation in the hippocampus.

Introduction

The receptors for the excitatory amino acids have been classified into at least 3 subtypes (N-methyl-D-aspartate (NMDA), kainate and quisqualate) as a result of pharmacological investigations (for reviews see Watkins & Evans 1981; McLennan 1983; Stone & Connick 1985; Mayer & Westbrook 1987; Stone & Burton, 1988). Responses involving the activation of the N-methyl-D-aspartate (NMDA) receptor are very sensitive to changes in the extracellular concentration of magnesium (Evans *et al.*, 1977). This antagonism is thought to result from a voltage-dependent blockade of the ionic channels activated by the NMDA receptor (Nowak *et al.*, 1984; Mayer *et al.*, 1984). In the rat neocortex the relief of this blockade upon removal of magnesium from the artificial cerebrospinal fluid (ASCF) also gives rise to potentiation of responses due to NMDA receptor-mediated processes (Thomson & West, 1986). This implies that when magnesium is at physiological levels (approximately 1–2 mM) (Davson *et al.*, 1987) there will be substantial, but not necessarily total, antagonism at the NMDA receptor.

Several groups of investigators have recently observed that slices of rat or mouse neocortex maintained in a twin compartment, grease gap bath (Harrison & Simmonds, 1985) produce spontaneous depolarizing shifts often with rhythmic after potentials when the magnesium content of the superfusing medium is lowered from a normal content of 1 mM to 0 mM (Horne *et al.*, 1986, Burton *et al.*, 1987). This phenomenon is also seen in human cortex (Avoli *et al.*, 1987). These depolarizing shifts usually appear within 30–60 min of superfusion with magnesium-free ACSF and gradually increase in both size and

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frequency over the following 1 to 2 h (Burton et al., 1987). Not all slices exhibit this activity spontaneously although most can be induced into producing depolarizing shifts following depolarization with an NMDA receptor agonist. Such depolarizing shifts are sensitive to NMDA antagonists such as 2-amino-5-phosphonopentanoate (APV), 2-amino-7-phosphonoheptanoate (APH), kynurenic acid, and ketamine, and are abolished by tetrodotoxin (Horne et al., 1986; Burton et al., 1987).

Similar paroxysmal depolarizations also appear after 15–20 min perfusion with 50 μM bicuculline in normal (1 mM magnesium) Krebs medium (Horne et al., 1986). These depolarizing shifts were also abolished by NMDA antagonists. It is therefore possible that changes in the concentration of GABA in a cortical perfusate could also contribute to the epileptiform activity seen in zero magnesium medium (Heyer et al., 1982).

Since other forms of self perpetuating depolarizing activity such as long term potentiation (LTP) have been associated with an increase in glutamate release (Dolphin et al., 1982) we have endeavoured to examine the changes in endogenous amino acid release concomitant with the onset of spontaneous depolarizing activity initiated by a lowering of extracellular magnesium. To date there have been no reports on the effect of removal of magnesium ions on the release of the endogenous excitatory amino acids, and only few reports on its effect on γ-aminobutyric acid (GABA) release (e.g. Drejer et al., 1987). Some of the present results have been given previously in a preliminary form (Smith & Stone, 1988; Connick et al., 1988).

Methods

Full details of the preparation and a qualitative description of its properties appear elsewhere (Burton et al., 1987).

Tissue preparation

Male MF1 mice (4–8 weeks old) were killed by cervical dislocation followed by decapitation and the whole brain removed quickly into ice-cold artificial cerebrospinal fluid (ACSF). Coronal sections about 500 μm thick were cut with a Vibroslice (Campden Instruments). Acceptable sections had as rostral and caudal limits the genu of the corpus callosum and the rostral region of the cerebral ventricles respectively. These were transferred to an incubation chamber containing a humidified 95% O_2, 5% CO_2 atmosphere at room temperature (20–24°C). A suitable slice was subsequently divided in the midline and further cuts were made to produce several wedge shaped pieces of tissue about 1.5 mm wide at the pial surface and about 1 mm wide at the corpus callosum. One such wedge was transferred to a two chamber bath, so that most of the cortical tissue was contained in one chamber, and the corpus callosum and a little cortical tissue contained in the other. A high resistance seal between the two compartments was achieved by use of high vacuum silicone grease (BDH). Each chamber was continuously perfused at 2.5 ml min^{-1} (or 2.0 ml min^{-1} for release studies) via droppers with ACSF gassed with a mixture of 95% O_2 and 5% CO_2. The callosal compartment of the bath was perfused with magnesium containing ACSF at all times. Where reference is made to a change from magnesium-containing to magnesium-free ACSF this only applies to the cortical (pial) compartment.

ACSF composition

ACSF normally had the following composition (in mM): NaCl 123.8, KCl 3.3, $MgSO_4 \cdot 7H_2O$ 1.0 (or as stated in the text), KH_2PO_4 1.22, $NaHCO_3$ 25.0, $CaCl_2$ 2.5, D-glucose 10.0, and was continuously bubbled with a 95% O_2, 5% CO_2 mixture to bring the pH to about 7.4. The ACSF was approximately 325 mOsm. The magnesium content of the nominally Mg-free ACSF, prepared by omission of $MgSO_4$, was not greater than 11.3 μM (estimated from manufacturer's data) and typically was 2–3 μM (estimated by Atomic Absorption spectroscopy).

Experimental protocol

The d.c. potential between the two compartments was continuously monitored via Ag/AgCl electrodes and a high input impedance amplifier. It was displayed on a storage oscilloscope and for permanent records a chart recorder. Unless otherwise stated, after an incubation of at least 1 h in ACSF the cortical chamber was perfused with nominally Mg-free ACSF.

Amino acid release After 1 h in ACSF (1 mM magnesium) the cortical side of the bath was perfused with Mg-free ACSF. Samples of perfusate (2 min) were collected and 500 pmol of D-α-aminoadipic acid was added as an internal standard prior to concentration in a vacuum centrifuge. Fractions were resuspended in 100 μl of water and the endogenous amino acids analysed by high performance liquid chromatography (h.p.l.c.) using a method based upon that of Turnell & Cooper (1982).

o-Phthalaldehyde (OPT)/2-mercaptoethanol derivatives were produced by taking 100 μl of OPT reagent solution (Sigma, UK) and mixing with 100 μl of amino acid mixture (standards or sample). The mixture was immediately vortexed to produce the 1-

alkyl-thio-2-alkyl-substituted isoindole derivative (Roth, 1971). After 30 s, 20 μl of the mixture was injected onto the chromatographic column for analysis.

A Gilson gradient system was used, and detection performed by a Gilson Spectra Glo fluorimeter with an excitation wavelength of 390 nm and emission cut off filter at 475 nm, at maximum sensitivity. Separation of the derivatised amino acids was performed on a reverse phase 'Resolve' C_{18} analytical column (Waters, UK) fitted with a C_{18} 'guard pac' pre-column (Waters, UK).

Changes in magnesium concentration After 1 h in normal ACSF the concentration of magnesium in the ACSF superfused over some slices was lowered in a stepwise fashion, while with other slices the magnesium concentration was reduced to 0 in one step. Upon achieving a nominally magnesium-free state, slices were equilibrated for approximately 1 h before increasing the magnesium content of the ACSF. Slices were then returned to zero magnesium ACSF as a recovery control. Each magnesium concentration change was allowed to equilibrate for 30 min.

Atomic absorption spectroscopy Samples of perfusate (200 μl) were collected throughout the normal washout of magnesium from the bath (2 mM to 0 mM magnesium in one step). These samples were then analysed for magnesium content in a Pye Unicam SP9 atomic absorption spectrophotometer with a magnesium vapour lamp (285.2 nm) against a calibration curve of $MgSO_4$ in reagent grade water.

Materials

All chemicals were of 'Analar' grade and were obtained from BDH Ltd. Amino acids were from the Sigma Chemical Co. Reagent grade water was prepared by running tap water through Milli RO4 and Milli Q water purification systems (Millipore, UK).

Statistics

Throughout this study, statistical significance has been assessed relative to control conditions by use of either a paired or unpaired Student's t test as appropriate.

Results

In none of the experiments in which tissues were bathed continuously in magnesium-containing ACSF were depolarizing shifts obtained (Burton *et al.*, 1987). Spontaneous depolarizing shifts appeared within about 1 h of superfusion with magnesium-free ACSF in about 60% of the viable slices prepared (mean $\pm$ s.e.mean; 67.6 $\pm$ 7.1 min to approx 50% final amplitude; $n = 13$). The depolarizing shifts occurred with or without rhythmic after potentials (see Figure 1). Each depolarizing shift consisted of an initial brief (50–100 ms) negative going potential in the pial compartment followed by a slow repolarization over the next 2–10 s. In most wedges a series of repetitive negative going potentials were superimposed on the slow repolarising phase.

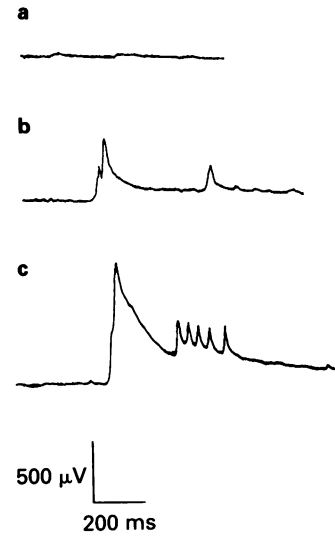


Figure 1 Traces of spontaneous activity in mouse neocortical slice; (a) before introduction of magnesium-free ACSF; (b) during the development of spontaneous depolarizing activity; (c) fully developed depolarizing shift showing rhythmic after potentials.

Effect of incremental rise in magnesium concentration

In seven slices the magnesium concentration was reduced in one single step to zero, when depolarizing shifts occurred. The magnesium concentration was then raised to assess the sensitivity of the spontaneous activity to magnesium concentration. When incubated with ACSF containing 200 μM magnesium (slices were allowed to equilibrate for 30 min following the change in magnesium concentration) 3 slices showed increased depolarizing shift amplitude (109.8 $\pm$ 3.9% of controls; mean $\pm$ s.e.mean) although at all magnesium concentrations the frequencies of the shifts were reduced. It is interesting to note that of the original seven slices, five were still spontaneously active when the magnesium concentration was raised above control (1 mM) levels; in

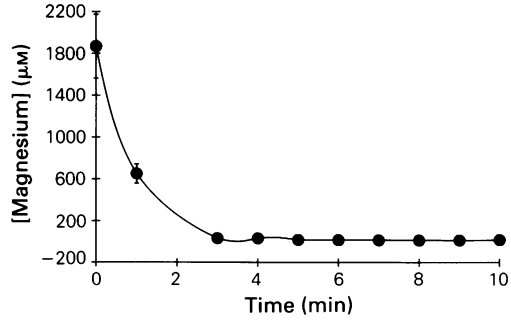


Figure 2 Graph of washout of magnesium from the cortical chamber of the two compartment grease gap bath. The initial magnesium concentration is 2 mM and the magnesium concentration was measured by atomic absorption spectroscopy (see text). Each point is the mean from three separate experiments with s.e.mean shown by error bars except where smaller than symbol.

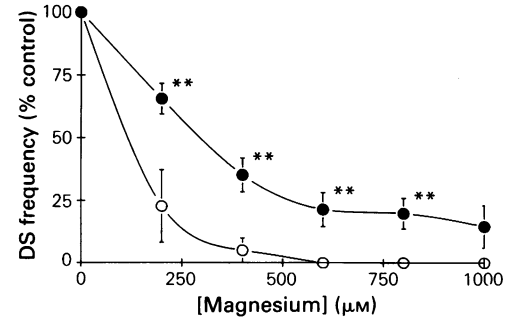


Figure 4 Graph to show hysteresis of spontaneous depolarizing shift (DS) frequency in mouse neocortical slices to decreasing (open circles) and increasing (filled circles) magnesium concentrations. Each point represents the mean number of depolarizations per minute over the last 5 min of each 30 min step at each individual magnesium concentration; s.e.mean shown by vertical bars. $^{**}P < 0.01$. Magnesium concentrations given in μM.

addition, the spontaneous activity in two slices was not blocked at 2.6 mM magnesium. In the two slices that were still active at 2.6 mM, the washout of magnesium was rapid (see Figure 2) and the depolarizing shifts increased in frequency and amplitude in a similarly rapid manner following the final removal of magnesium from the ACSF (see Figure 3).

Effect of a stepwise decrease and increase of magnesium concentration

The effect of reducing the magnesium content of the ACSF in steps of 200 μM, each lasting 30 min, on the frequency and amplitude of the depolarizing shifts can be seen in Figures 4 and 5. The onset of depolarizing shifts was only apparent at concentrations of magnesium of 400 μM or less in the decremental part of the experiment. In one slice, depolarizing shifts began at 400 μM (frequency 40% of value obtained in magnesium-free solution) but were of very small amplitude (19% of control value obtained in magnesium-free solution). Two other slices also began to show spontaneous activity at 200 μM magnesium, the amplitudes now being greater (28% and 63% of magnesium-free values) and the frequencies more variable (5% and 71% respectively). When the slices were subsequently perfused with nominally magnesium-free ACSF, they developed their depolarizing shifts as previously reported (Horne *et al.*, 1986; Burton *et al.*, 1987). After incubation in nominally magnesium-free ACSF, the reintroduction

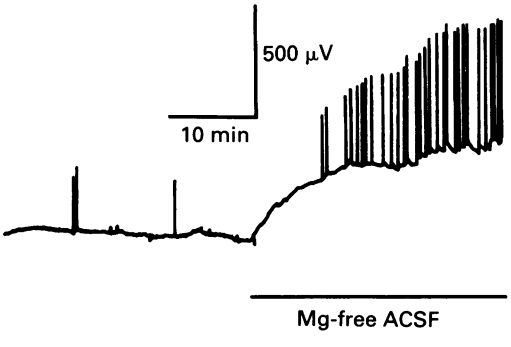


Figure 3 Reappearance of spontaneous depolarizing shifts in mouse neocortical slice following washout of 2.6 mM magnesium. Note rapid return of both frequency and amplitude to near control levels. (Retouched)

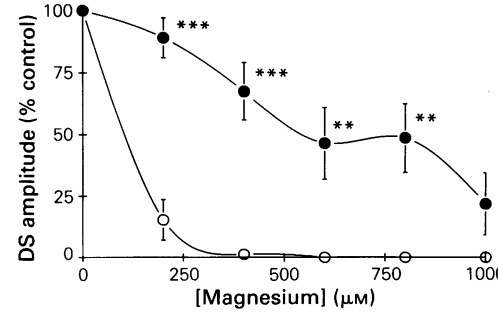


Figure 5 Graph to show hysteresis of spontaneous depolarizing shift amplitude to decreasing (open circles) and increasing (filled circles) magnesium concentrations in mouse neocortical slices. $^{**}P < 0.01$; $^{***}P < 0.001$. Concentrations of magnesium given in μM.

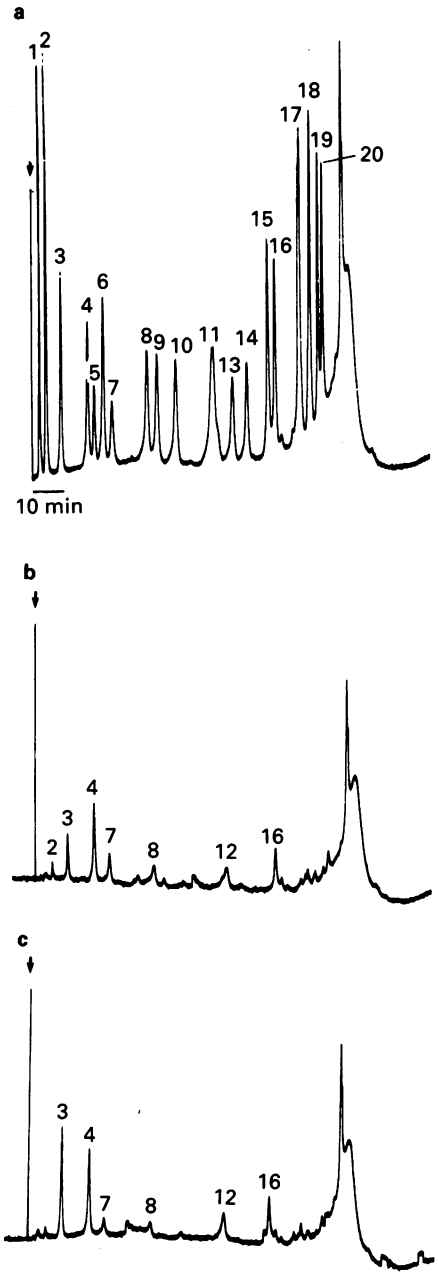


Figure 6 High performance liquid chromatography (h.p.l.c.) chromatograms of (a) a standard injection of 20 μl of a mixture of 25 pmol each of (1) phosphoserine, (2) aspartic acid, (3) glutamic acid, (4) α-aminoadipic acid, (5) asparagine, (6) serine, (7) glutamine, (8) glycine, (9) threonine, (10) citrulline, (11) alanine, (13) taurine, (14) aserine, (15) β-aminoisobutyric acid, (16) tyrosine, (17) methionine, (18) valine, (19) tryptophan and (20) phenylalanine. (b) A typical trace of amino acids released from a slice at t_{-28}; peak 12 is that of GABA. (c) A typical trace of amino acids released from a slice at t_{60}. The traces in (b) and (c) are efflux from the same slice. The broad peak at the end of each chromatogram represents the elution of a number of unresolved compounds corresponding to the rapid increase in the elution gradient. Chromatographic conditions were as follows: Solvent (a): 0.05 M sodium acetate, 0.05 M disodium hydrogen phosphate, pH 7.4: methanol: tetrahydrofuran, 96:2:2. This mixture was then filtered through a 0.45 μm Durapore filter (Millipore, UK) under vacuum. Solvent (b) consisted of 65% methanol in water. This was premixed and filtered under vacuum. Chromatographic conditions: The gradient programme, expressed as time in minutes from injection (% solvent b) was; 0(0), 4.5 (3), 5.4 (6), 6.4 (7), 7.3 (8.6), 9.1 (10), 10 (10.7), 10.9 (11.4), 11.8 (12.5), 12.7 (13), 13.6 (14.3), 27.3 (23), 47 (100). The flow rate was 1.5 ml min^{-1} at room temperature.

of ACSF containing magnesium led to an overall loss of frequency and amplitude, although in 2 of 8 slices the amplitude was greater than that of control (105% and 103% of controls). All the slices showed reduced depolarizing shift frequencies. At all higher magnesium concentrations both frequency and amplitude were depressed below the values in zero magnesium although at all levels of magnesium below 1 mM, the frequency and amplitude were significantly higher than observed during the progressive reduction of magnesium concentration. Graphs of the amplitude and frequency of depolarizing shifts therefore exhibit a hysteresis phenomenon in which their values at any given magnesium concentration depend on the direction of magnesium concentration change (Figures 4 and 5).

Amino acid release

Although the detection system used was routinely capable of resolving 10 to 50 pmol of up to 23 amino acids, analysis of the ACSF superfusing the pial area of the slice showed a detectable efflux of only aspartate, glutamate, glutamine, glycine, GABA and tyrosine. These amino acids were identified on a basis of their retention times and their concentration assessed by measurement of their peak height relative to the internal standard as previously described (Connick & Stone, 1988). In addition, the identities of individual peaks were confirmed by 'spiking' the sample with specific amino acids with the exception of tyrosine which was identified by its retention time alone.

Typical chromatograms of amino acid standards (Figure 6a), and typical samples from perfusates taken 28 min before and 60 min after removal of magnesium are shown in Figure 6b and c respectively. The only amino acid to change in the profile

detected was glutamic acid. The efflux of the compound was typically between 100 to 150 pmol ml^{-1} in the superfusing ACSF. A more exact measurement of the release of glutamate in terms of tissue weight was not possible because of the nature of the preparation. Typically the total weight of the cortical slice was between 2 and 3 mg with a variable proportion of the slice lying on the callosal side of the bath. The efflux of glutamate therefore represents that from approximately 1–2 mg of tissue.

During the course of the experiment the amounts of aspartic acid, glutamine and glycine usually declined however, this was not the result of the removal of magnesium from the medium. The efflux of GABA and tyrosine remained at a constant low level throughout the experiment.

In the presence of magnesium no spontaneous activity was detectable (Figure 1a) and glutamate release was stable at 99.7 ± 5.4% of the mean release ($n = 3$ to 5) at time 0 (t_0) (Figure 7). Upon removal of magnesium at t_0, there was an immediate increase in glutamate release (Figure 7) corresponding to the washout of magnesium from the bath (Figure 2). Glutamate release was increased to 352.5 ± 23.8% between t_0 and t_{40} ($n = 3$ to 5), the large s.e.mean representing the very large increase in release found in some slices and the difficulty in normalizing data from slices with varying frequency, amplitude and time of onset of their depolarizing shifts. Comparing the six fractions immediately either side of t_0, there was an increase in release of 266% ($P < 0.05$), that is within 12 min of the change to magnesium-free solution.

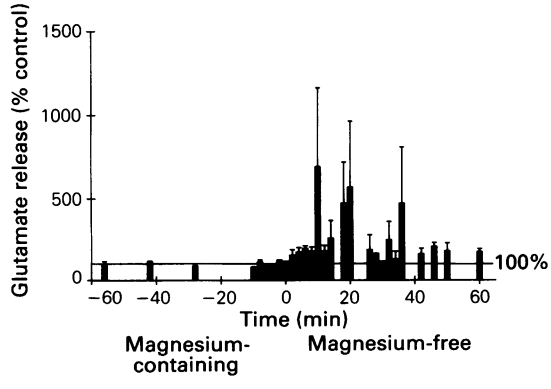


Figure 7 Histogram of glutamate release as a % of release at time zero (t_0). Each column represents mean (s.e.mean shown by vertical bars) from between 3 and 5 experiments. Magnesium-containing ACSF (1 μM) was exchanged for magnesium-free ACSF at t_0. Spontaneous depolarizing activity appeared 56.4 ± 8.6 min (mean ± s.e.mean) after the change to zero magnesium ACSF.

After the initial dramatic increase in glutamate release up to 40 min after t_0 (354% of the resting efflux; $P < 0.05$) the efflux stablized between 40 and 60 min corresponding to the maturation of the depolarizing shifts and the development of numerous afterpotentials superimposed upon each depolarizing shift (Figure 1c). The average release of glutamate between 40 and 60 min was 181.4 ± 9.5% and although less than that occurring between 0 and 40 min, it was still substantially higher (182%; $P < 0.001$) than the release into magnesium containing ACSF immediately before the change to zero magnesium buffer.

Discussion

The spontaneous depolarizing shifts described here are similar to those seen in the neocortical slice preparation of the rat (Harrison & Simmonds, 1985; Horne *et al.*, 1986). The fast rise time of the initial peak (around 50 ms) and the appearance of rhythmic afterpotentials are characteristic of both preparations. In this paper we are primarily concerned with the initial event as the appearance and frequency of afterpotentials per depolarizing shift was found to be inconsistent between preparations.

It is interesting to speculate as to the origin and mechanism of these depolarizing shifts in the light of the results presented in this paper. We are unable to give any information as to the anatomical site of origin of the spontaneous depolarizing shifts owing to the non-focal nature of the grease gap recording technique. However, a recent paper (Aram & Lodge, 1988) in which the whole neocortical slice preparation of the rat and microelectrode recording techniques were used, has shown that in magnesium-free conditions the greatest amplitude of these spontaneous epileptiform events was found in layers II/III. This of course does not necessarily reflect origin but probably reflects the greatest concentration of cells involved in the spontaneous activity. In fact these workers note that there are multiple foci from which these epileptiform bursts spread to both contra- and ipsilateral cortices. One would expect the mouse neocortex to behave similarly owing to the close phylogenetic relationship between the two species.

The depolarizing shifts seen on lowering extracellular magnesium levels, may be attributable to 3 mechanisms. A general increase in the level of cell excitability due to the removal of magnesium could be the cause although Horne *et al.* (1986) found that if magnesium, after removal, was subsequently replaced by an equivalent concentration of calcium, then the frequency of the depolarizing shifts did not change. This may indicate that the general level of cell excitability is not the major mechanism of depolarizing shift genesis.

Another mechanism by which the initiation and maintenance of spontaneous depolarizing shifts may be explained could be by regional anoxia in the area around the grease gap (Rothman & Olney, 1986). However this too would seem unlikely as Aram & Lodge (1988) have demonstrated that the spontaneous depolarizing shifts can be initiated by low magnesium levels in a standard slice (i.e. not grease gap) bath and with orthodox methods when the neurones showed no signs of deterioration.

An alternative cause of the spontaneous activity could be the reduction of the magnesium blockade of the ion channel associated with the NMDA receptor, an effect which has been suggested by results obtained by Coan & Collingridge (1987) in the hippocampus. This unmasking of the NMDA receptor would lead to an increased level of excitatory tone in all neurones receiving an NMDA receptor-mediated excitatory input.

Since an increase of extracellular magnesium levels has been shown to induce a reduction of neurotransmitter release (del Castillo & Engbaek, 1954), the removal of magnesium could also lead to an increase in neurotransmitter release acting at those receptors (Jenkinson, 1957; Richards & Sercombe, 1970). Although one would expect this to apply to the release of inhibitory neurotransmitters (Drejer *et al.*, 1987) as well as excitatory ones, the present results indicate that the only increase in putative neurotransmitter efflux is that of glutamate. This result would suggest that depolarizing shifts are due to an increase in glutamate release but would not necessarily exclude the unmasking of the NMDA receptor channel also having an important role. Indeed the fact that the increase in glutamate release does not follow the same time course as the development of spontaneous activity may even suggest a model which includes the involvement of the NMDA receptor channel in the genesis and maintenance of depolarizing shifts. It is important to note that the initial increase in glutamate release peaks before the full development of the depolarizing shifts and then tails off whilst remaining significantly higher than the resting release which occurs during the control period. Furthermore the effects of magnesium on the depolarizing shifts are different when investigated before or after the full development of the spontaneous activity. It is feasible that the removal of magnesium initially causes an increase in transmitter release (in this case presumably glutamate) as indicated by the results. This increased level of glutamate in turn increases the excitatory tone of neurones which should be self perpetuating as raised levels of glutamate would in turn cause increased glutamate release (Connick & Stone, 1988). The increase in excitatory tone would also tend to overcome the voltage-dependent magnesium blockade of the NMDA receptor-associated ion channel. This in turn would lead to recruitment of a suitable proportion of neurones that presumably fire synchronously via synaptic interconnections. Alternatively a discrete population of sensitive 'initiator' neurones may experience enhanced excitatory tone and presumably recruit other neurones eventually leading to fully developed depolarizing shifts.

The potentiated involvement of NMDA receptors could explain the observed hysteresis in the susceptibility of the depolarizing shifts to blockade by reintroduction of magnesium into the bath. Whilst it is possible that the hysteresis could simply reflect a lag between the change of magnesium concentration and equilibrium in the 30 min steps used, this is unlikely since depolarizing shifts typically appear after about 30 min, reaching half their maximum amplitude after 67 min. In addition, the fact that some slices still showed spontaneous activity at up to 2.6 mM magnesium following the full development of the depolarizing shifts argues against this explanation. The observed hysteresis is similar to an effect noted previously by Anderson *et al.* (1986).

NMDA receptor antagonists are able to prevent the initiation of long term potentiation (LTP) in both the cortex and hippocampus (Bliss & Lomo, 1973; Artola & Singer, 1987), but once established these antagonists have no effect on the perpetuation of LTP. Huang *et al.* (1987) have shown that the induction of LTP is facilitated by lowering magnesium concentrations. Skrede & Malthe-Sorenssen (1981) found that high frequency stimulation of afferent fibres to CA1 neurones in the hippocampus (conditions similar to that needed for the induction of LTP) caused a long lasting increase in the resting release of [^{3}H]-D-aspartate from pre-loaded cells. Furthermore Dolphin *et al.* (1982) using a push-pull perfusion technique, found that LTP induced in the perforant path/dentate gyrus granule cell pathway of the hippocampus produced a long lasting increase in the release of newly synthesized glutamate. The present finding of an increased release of glutamate is therefore consistent with the proposal that the maintenance of depolarizing shifts is partly due to a long term change of excitability akin to LTP.

The role of GABA in the genesis of the depolarizing shifts is unclear. Removal of magnesium has been reported to enhance NMDA-induced but not glutamate-induced release of GABA from cultured cortical tissue (Drejer *et al.*, 1987). The present failure to detect any change of GABA release is therefore entirely consistent with our demonstration of the selective release of glutamate by the slices.

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m-Octopamine injected into the paraventricular nucleus induces eating in rats: a comparison with noradrenaline-induced eating

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1 The effects on food intake in rats of injection of *m*- and *p*-octopamine into the paraventricular nucleus (PVN) of the hypothalamus were examined, and compared to the effects of noradrenaline (NA).

2 *m*-Octopamine injected into the PVN induced a dose-dependent increase in food intake, with the maximal effect occurring at a dose of 25 nmol. *p*-Octopamine did not elicit eating unless it was administered to animals pretreated with the monoamine oxidase inhibitor, pargyline.

3 The effects of pretreatment with various adrenoceptor antagonists, injected into the PVN, on the eating responses induced by 25 nmol *m*-octopamine and NA were examined. The α_1-adrenoceptor antagonist, corynanthine, and the β-adrenoceptor antagonist, propranolol, failed to alter the eating induced by *m*-octopamine or NA. The effects of these two amines were susceptible to blockade of α_2-adrenoceptors. Idazoxan reversed the eating induced by *m*-octopamine and noradrenaline. However, yohimbine was effective only against the eating induced by *m*-octopamine. Thus, both *m*-octopamine and NA appear to act via α_2, but not α_1 or β-adrenoceptors.

4 Injection of α-methyl-*p*-tyrosine into the PVN attenuated the effect of *m*-octopamine, but not of NA. This result suggests that *m*-octopamine elicits eating, at least in part, by releasing endogenous NA.

5 The NA and octopamine uptake inhibitor, desipramine, significantly potentiated the eating induced by a low dose of *m*-octopamine. This effect may occur because desipramine would prolong the synaptic activity of released NA.

6 The results indicate that *m*-octopamine elicits a marked and reliable eating response which is mediated largely by a release of endogenous NA, which acts at α_2-receptors. These results are consistent with the view that octopamine may function as a modulator of NA activity in the central nervous system.

Introduction

Octopamine is a biogenic amine which occurs naturally in invertebrates and vertebrates (see review by Robertson & Juorio, 1977; David & Coulon, 1985; Evans, 1985; Williams *et al.*, 1987). In insects, specific octopamine neurones and receptors have been identified and this amine appears to be an important neuroactive substance (Evans, 1985). However, in vertebrates the functional significance of octopamine in controlling neural activity is unknown.

In the rat, octopamine [in two isomeric forms, *para*- (*p*-) and *meta*- (*m*-)] is present in very low concentrations in several sympathetically innervated organs (Molinoff & Axelrod, 1969; 1972; Ibrahim *et al.*, 1985) and in the brain (Buck *et al.*, 1977; Danielson *et al.*, 1977; David & Delacour, 1980). Several similarities between octopamine and noradrenaline (NA) have been noted. The distribution of octopamine in the peripheral and central nervous system roughly parallels that of NA. Both amines have similar uptake and release characteristics (Baldessarini & Vogt, 1971; Baldessarini, 1980; Reimann, 1984). Drugs affecting endogenous NA levels affect octopamine levels in a similar fashion (Harmar & Horn, 1976; Buck *et al.*, 1977; Danielson *et al.*, 1977; Harmar, 1980). Both the *m*- and *p*-isomers of octopamine have significant activities at

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α_1, α_2 and β-adrenoceptors (Fregly *et al.*, 1979; 1984; Reiman, 1984; Jordan *et al.*, 1987; Brown *et al.*, 1988). It is not surprising then, that attempts to ascribe a functional role to octopamine have focussed on this relationship to noradrenergic systems. Thus, it has been suggested that octopamine may function as a 'false' transmitter within the noradrenergic system (Kopin *et al.*, 1965), or as a co-transmitter located in noradrenergic neurones (Axelrod & Saavedra, 1977), which can serve as a modulator of noradrenergic function (Jones, 1982).

Other lines of evidence have indicated that octopamine may function independently of the noradrenergic system. Several studies have identified single neurones in the spinal cord (Hicks & McLennan, 1978a), cerebral cortex (Hicks & McLennan, 1978b; Jones, 1982) and thalamus (Dao & Walker, 1980) which respond differently to iontophoretically applied *p*-octopamine and NA. David and colleagues have provided behavioural and neurochemical evidence that octopamine may play an important role in neurovegetative function, which is not mediated via changes in NA activity. In these experiments rats of the Roman strain, selected for high avoidance (RHA) and low avoidance (RLA) responding were used. It was found that hypothalamic and brain stem levels of *p*- and *m*-octopamine were higher in the RHA strain than in the RLA strain (David & Delacour, 1980; Delacour *et al.*, 1983). No differences in NA levels between the strains were observed. These authors suggested that the strain difference in avoidance responding may be related specifically to endogenous octopamine levels. This idea is supported by the finding that octopamine, administered intraventricularly, temporarily suppressed the strain difference in *p*-octopamine levels, without affecting NA levels, and facilitated avoidance responding to a greater degree in RLA rats, so that the strain difference in avoidance conditioning was no longer apparent (Delacour *et al.*, 1983).

The present experiments were designed to examine further the behavioural effects of intracerebrally administered *m*- and *p*-octopamine, and to compare them with those induced by NA. Specifically, the experiments examined the effects of these amines on food intake in satiated rats following microinjection into the paraventricular nucleus of the hypothalamus. This paradigm was chosen for two reasons. Firstly, in the rat brain octopamine content is highest in the hypothalamus (Danielson *et al.*, 1977). Secondly, injection of NA into the PVN induces a robust eating response (Leibowitz, 1978) which is mediated by α_2-adrenoceptors (Goldman *et al.*, 1985). Further, endogenous NA in this region appears to be specifically involved in the normal control of feeding behaviour (Leibowitz, 1980). Initial experiments yielding dose-response curves to *p*- and *m*-octopamine revealed that *m*-octopamine was the more potent isomer; *p*-octopamine induced eating only in the presence of a monoamine oxidase inhibitor (MAOI). Further experiments which were undertaken to explore the interaction of octopamine with the various adrenoceptor sub-types, and with endogenous NA, used *m*-octopamine only, since it was reasoned that increases in endogenous amine levels following MAO inhibition would confound the results.

Methods

Male Wistar rats weighing 270–310 g at the time of surgery were individually housed in hanging wire mesh cages in a temperature controlled room (22 ± 2°C), under a 12 h light-dark cycle (lights on at 08 h 00 min). Food and water were available at all times. Testing was generally conducted in the afternoon between 13 h 00 min and 17 h 00 min.

Surgery

Rats were anaesthetized with 40–50 mg kg^{-1} sodium pentobarbitone (Nembutal, Abbot Laboratories) and placed in a stereotaxic frame. A 22 g stainless steel guide cannula (Plastic Products, Roanoke, VA) cut to 15 mm in length was aimed at a site 4 mm above the left PVN. With the incisor bar set at 3.3 mm below the interaural line the following co-ordinates were used (relative to bregma): AP −1.3 mm, L +0.4 mm, V −4.0 mm. Each cannula was secured by acrylic cement which was held in place by 4 stainless steel screws drilled into the skull. A 28 g stainless steel stylet served to keep the cannula patent. Following surgery the skin wound was treated with xylocaine. Animals were allowed at least 7 days to recover. During this period each animal was handled frequently, and familiarised with the injection procedure.

Test procedure

The general test procedure was identical for all experiments. On test days fresh food was placed on the cage floor 1 h prior to drug injection. Drugs were injected directly into the brain through a glass capillary tube (70–90 μm diameter) connected to a 5 μl Hamilton syringe via a length of plastic tubing. The glass capillary was housed inside a 15 mm length of 28 g stainless steel tubing, and could be extended 4 mm beyond the end of the guide cannula to penetrate the PVN (see Azami *et al.* (1980) for full details of the microinjection assembly). Each solution was injected over a period of 1–2 min depending on drug volume and the needle left in place for a further 30 s. The rat was then replaced in the home cage, and a

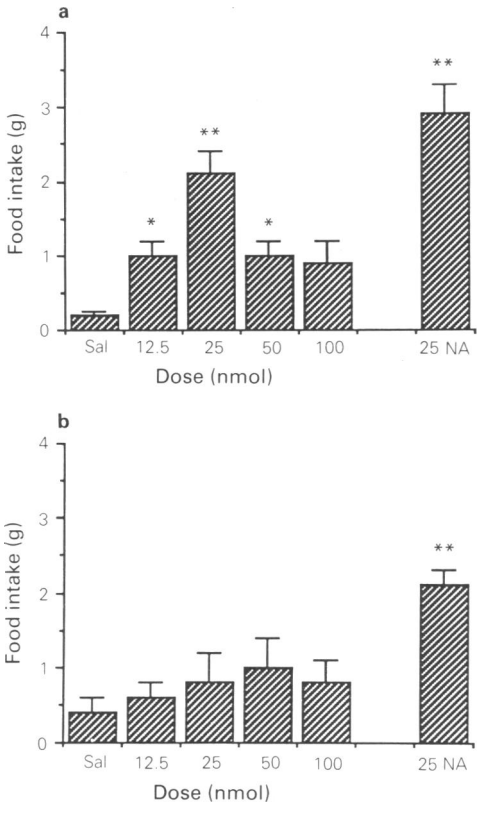


Figure 1 The effects of increasing doses of *m*-octopamine (a) and *p*-octopamine (b) and of 25 nmol noradrenaline (NA) injected into the paraventricular nucleus (PVN) on food intake of rats. Values represent the mean food intakes (g) of 9 rats for *m*-octopamine and 6 animals for *p*-octopamine; vertical bars show s.e.mean. $*P < 0.05$, $**P < 0.01$ compared to saline treatment.

weighed amount of food was placed on the cage floor. Food intakes were determined after 1 h by reweighing the remaining food, plus any spillage collected on paper towels placed beneath the cage floor.

Experiments

The first series of experiments used two separate groups of rats to investigate the effects of (±)-*m*-octopamine [(±)-norphenylephrine HCl, Aldrich] and (±)-*p*-octopamine HCl (Sigma Chemical Co., St. Louis, MO, U.S.A.) on food intake ($n = 9$ and 6 respectively). Within these two groups each animal was tested once with 12.5, 25, 50 and 100 nmol of the appropriate octopamine isomer, and sterile saline. The treatments were administered in a randomised order with at least 3 days between successive tests. In addition, each animal was also tested for its response to a PVN injection of 25 nmol (−)-noradrenaline bitartrate (arterenol, Sigma). Twelve other animals were injected (i.p.) also with 50 mg kg^{-1} pargyline HCl (Sigma). Twenty-four hours later the effects of 25 nmol *p*-octopamine ($n = 6$) and 0.9% saline ($n = 6$) injected into the PVN on food intake were examined. All PVN injections were made in a volume of 0.4 μl.

The second series of experiments examined the impact of a variety of adrenoceptor antagonists injected into the PVN on the feeding response elicited by 25 nmol *m*-octopamine, and 25 nmol noradrenaline. The antagonists used were the α_2-antagonists yohimbine HCl (Sigma) and idazoxan (Reckitt and Colman, Hull), the α_1-antagonist corynanthine HCl (Sigma) and the β-antagonist (±)-propranolol HCl (Sigma). For each antagonist study a separate group of animals was used ($n = 7$–10), and tested under each combination of antagonist dose, and its vehicle, followed by *m*-octopamine and noradrenaline. The order of treatments was randomised, with at least 3 days between successive tests. Idazoxan, corynanthine and propranolol were dissolved in sterile saline. Idazoxan and propranolol were administered in a volume of 0.5 μl, and corynanthine was administered in a volume of 1 μl. Yohimbine was dissolved in 1% sodium bisulphite; the 12.5 nmol dose was injected in a volume of 0.5 μl, and the 25 nmol dose in a volume of 1 μl. All antagonists were injected 30 min before *m*-octopamine or NA (food intakes were determined at the end of this period).

A third series of experiments examined the effects of depleting endogenous NA with α-methyl-*p*-tyrosine (AMPT), and of inhibiting the NA re-uptake system with desipramine, on the feeding response elicited by 25 nmol *m*-octopamine. Eight rats were injected on two separate occasions, spaced 5 days apart, with 25 nmol of *m*-octopamine; on one occasion they also received an injection of 200 nmol AMPT methyl ester (Sigma) into the PVN 4 h previously; on the other occasion they received 1 μl of saline. A further 8 rats were treated likewise except that they were injected with 25 nmol NA rather than *m*-octopamine. For the experiment involving desipramine, 8 rats were tested twice with 12.5 and 25 nmol *m*-octopamine and saline. At each dose the animals were also pretreated 30 min earlier with 100 nmol desipramine HCl or saline (1 μl).

Histology

At the end of each experiment all rats were deeply anaesthetized and injected with 0.5 μl of fast green dye in the PVN. The rats were then perfused with 4% formalin, and the brain removed. Standard histological procedures were used to locate the injection

site. Any animal having an injection site more than 0.3 mm distant from the PVN was excluded from data analysis. In addition any rat eating less than 1.0 g of food following 25 nmol NA was excluded.

Results

The effects of various doses of *m*- and *p*-octopamine injected into the PVN on food intake are shown in Figure 1. One way analysis of variance revealed a significant effect of *m*-octopamine dose [$F(4,32) = 7.7$, $P < 0.001$]. The effect of *m*-octopamine was dose-dependent, with a peak effect occurring at 25 nmol. *p*-Octopamine failed to increase food intake [$F(4,20) = 0.3$, $P > 0.8$]. In both groups of animals 25 nmol NA elicited a strong increase in food intake. When rats were pretreated with 50 mg kg^{-1} pargyline, to retard the oxidative deamination of *p*-octopamine, this isomer of octopamine (25 nmol) elicited a significant mean eating response of 2.9 ($\pm$0.7) g compared to 0.3 ($\pm$0.1) g after saline [$t(13) = 3.4$, $P < 0.01$].

Figure 2 illustrates the effects of pretreatment with 4 adrenoceptor antagonists on the eating responses induced by 25 nmol *m*-octopamine and NA. Separate analyses of variance were used to examine the effects of these antagonists against octopamine and NA. Following significant treatment effects, Tukey's test was used to compare differences between means ($\alpha = 0.05$). In the case of yohimbine, significant treatment effects were observed for both the *m*-octopamine [$F(3,27) = 38.8$, $P < 0.0001$] and noradrenaline [$F(3,27) = 13.4$, $P < 0.001$] conditions. Yohimbine, at both doses strongly attenuated the effect of octopamine, but not the effect of NA. Following idazoxan, significant treatment effects were observed for both octopamine and NA conditions [$F(3,21) = 14.6$ and 35.9, respectively, both $P < 0.001$]. Both doses of idazoxan significantly attenuated the eating responses induced by *m*-octopamine and NA. Analysis of the results obtained with corynanthine and propranolol also revealed significant treatment effects [smallest $F(3,18) = 9.3$, $P < 0.001$]. However, neither of these antagonists significantly attenuated the eating elicited by *m*-octopamine or NA. It is important to note that none of these antagonists was found to alter baseline food intake when injected alone (data not shown; Goldman *et al.*, 1985). However, following treatment with corynanthine, some animals were observed to eat in the 30 min pretreatment interval. This effect has been noted previously (Goldman *et al.*, 1985), though at doses higher than those used here.

Injection of AMPT into the PVN before *m*-octopamine resulted in a significant attenuation of the effect of *m*-octopamine [$t(7) = 2.6$, $P < 0.05$] as

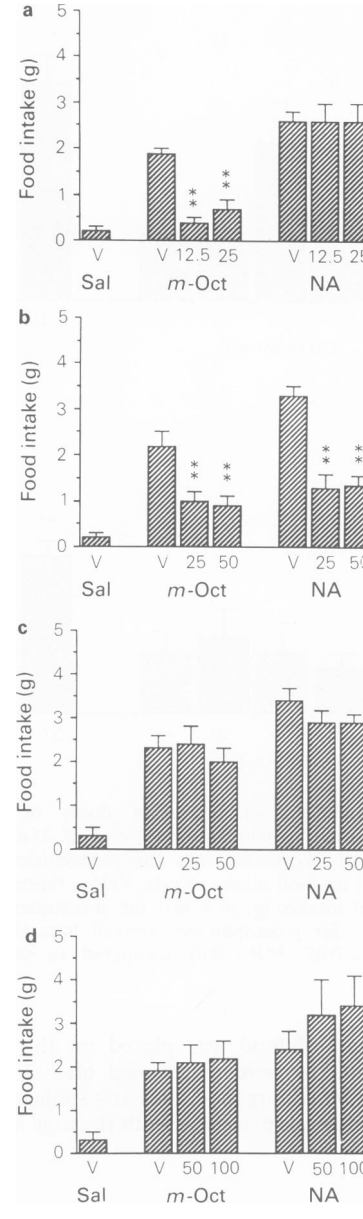


Figure 2 The effects of pretreatment with various doses of adrenoceptor antagonists: (a) Yohimbine, (b) idazoxan, (c) corynanthine, (d) propranolol, and their respective vehicle (V) solutions on the eating responses induced by 25 nmol *m*-octopamine (*m*-Oct), 25 nmol noradrenaline (NA) and saline (Sal): In all experiments the pretreatment interval was 30 min, and all compounds were injected into the paraventricular nucleus. Values represent the mean food intakes (g) of 7–10 rats per group; vertical bars show s.e.mean. $^{**}P < 0.01$ compared to appropriate vehicle (V) pretreated group.

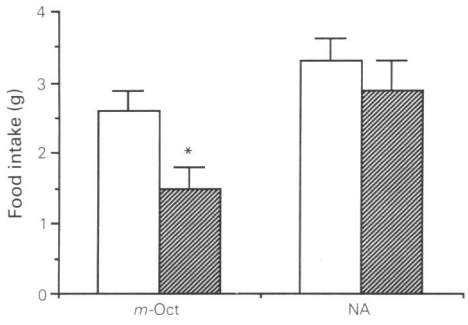


Figure 3 The effect of 200 nmol α-methyl-*p*-tyrosine (hatched columns) or saline (open columns) on the eating response induced by injection into the paraventricular nucleus of 25 nmol *m*-octopamine (*m*-Oct) or 25 nmol noradrenaline (NA). Values represent the mean food intakes (g) of 8 rats in each group; vertical bars show s.e.mean. $*P < 0.05$ compared to saline pretreatment.

shown in Figure 3. However, AMPT did not alter the action of NA [$t(7) = 1.1$, $P > 0.5$].

The effects of 100 nmol desipramine on the eating response induced by 12.5 and 25 nmol *m*-octopamine and saline are shown in Figure 4. Two way analysis of variance (using desipramine pretreatment and *m*-octopamine dose as factors) revealed a significant main effect of *m*-octopamine dose [$F(2,14) = 31.9$, $P < 0.001$], with both doses reliably increasing food intake relative to saline treatment. Neither the main effect of desipramine pretreatment [$F(1,7) = 1.3$, $P > 0.3$] nor the despramine × *m*-octopamine dose

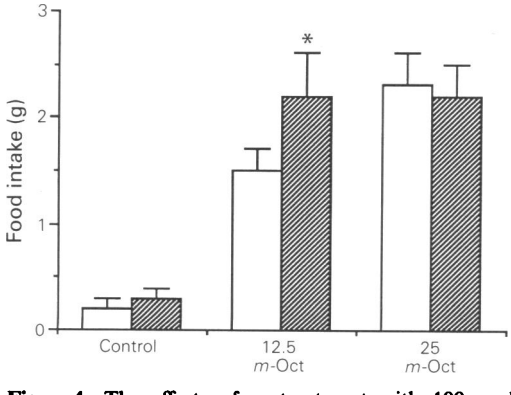


Figure 4 The effects of pretreatment with 100 nmol desipramine (hatched columns) or saline (open columns) injected into the paraventricular nucleus on the eating induced by various doses of *m*-octopamine (*m*-Oct). Values represent the mean food intakes (g) of 8 rats; vertical bars show s.e.mean. $*P < 0.05$ compared to corresponding saline pretreated group.

interaction [$F(2,14) = 2.3$, $P > 0.05$] reached significance. However, multiple comparison tests (Duncan's multiple range, $\alpha = 0.05$) showed that desipramine significantly enhanced the effect of 12.5 nmol *m*-octopamine.

Throughout the course of these experiments several qualitative similarities were observed between the behaviours of animals treated with *m*-octopamine and NA. Although not measured formally, it was observed that eating in response to *m*-octopamine began within several minutes of injection and was often preceded by a bout of drinking. Both of these actions are typical of NA treatment (Leibowitz, 1978).

Discussion

Direct injection of *m*-octopamine into the PVN of satiated rats induced eating. This effect was dose-dependent, with the peak effect occurring at 25 nmol; higher doses resulted in a diminuation of the response. In contrast to the robust effect observed with *m*-octopamine, *p*-octopamine failed to induce eating unless animals were pretreated with the MAOI, pargyline. These results are similar to those reported by Fregly *et al.* (1984) who found that intraventricular administration of *m*- but not *p*-octopamine attenuated angiotensin II-induced drinking. Similarly Booth (1968) found that octopamine (presumably the *para*-isomer) failed to elicit eating when injected close to the PVN. The different potencies of the octopamine isomers may indicate that they act through different mechanisms to affect feeding (see below).

Observation of animals treated with *m*-octopamine showed that eating began within several minutes of injection, and that eating was often preceded by a bout of drinking. Animals injected with NA behave in this way (Leibowitz, 1978), and so it is likely that *m*-octopamine and NA act through the same or a similar mechanism to elicit eating. The results of several antagonist studies support this possibility. The α_1-receptor antagonist corynanthine, and the β-receptor antagonist propranolol failed to attenuate eating induced by *m*-octopamine or NA. Therefore, an action at α_1- and β-adrenoceptors is unlikely. The eating response induced by *m*-octopamine or NA was susceptible to blockade of α_2-adrenoceptors. Idazoxan attenuated the eating induced by *m*-octopamine and NA; yohimbine attenuated the action of *m*-octopamine, but not of NA. This failure of yohimbine to reverse NA-induced eating is surprising in view of the previous report that 12.5 nmol of the antagonist reduced the eating induced by 40 nmol NA by 50% (Goldman *et*

al., 1985). These discrepant results may relate to procedural differences such as the use of a more palatable diet, yielding higher food intakes, by Goldman *et al.* (1985). However, the positive results obtained with idazoxan support the contention that NA acts via α_2-receptors in the PVN to induce eating. The finding that *m*-octopamine acts via α_2-receptors is similar to the finding that the attenuation of angiotensin II-induced drinking is reversed by yohimbine (Fregly *et al.*, 1984).

The results of these antagonist studies do not provide any insight into the mode of action of *m*-octopamine at α_2-receptors. It is possible that *m*-octopamine acts directly to stimulate these receptors or it could act indirectly to release endogenous NA which then activates α_2-receptors. The ability of *m*-octopamine to induce eating was reduced in animals which had previously received an injection of AMPT into the PVN. Since AMPT inhibits tyrosine hydroxylase activity, thus depleting endogenous NA stores, these results suggest that *m*-octopamine acts to release endogenous NA. However, since the reversal of *m*-octopamine-induced eating was incomplete, and since the extent of the NA depletion in the PVN was not determined, it is not possible to conclude that the NA releasing action of *m*-octopamine accounts wholly for the behavioural response. The finding that *m*-octopamine releases NA following injection into the PVN is consistent with the observation that *m*-octopamine has an indirect action in rat isolated atria (Celuch & Juorio, 1987). This study found also that *p*-octopamine did not have an indirect action when administered in the absence of a MAOI. If this situation occurs in the PVN, then the lack of indirect action of *p*-octopamine may explain the failure of *p*-octopamine to elict eating, when administered in the absence of pargyline. In turn this would indicate that the NA releasing action of *m*-octopamine is more important than direct receptor activation in mediating the eating response. However, this dual action of *m*-octopamine may explain the inverted U shape of the dose-response curve obtained for *m*-octopamine (Figure 1). At low doses the NA-releasing action of *m*-octopamine may predominate, resulting in eating at doses up to 25 nmol. Above this dose the weak agonist action of *m*-octopamine at α_2-adrenoceptors may be sufficient to prevent the activation of these receptors by the endogenously released NA.

The eating induced by 12.5 nmol *m*-octopamine was enhanced by pretreatment with desipramine. Previously despiramine has been reported to enhance the effect of a low dose of NA injected close to the PVN (Slangen & Miller, 1969). Presumably this occurs because desipramine inhibits NA uptake, thereby prolonging the synaptic action of the exogenously applied NA. Since *m*-octopamine induces eating, by releasing endogenous NA, the potentiating effect of desipramine probably occurs in a similar fashion by prolonging the synaptic action of this endogenous NA. However, desipramine did not potentiate the effect of 25 nmol NA. Since desipramine also inhibits octopamine uptake (Baldessarini & Vogt, 1971), this would have the effect of retarding the oxidative deamination of *m*-octopamine injected into the PVN. This would enhance the weak, direct action of *m*-octopamine at α_2-adrenoceptors, thereby masking any potentiation of the effects of endogenous NA by desipramine.

It has been suggested that the comparatively low activities of octopamine isomers at adrenoceptors militates against a direct physiological role for octopamine at these sites (Williams *et al.*, 1987). The present study does not address directly the issue of the physiological role of octopamine in the central nervous system. This is primarily because the octopamine doses were high in comparison to the endogenous level of octopamine; it should be noted, however, that the doses employed were relatively low in comparison to the doses of amines used in many other behavioural studies. However, the finding that octopamine elicits a marked and reliable behavioural response which is mediated via an interaction with the noradrenergic system supports the hypothesis that octopamine may serve as a modulator of NA activity.

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Secretion of acetylcholinesterase and butyrylcholinesterase from the guinea-pig isolated ileum

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1 Strips of longitudinal muscle from guinea-pig ileum, retaining Auerbach's plexus, were superfused with oxygenated Krebs solution. Addition of 50 mM KCl led to a pronounced Ca^{2+}-dependent increase in the activities of both acetylcholinesterase and non-specific cholinesterase (butyrylcholinesterase) in the perfusate but with no change in lactate dehydrogenase activity.

2 No release of acetylcholinesterase, either spontaneous or K^+-evoked was observed in tissue freed of the nerve plexus, although release of butyrylcholinesterase still occurred.

3 Carbachol induced a marked Ca^{2+}-dependent increase in the release of acetylcholinesterase but had no effect on the release of butyrylcholinesterase or lactate dehydrogenase. This carbachol-evoked increase in acetylcholinesterase release was blocked by hexamethonium but not by atropine.

4 Four readily soluble molecular forms of acetylcholinesterase and three soluble molecular forms of butyrylcholinesterase were present in innervated longitudinal muscle strips, but insignificant amounts of acetylcholinesterase were detected in denervated strips of muscle. Only one of the four molecular forms of acetylcholinesterase was recovered in the perfusates.

5 It is concluded that acetylcholinesterase is secreted from the nerves of Auerbach's plexus in response to depolarizing stimuli or to nicotinic cholinergic stimulation, while butyrylcholinesterase is secreted from non-neural elements, possibly the longitudinal muscle cells, of guinea-pig ileum in response to a depolarizing stimulus.

Introduction

Neurones within the central nervous system can release acetylcholinesterase (EC 3.1.1.7; AChE) upon stimulation (Chubb *et al.*, 1976; Greenfield & Smith 1979; Greenfield, 1984; Vogt *et al.*, 1984; Appleyard *et al.*, 1988; Appleyard & Smith, 1987a). The release of AChE has also been demonstrated in the periphery, e.g. from the adrenal medulla (Chubb & Smith, 1975b), the phrenic nerve-diaphragm preparation (Skau & Brimijoin, 1978), sympathetic ganglia (Gisiger & Vigny, 1977), fibroblast cells (Bartos & Glinos, 1976) and platelets (Chuang *et al.*, 1976).

The gastro-intestinal tract is very rich in AChE (Koelle, 1953) and certain clinical observations have raised the possibility that both AChE and non-specific cholinesterase (EC 3.1.1.8, butyrylcholinesterase, BuChE) might be released from the gastro-intestinal tract. Thus several groups who have examined the cholinesterase content of amniotic fluid have detected the presence of AChE (Wald *et al.*, 1980; Dale, 1980; Voigtlander *et al.*, 1981; Garry *et al.*, 1981; Crandall *et al.*, 1982; Read *et al.*, 1982) in cases of foetal exomphalos. It has been suggested by Wald *et al.* (1980) that the AChE is derived from peripheral nervous tissue such as the intestinal nerve plexi which are exposed to the amniotic fluid in this condition. Indeed soluble AChE has been found in rectal biopsy material from children with Hirschsprung's disease (Dale *et al.*, 1979). Secretion of BuChE from intestinal tissue in cases of exomphalos is also suggested, both by the occasional presence of additional electrophoretic bands of BuChE (Wald *et al.*, 1980) and by the higher ratio of BuChE to AChE activities in amniotic fluid in cases of exomphalos compared to cases with neural tube defects, in the absence of any visible blood contamination (Goldfine *et al.*, 1983; Wald *et al.*, 1984; Burton, 1986).

In the present study we have examined whether AChE and BuChE can be secreted from an intestinal tissue, namely the longitudinal muscle layer of the

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guinea-pig small intestine, that can be isolated either with, or without, its associated myenteric nerve plexus (Ambache & Freeman 1968; Paton & Abou Zar, 1968). In this tissue the bulk of AChE present is confined to the nerve plexus, whereas the BuChE is confined to the muscle (Ambache *et al.*, 1971). A preliminary account of these results has been given (Appleyard & Smith, 1987b).

Methods

Tissue preparation

Albino guinea-pigs (200–300 g) of either sex were stunned by a blow on the head, then decapitated. The small intestine was removed, about 8–12 cm of the terminal ileum being discarded, and placed in warmed oxygenated Krebs solution containing (mM): NaCl 118, KCl 4.7, $MgSO_4$ 1.14, KH_2PO_4 1.18, $NaHCO_3$ 25, $CaCl_2$ 2.52 and glucose 11.1; gassed with 95% O_2, 5% CO_2. Longitudinal muscle strips were prepared by the method of Rang (1964), and Paton & Aboo Zar (1968), with slight modifications to minimize damage to the plexus. A piece of ileum, approximately 10 cm long, was stretched over a glass pipette of 7 mm diameter and the mesentery was cut away. The longitudinal muscle layer was separated from the underlying circular muscle by stroking tangentially away, in both directions, from the mesenteric attachment at one end of the strip with a small piece of cotton wool, followed by gentle traction to strip it from the whole length. The whole procedure was carried out under warmed oxygenated Krebs solution to minimize the damage to the plexus which occurred if the preparation were not kept moist. This method of preparation yielded strips retaining Auerbach's plexus.

To obtain plexus-free strips, a longer strip of longitudinal muscle was pulled off, the ileum being pulled upwards on the supporting glass pipette by applying gentle traction at the proximal end. The plexus-retaining proximal portions could be identified by their striated appearance under oblique light, while the distal plexus-free strips had a smooth appearance.

In vitro *superfusion*

Sections of longitudinal muscle, weighing approximately 150 mg were superfused with oxygenated Krebs solution at 37°C in 0.4 ml chamber at a rate of 0.25 ml min^{-1}. After an initial washout period of 10 min, fractions of 0.5 ml were collected every two minutes into vials containing 25 μg bovine serum albumin to preserve the enzymes.

K^+-evoked release was studied by replacing an equivalent amount of NaCl by 50 mM KCl in the perfusing medium for 4 min, 10 min after starting fraction collection. The effects of K^+ stimulation upon plexus-free strips were also studied.

In a further series of experiments carbachol (0.01 mM) was added to the perfusion fluid for 4 min, 10 min after starting the collection of fractions. The effects of the cholinoceptor antagonists hexamethonium bromide (1×10^{-3} M) and atropine sulphate (0.5×10^{-6} M) upon carbachol stimulation were also studied, these drugs being present in the perfusion fluid from the start of the superfusion.

All experiments were repeated in Ca^{2+}-free medium containing 1.0 mM EDTA.

Biochemical analysis

All perfusates were assayed for AChE, BuChE and lactate dehydrogenase (LDH) activities. Acetylcholinesterase and BuChE were assayed by a modified version (Chubb & Smith, 1975a) of the Ellman method (Ellman *et al.*, 1961) using 1 mM acetylthiocholine as substrate at pH 7.0 (30°C); the two activities were distinguished (Silver, 1974) by use of the specific AChE inhibitor BW284c51 (1,5-*bis*-(4 allyldimethylammonium phenyl)-pentone-3-one dibromide 1.5×10^{-6} M) and the butyrylcholinesterase inhibitor ethopropazine (10^{-5} M). It should be noted that the activities given for butyrylcholinesterase are submaximal since the substrate was acetylthiocholine, not butyrylthiocholine, and the substrate concentration was below the value that gives V_{max}; however, the substrate concentration was optimal for AChE. Lactate dehydrogenase (LDH) was assayed fluorometrically (Brooks & Olken, 1965).

At the end of the perfusion the tissue samples and the contents of the chamber were homogenized in 1.0 ml of 50 mM phosphate buffer pH 7.0 at 0°C. Samples were centrifuged (1,000 *g* for 10 min) to remove particulate matter and then assayed for choline acetyltransferase (Fonnum, 1975) and AChE activity. Aliquots of homogenate were further centrifuged (at 100,000 *g* for 1 h) to sediment the membranes; the supernatant was then subjected to polyacrylamide gel electrophoresis (Chubb & Smith, 1975a). The perfusates from a number of identical experiments, for both the pre-stimulation and the post-stimulation fractions, were combined and concentrated by vacuum dialysis against 0.39 M Tris/glycine buffer (pH 8.1) and were then analysed by polyacrylamide gel electrophoresis. The gels were incubated with acetylthiocholine to reveal bands of cholinesterase activity (Chubb & Smith, 1975a) using BW 284c51 to distinguish between AChE and BuChE.

Analysis of results

Graphical representation of results To minimize the effect of experimental variation in the resting levels of enzymes in the perfusates, the results of each experiment were standardised for graphical presentation. Basal levels of release for each experiment were determined by calculating the mean value of the activities of the pre-stimulation fractions for the time period during which the release had reached a constant level. The enzyme activity in each sample for a particular experiment was expressed as a percentage of this mean basal release, which was taken as 100%. To obtain combined results for each group of experimental animals the mean ± s.e.mean of these percentages was then calculated for each sample.

Statistical procedures Statistical analysis of the raw data was performed by the use of a one-tailed permutation test (Lindgren, 1976), which has the effect of comparing the mean of the enzyme activity in the last three pre-stimulation fractions with the mean of that in the first three post-stimulation fractions.

For each piece of tissue, the difference (mean fraction after minus mean fraction before) was calculated for the 20 different ways of choosing three fractions from the six as 'before' and three fractions from the six as 'after'. These twenty values were then ranked and thus a rank was assigned to the actual experimental result. A statistic could then be formed by summing the ranks for each piece of tissue. The distribution of this statistic could then be calculated under the null hypothesis that the six fractions are all the same value against the alternative hypothesis that the values for the three fractions after stimulation are larger than the values for the three fractions before stimulation. The result was considered to be significant if it fell within the lower 5% of the distribution, since lower values are more likely under the alternative hypothesis.

Correlations between the activities of AChE and BuChE in perfusates and the tissue contents of these enzymes and choline acetyltransferase were analysed using the Spearman-Rank correlation test.

Results

Basal release of AChE, BuChE and LDH and the effect of depolarizing concentrations of K^+

There was a rapid washout of all three enzymes over the first six minutes of the perfusion; ten minutes after the fraction collection began all enzyme levels were low and relatively constant (Figure 1)

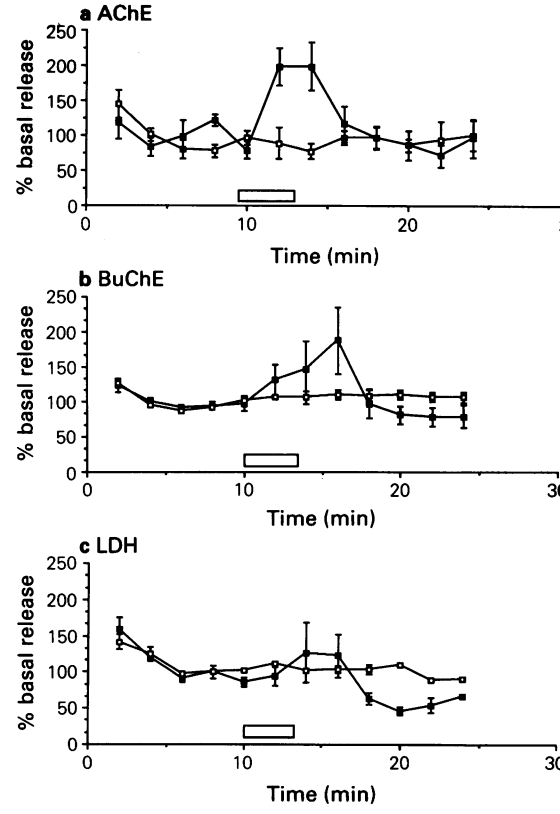


Figure 1 Activities of (a) acetylcholinesterase (AChE), (b) butyrylcholinesterase (BuChE) and (c) lactate dehydrogenase (LDH) in superfusates of Auerbach's plexus showing both the spontaneous release and the secretion of the cholinesterases evoked by depolarizing K^+ stimulation. (□) Unstimulated controls, $n = 4$. (■) Stimulated with 50 mM K^+ during period denoted by the bar, $n = 7$. Activities are expressed as a percentage of the mean basal release in each case. Each point represents the mean and vertical lines show s.e.mean.

with the following basal activities ($n = 11$): AChE = 1.99 ± 0.88, BuChE = 14.1 ± 4.7, LDH = 59.1 ± 3.5 mu mg^{-1} tissue ml^{-1} perfusion fluid. The hydrolysis of acetylthiocholine that was insensitive to BW284c51 was inhibited by ethopropazine (10^{-5} M), thus classifying the enzyme as butyrylcholinesterase (Silver, 1974). The basal levels of AChE and BuChE in perfusates were both significantly correlated with the tissue contents of AChE ($P < 0.013$) and BuChE ($P < 0.007$), respectively, but there was no correlation between basal LDH and the tissue content of LDH.

Introduction of 50 mM KCl into the perfusion fluid, ten minutes after starting superfusion, led to a

Table 1 Effect of depolarising concentration (50 mM) of K^+ upon the release of acetylcholinesterase (AChE), butyrylcholinesterase (BuChE) and lactate dehydrogenase (LDH) from Auerbach's plexus when Ca^{2+} was omitted from the perfusion medium

Enzyme	*Pre-stimulation*	*Post-stimulation*
AChE	95.9 ± 19.0	95.8 ± 13.2
BuChE	102.4 ± 6.6	140.9 ± 18.5
LDH	89.4 ± 5.2	128.2 ± 21.5

All activities are expressed as a percentage of the mean basal release. Pre-stimulation values are the mean ± s.e.mean of the three fractions immediately preceding stimulation, post-stimulation values are the mean ± s.e.mean of the three fractions immediately following stimulation, $n = 4$. None of the post-stimulation values was significantly different from the corresponding pre-stimulation value.

rapid and statistically significant ($P = 0.001$) increase in the AChE activity in the perfusate, the levels returning to normal upon restoration of normal K^+ concentrations (Figure 1). A less marked (but statistically significant with $P < 0.005$) rise in BuChE activity of slower onset also occurred in response to depolarising concentrations of K^+, but there was no change in the LDH activity of the perfusates (Figure 1). These K^+-evoked increases in AChE and BuChE activities were Ca^{2+}-dependent since there were no significant increases of AChE and BuChE activities in response to 50 mM K^+ in calcium-free perfusates (Table 1).

When plexus-free tissue was used no release of AChE, either basal or K^+-evoked, could be detected. However, BuChE activities in the perfusates, both basal and K^+-evoked, were comparable to those of plexus-retaining tissue, as were levels of LDH (data not shown). Classification of the tissue as denervated was shown to be correct by the absence of significant choline acetyltransferase activity in the tissue homogenates. AChE activity was also negligible in the same tissue homogenates compared to the high activities detected in plexus-retaining tissue.

Effect of carbachol on the release of AChE, BuChE and LDH

Introduction of 0.01 mM carbachol into the perfusion medium led to a significant ($P = 0.002$) increase in AChE release that was rapid in onset. However, there was no significant accompanying change in BuChE activity or LDH activity in the perfusates (Figure 2). No increase in the release of AChE was observed in response to carbachol stimulation when

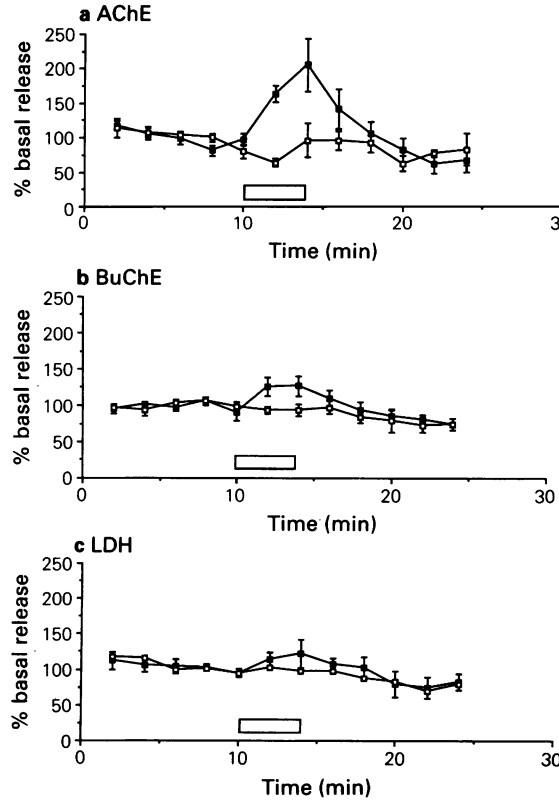


Figure 2 Effect of carbachol (0.01 mM) stimulation (bar) upon the release of (a) acetylcholinesterase (AChE), (b) butyrylcholinesterase (BuChE) and (c) lactate dehydrogenase (LDH) into superfusates of Auerbach's plexus in medium containing 2.52 mM Ca^{2+} (■) ($n = 8$) and with no added calcium salts (□) ($n = 5$). Activities are expressed as a percentage of the mean basal release in each case. Each point represents the mean and vertical lines show s.e.mean.

Ca^{2+} was omitted from the perfusion fluid (Figure 2). The carbachol-evoked release of AChE was not blocked by the addition of 0.005 mM atropine to the perfusion fluid (Figure 3); again release was found to be Ca^{2+}-dependent, and there was no accompanying change in BuChE and LDH activities of the perfusates (Table 2). The addition of hexamethonium (1 mM) blocked the carbachol-evoked release of AChE (Figure 3).

The tissue samples were analysed for their choline acetyltransferase activity, as a measure of the extent of the cholinergic innervation. There was significant correlation (Spearman-Rank, $P < 0.05$) between the choline acetyltransferase activity of the tissue and the maximum amount of AChE released by the tissues in response to stimulation with either K^+ or carbachol (Figure 4).

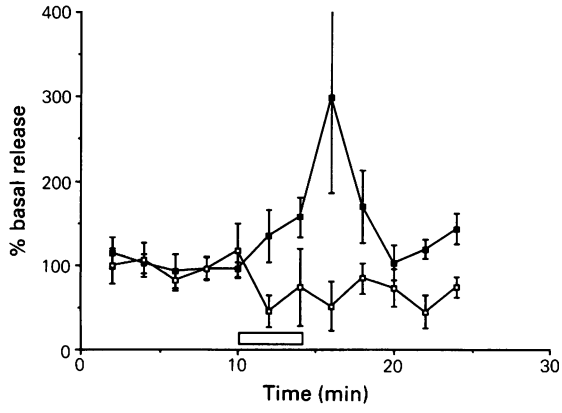


Figure 3 Effect of the cholinoceptor antagonists atropine (■) ($n = 4$) and hexamethonium (□) ($n = 4$) on the carbachol-evoked (bar) release of acetylcholinesterase (AChE) into superfusates of Auerbach's plexus. Activities are expressed as a percentage of the mean basal release. Each point represents the mean and vertical lines show s.e.mean.

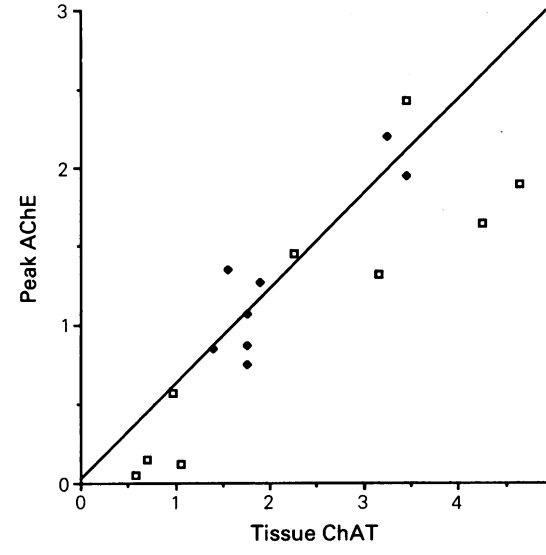


Figure 4 Correlation between peak levels of acetylcholinesterase (AChE) in the perfusate and the tissue content of choline acetyltransferase (ChAT) in superfused strips of Auerbach's plexus stimulated with a depolarizing concentration of K^+ (□) or 0.01 mM carbachol (◆).

Isoenzymes of AChE in tissue and perfusates

Electrophoretic analysis of the soluble fraction of homogenates of plexus-retaining longitudinal muscle strips revealed five bands of cholinesterase activity. Two of these bands disappeared when the gels were

Table 2 Effect of various stimulating agents upon the release of butyrylcholinesterase (BuChE) and lactate dehydrogenase (LDH) from Auerbach's plexus

	BuChE	
Stimulation	*Pre-stimulation*	*Post-stimulation*
Carbachol 0.01 mM + Hexamethonium 1 mM ($n = 4$)	94.0 ± 6.4	118.9 ± 21.6
Carbachol 0.01 mM + Atropine 500 nM ($n = 4$)	102.0 ± 4.1	95.6 ± 4.0
	LDH	
Stimulation	*Pre-stimulation*	*Post-stimulation*
Carbachol 0.01 mM + Hexamethonium 1 mM ($n = 4$)	96.9 ± 6.2	124.3 ± 17.1
Carbachol 0.01 mM + Atropine 500 nM ($n = 4$)	97.1 ± 1.3	92.6 ± 11.7

All activities are expressed as a percentage of the mean basal release. Pre-stimulation values are the mean ± s.e.mean of two fractions immediately preceding stimulation, post-stimulation values are the mean ± s.e.mean of the three fractions immediately following stimulation.
None of the post-stimulation values was significantly different from the corresponding pre-stimulation values.

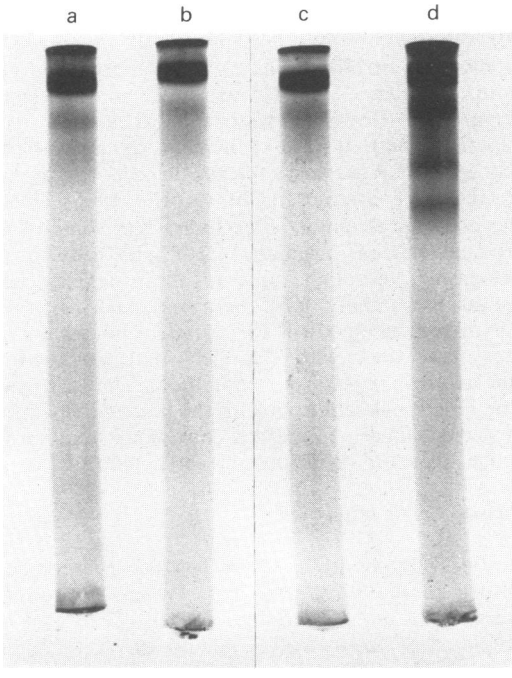


Figure 5 Polyacrylamide gels of high-speed supernatants of tissue homogenates of innervated and denervated strips of guinea-pig longitudinal muscle stained to reveal cholinesterase activity. The samples were applied at the top and the direction of migration was towards the anode. Gels (a) and (b) are from the homogenates of longitudinal muscle with nerve plexus removed whilst gels (c) and (d) are from homogenates of longitudinal muscle with the plexus intact. Gels (a) and (c) were stained in the presence of the acetylcholinesterase inhibitor BW284c51.

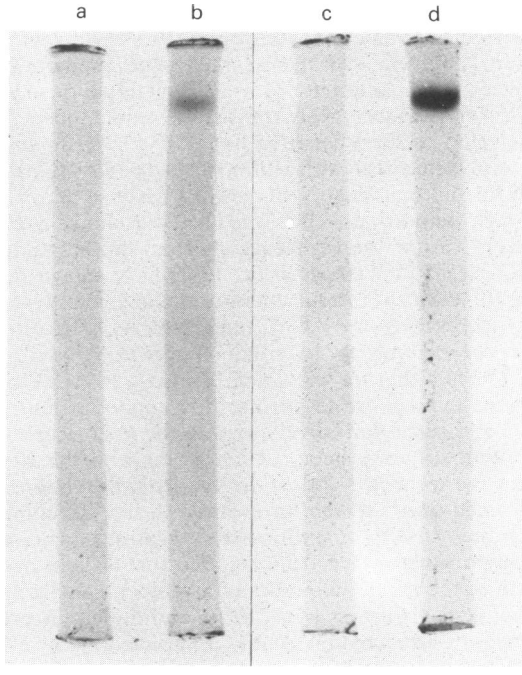


Figure 6 Polyacrylamide gels of superfusates of Auerbach's plexus before (a, b) and after (c, d) carbachol stimulation, stained to reveal cholinesterase activity. The samples were applied at the top and the direction of migration was towards the anode. Gels (a) and (c) were incubated in the presence of the acetylcholinesterase inhibitor BW284c51, whilst gels (b) and (d) were incubated in the absence of inhibitor.

incubated in the presence of the acetylcholinesterase inhibitor BW284c51 and two were reduced in intensity of staining, suggesting that the tissue contains up to four molecular forms of soluble AChE and three of BuChE (Figure 5). No isoenzymes of AChE could be detected in homogenates of denervated tissue. BuChE was present in both preparations: since there was no noticeable difference in the intensity of staining in the presence of BW284c51 for both innervated and denervated tissue, BuChE appears to be confined to the non-nervous elements of the tissue.

Electrophoretic analysis of the perfusates for AChE revealed that the AChE activity, both basal and carbachol-evoked, was attributable to a single molecular form (Figure 6).

Discussion

The results indicate that both AChE and BuChE are secreted from the guinea-pig ileum. Secretion of AChE could be evoked in a Ca^{2+}-dependent manner by both depolarizing concentrations of K^+ and the cholinoceptor agonist carbachol. In neither case was the increase in activity due to non-specific tissue damage since levels of the soluble cytoplasmic marker LDH remained unchanged. Furthermore, only one of the four soluble isoenzymes of AChE present in Auerbach's plexus was present in the perfusate. This is analogous to the situation in the adrenal medulla (Chubb & Smith, 1975b) and brain (Chubb *et al.*, 1976; Greenfield & Shaw, 1982) where only one of several soluble isoenzymes present in the tissue is secreted.

Origin of the secreted cholinesterases

The secreted AChE would appear to be of neuronal origin, rather than deriving from the longitudinal muscle, since no AChE release, either spontaneous or evoked, could be demonstrated from tissue from which the nerve plexus had been removed. Indeed, denervated tissue was found to contain negligible

AChE activity; the small amount present can be attributed to the presence of nervous elements which were not removed during the tissue preparation, since small amounts of the cholinergic neurone marker choline acetyltransferase could also be detected in the denervated tissue. The latter findings are in agreement with earlier histochemical (Koelle, 1955) and manometric studies (Ambache *et al.*, 1971) which found that AChE was localised almost exclusively in the nerve plexus. Histochemical studies (Koelle, 1955; Garrett *et al.*, 1972) have shown that neurones of the intermyenteric ganglion stain weakly to moderately for AChE, and that AChE-positive nerves innervate the longitudinal muscle.

The fact that the maximum amounts of AChE released in response to depolarizing concentrations of K^+ and carbachol correlated with the tissue levels of choline acetyltransferase is consistent with the view that the secreted AChE is derived from the cholinergic neurones of the myenteric plexus. Immunostaining with antibodies against choline acetyltransferase has demonstrated that at least one-fifth of the nerve cell bodies of myenteric ganglia are cholinergic (Furness *et al.*, 1983), and these cells presumably also contain AChE. Pharmacological evidence demonstrates two types of intrinsic cholinergic neurone within the enteric nervous system (Furness & Costa, 1980): those which act upon the smooth muscle (Bennett & Burnstock, 1968) and cholinergic interneurones with inputs onto neurones of the myenteric plexus (Hirst *et al.*, 1974). In addition, there are extrinsic cholinergic inputs to the enteric nervous system, arising from the bulbar and sacral outflow of the central nervous system (Furness & Costa, 1980). The secreted AChE could derive from the processes and axons of any of these cholinergic nerves. In addition AChE is present in non-cholinergic peripheral neurones (Silver, 1974) and these could contribute to the secreted AChE.

The carbachol-evoked secretion of AChE would appear to involve nicotinic receptors since it is blocked by hexamethonium but not by atropine. Extracellular and intracellular recording studies have demonstrated a population of neurones in the myenteric plexus which have an excitatory nicotinic synaptic input (North, 1982). It is possible that these include AChE-staining cells; AChE could therefore be secreted from these cells in response to stimulation of the cholinergic input.

Most, if not all, of the secreted butyrylcholinesterase seems to derive from non-nervous tissue, such as the longitudinal muscle, rather than from the nerve plexus, since removal of the nerve plexus did not greatly affect either the spontaneous or the K^+-evoked secretion of BuChE. Biochemical analysis has demonstrated that the bulk of BuChE in the guinea-pig remains after denervation of the longitudinal muscle (Ambache *et al.*, 1971); this agrees with our observation that no appreciable difference in intensity of BuChE stains could be detected in gels from innervated and denervated muscle strips (Figure 5). However, histochemical studies have demonstrated that most of the neurones and interstitial cells of Auerbach's plexus in cat, rabbit and monkey also contain BuChE (Koelle, 1955). Hence, the possibility cannot be excluded that some of the secreted BuChE originates from glial cells in the guinea-pig, although most of these satellite cells would presumably have been removed when the plexus was stripped off the muscle. Our results are most consistent with the view that the secreted BuChE originates from longitudinal muscle cells. The functional significance of the secreted BuChE is at present unclear; indeed a convincing function for BuChE is yet to be proposed (Kutty, 1980).

Possible implications

Acetylcholinesterase secretion has also been demonstrated in the substantia nigra, where it produces behavioural and electrophysiological effects that are unrelated to its hydrolytic action on acetylcholine (Greenfield, 1984). For example, application of AChE decreases the firing rate of pars compacta neurones (Greenfield *et al.*, 1981). Therefore secreted AChE might possibly have similar non-cholinergic actions within the gut. Consistent with such a notion is the finding by Ambache *et al.* (1986) of a proteinaceous component derived from Auerbach's plexus that will contract longitudinal muscle from the guinea-pig ileum.

A function of secreted AChE within the gut is also suggested by the fact that parasitic intestinal nematodes secrete large amounts of AChE but only during their gut-dwelling life-stages (Philip, 1984). It has been proposed that AChE secretion has survival value for the parasites; AChE may act as a biochemical 'holdfast' by inhibiting locally the peristaltic movements of the gut during the expulsion process (Ogilvie & Jones, 1971).

The secretion of acetylcholinesterase from the gut could have implications in disease states such as Hirschsprung's disease. Many studies have shown markedly elevated levels of soluble AChE within aganglionic rectal mucosal tissue of patients with this disease (Dale *et al.*, 1979; Bonham *et al.*, 1985); secretion of AChE could therefore be affected and contribute to the spasticity of the affected region. There have also been accounts of abnormal forms of AChE in rectal biopsies from patients with Hirschsprung's disease (Bajgar & Hak, 1979).

Finally our observations may account for certain findings on human amniotic fluid in cases where the foetus is abnormal. Secretion of AChE from the

exposed gastrointestinal tissue could account for its presence in the amniotic fluid in cases of exomphalos (Wald *et al.*, 1980; Dale, 1980; Voigtlander *et al.*, 1981; Garry *et al.*, 1981; Crandall *et al.*, 1982; Read *et al.*, 1982). Secretion of BuChE, in addition, would explain why the ratio of BuChE to AChE in amniotic fluid is higher in cases of foetal exomphalos (Goldfine *et al.*, 1983; Wald *et al.*, 1984), compared to cases of foetal neural tube defects where the main change is a greatly increased amount of AChE derived from cerebrospinal fluid (Smith *et al.*, 1979).

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Identification of M_1 muscarinic receptors in pulmonary sympathetic nerves in the guinea-pig by use of pirenzepine

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1 The effect of pirenzepine, a muscarinic antagonist considered to be selective for M_1 receptors, was studied on bronchoconstriction and bradycardia elicited by preganglionic stimulation of the parasympathetic vagal nerves and by i.v. injections of acetylcholine (ACh) in anaesthetized guinea-pigs.

2 Pirenzepine was equipotent in the heart and lung as an antagonist of the effects of i.v. ACh at postjunctional muscarinic receptors. Doses of pirenzepine in excess of $1\,\mu mol\,kg^{-1}$ abolished all muscarinic responses consistent with non-selective blockade of M_3 receptors on airway smooth muscle and M_2 receptors on atrial cells.

3 In the lung, low doses of pirenzepine ($1–100\,nmol\,kg^{-1}$) increased vagally-induced bronchoconstriction despite concurrent partial blockade of the postjunctional receptors. This suggests blockade of neuronal muscarinic receptors.

4 Propranolol ($1\,mg\,kg^{-1}$) increased control bronchoconstrictor responses elicited by ACh and vagal stimulation but did not alter the potency of pirenzepine for postjunctional receptors in heart or lung. However, pirenzepine-induced enhancement of vagally-induced bronchoconstriction was abolished by propranolol, suggesting that pirenzepine may be an antagonist for muscarinic receptors located in the sympathetic nerves innervating airway smooth muscle.

5 These results confirm that bronchoconstrictor stimuli indirectly initiate activation of an opposing sympathetic reflex in the guinea-pig lung. This response is facilitated by muscarinic receptors located in the sympathetic nervous pathway.

6 The high potency of pirenzepine for the neuronal receptors in the sympathetic nerves suggests that these are M_1 receptors. In contrast, the parasympathetic nerves innervating airway smooth muscle in this species contain M_2 receptors which inhibit neurotransmission.

Introduction

In the lung, the dominant nervous control of airway diameter is exerted by parasympathetic nerves which release acetylcholine (ACh) onto muscarinic receptors on the airway smooth muscle cell. Muscarinic receptors also exist on the post-ganglionic terminals of these cholinergic nerves. These neuronal receptors inhibit transmitter release (Fryer & Maclagan, 1984; 1987a; Faulkner *et al.*, 1986) and consequently their blockade with muscarinic antagonists increases vagally-induced bronchoconstriction. This may be a potential hazard when muscarinic antagonists such as ipratropium are used clinically as bronchodilators (Fryer & Maclagan, 1987b). As muscarinic antagonists affect both prejunctional and postjunctional muscarinic receptors, the degree of blockade of vagally-induced bronchoconstriction produced by these drugs is dependent on the balance between their pre- and postjunctional blocking activity. Thus, it is important to establish whether the neuronal muscarinic receptors belong to the same or to a different receptor subtype from the receptors on the smooth muscle cell.

Muscarinic receptors have been classified into three types on the basis of ligand binding and functional studies by use of selective antagonists. M_1 receptors exhibit a high affinity for pirenzepine and are located mainly in neural tissue (Hammer, 1982),

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whereas the muscarinic receptors located on nerves, glands, heart and smooth muscle exhibit a low affinity for pirenzepine. These have been further subdivided by the use of gallamine which is selective for the M_2 receptors in the heart (Riker & Wescoe, 1951) and by dicyclomine and 4-diphenylacetoxy-N-methyl piperidine (4-DAMP) which displays high affinity for the M_3 receptors on ileal smooth muscle and glands (de Jonge *et al.*, 1986; Birdsall & Hulme, 1984). The latter subtype includes the postjunctional receptors on airway smooth muscle (Barlow *et al.*, 1972).

The high potency of gallamine for the pulmonary neuronal receptors in the guinea-pig and cat (Fryer & Maclagan, 1987a; Blaber *et al.*, 1985) suggests that these receptors may resemble the cardiac M_2 muscarinic receptors. However, the effect of pirenzepine has not been tested in the pulmonary autonomic nerves in these species. The present experiments were carried out to examine the effect of pirenzepine in the guinea-pig in an attempt to clarify the subtype of muscarinic receptor found in the pulmonary autonomic nerves.

Methods

Guinea-pigs (250–300 g, either sex) of the Dunkin Hartley strain were anaesthetized with urethane ($1.5\,g\,kg^{-1}$, i.p.). The right carotid artery was cannulated for the measurement of blood pressure and heart rate was derived electronically from the blood pressure signal. A cannula was placed in the left jugular vein for the injection of drugs. Both vagi were cut and the distal ends placed on shielded platinum electrodes immersed in a pool of liquid paraffin. Each animal's body temperature was maintained at 37°C by use of a CFP 8185 homeothermic blanket control.

The anaesthetized animals were paralysed with suxamethonium chloride ($100\,\mu g\,kg^{-1}$) injected every 10 min and respired with positive pressure, constant volume ventilation from a Harvard Rodent respirator. Airflow ($\dot{V}$) and tidal volume (Vt) were recorded as described by Maclagan & Ney (1979). Pulmonary inflation pressure (Ppi) was measured with a Statham pressure transducer (PM5E): one port of the transducer was connected to a side arm of the tracheal cannula and the other port was open to the atmosphere. All signals were displayed on a Gould Recorder and arterial blood samples were taken at regular intervals and analysed for O_2 and CO_2 tension and pH with a Corning 166 microsample blood gas analyser. The tracheal cannula used had a resistance of $40–60\,cmH_20.1^{-1}\,s^{-1}$, so a positive pressure of approximately $80–100\,mmH_2O$ was needed at each stroke of the pump for adequate ventilation of the animal.

Bronchoconstriction elicited by i.v. ACh or by vagal nerve stimulation was recorded as described by Fryer & Maclagan (1984) as an increase in Ppi over the basal insufflation pressure change produced by the pump. The sensitivity of the method was increased by using a computerized device to subtract the basal tracheal insufflation pressure. The 'subtractor' was re-set before each bronchoconstrictor stimulus and the increase in Ppi which occurred during the ensuing bronchoconstriction was recorded on a second channel of the Gould recorder at higher amplification. The changes in pressure in the trachea recorded in this way probably reflect changes in both resistance and compliance components of the lungs.

Bronchoconstriction and bradycardia were elicited either by intravenous injection of ACh, or by preganglionic stimulation of both cervical vagus nerves (30 Hz, 0.2 ms, 5 s 10–20 V). Pirenzepine was injected i.v. in cumulative doses. Following each dose of pirenzepine, the responses to nerve stimulation and ACh were measured alternately at 2 min intervals.

All results were expressed as the ratio of the response to agonist or nerve stimulation after the antagonist (B2) divided by the response before administration of the antagonist (B1).

The experiments were repeated in a second group of animals which received propranolol ($1\,mg\,kg^{-1}$, i.v.) 30 min before administration of cumulative doses of pirenzepine.

Statistical procedures

Results are expressed as mean values ±s.e.mean from n experiments. Differences between means were tested for significance by use of the unpaired t test and values of $P < 0.05$ were taken as significant.

Drugs

Urethane and suxamethonium chloride (Sigma), acetylcholine bromide (BDH), pirenzepine hydrochloride (Boehringer) and propranolol hydrochloride (ICI) were used. All drugs were dissolved in 0.9% NaCl solution (saline).

Results

The effect of pirenzepine was studied on bronchoconstriction and bradycardia elicited either by intra-

venous injection of ACh, or by stimulation of both cervical vagus nerves. The responses induced by i.v. ACh were used as a measure of postjunctional muscarinic receptor activation in the lungs and heart.

Dose-response curves for bronchoconstriction induced by vagal nerve stimulation were obtained at the start of each experiment and stimulus parameters were then selected for the rest of the experiment (control response, see Figure 1) which produced approximately 50% of the maximal response. A dose of ACh was then selected to cause an increase in Ppi comparable to that produced by the control stimulus to the vagus nerve. In separate experiments saline injections were used instead of pirenzepine and the results showed that repeated alternating application of ACh or vagal nerve stimulation elicited reproducible bronchoconstrictor responses for several hours (Fryer & Maclagan, 1987a).

Administration of pirenzepine in cumulative doses reduced the bronchoconstriction induced by i.v. ACh, as shown by a decrease in the B2/B1 ratio, due to blockade of the muscarinic receptors located postjunctionally on the heart and on airway smooth muscle (Figure 1). In each experiment, the dose of pirenzepine that produced 50% inhibition of the

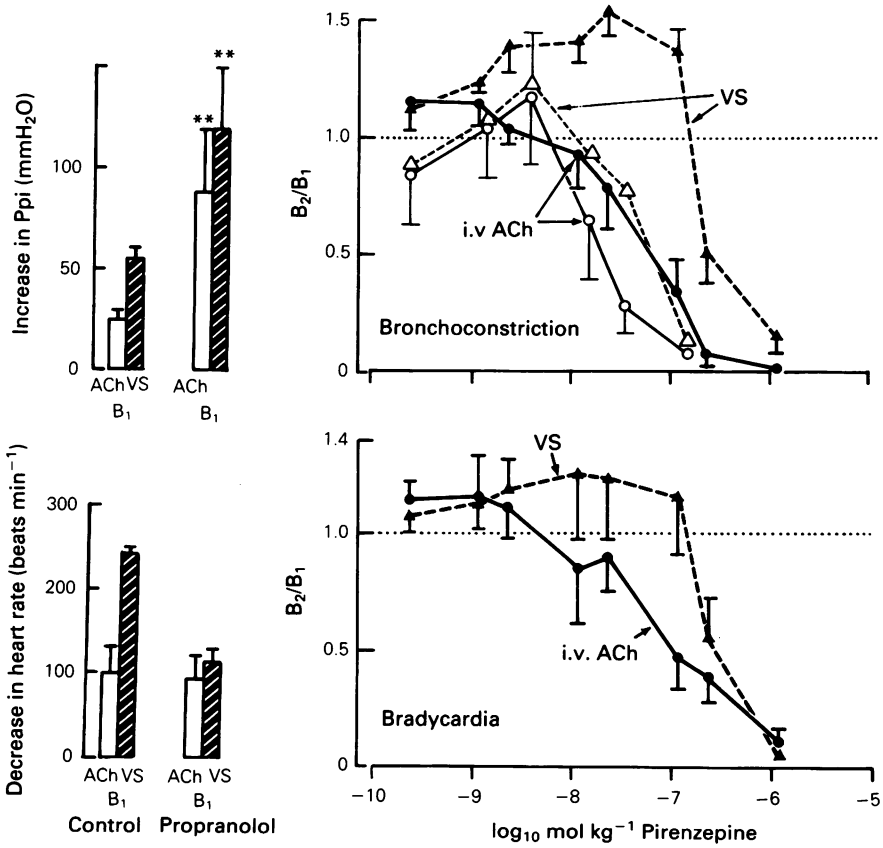


Figure 1 Summary of results showing the effect of pirenzepine on bronchoconstriction (measured as an increase in pulmonary inflation pressure (Ppi)) and bradycardia induced by vagal nerve stimulation (VS; 30 Hz, 0.2 ms, 3 s, 10–20 V; ▲) or by intravenous acetylcholine (ACh 1 $\mu g\,kg^{-1}$; ●). The control responses (B1) to VS (hatched columns) and ACh (open columns) before pirenzepine are shown on the left. Results are expressed in the graphs as the ratio B2/B1 where B2 is the response after pirenzepine.

A second group of guinea-pigs was pretreated with propranolol (1 $mg\,kg^{-1}$, open symbols), which abolished the pirenzepine-induced potentiation of vagally-induced bronchoconstriction. The effect of pirenzepine in the heart was not significantly altered by propranolol (not shown but see Table 1). Note that the control bronchoconstrictor responses were potentiated by propranolol. (Mean results with s.e.mean shown by vertical bars; $n = 6$).

Table 1 Antagonist activity of pirenzepine in lung and heart

Response	*Pirenzepine ED_{50}* ($nmol\,kg^{-1}$)	
Bronchoconstriction	*Control*	*After propranolol*
ACh-induced	69 ± 30	27 ± 13
Vagally-induced	382 ± 117	63 ± 20**
Bradycardia		
ACh-induced	116 ± 56	152 ± 70
Vagally-induced	491 ± 249	847 ± 429

Dose of pirenzepine causing 50% inhibition of responses induced by acetylcholine (ACh) and by vagal stimulation in heart and lung (ED_{50}, $nmol\,kg^{-1}$). Values were calculated for each individual guinea-pig and the table shows the mean ED_{50} ± s.e.mean ($n = 6$) for a control group of animals compared with a second group that received propranolol ($1\,mg\,kg^{-1}$) at the start of the experiment. Baseline values for the two groups, in absolute units, are given in Figure 1.
** Different from control: $P < 0.002$.

responses was calculated (ED_{50}) and the mean ED_{50} values for 5 experiments are summarized in Table 1. These results show that pirenzepine was approximately equipotent as an antagonist for the postjunctional muscarinic receptors located on airway smooth muscle and on the atrial cell.

Effect of pirenzepine on vagally-induced bronchoconstriction and bradycardia

In the same experiments, the effects of pirenzepine were tested on vagally-mediated responses (Figure 1). In the heart, vagally-induced bradycardia was progressively inhibited by increasing doses of pirenzepine in parallel with the inhibition of ACh-induced bradycardia. This is consistent with blockade of the postjunctional muscarinic receptors on the atrial cell.

However, in the lung vagally-induced bronchoconstriction was increased by low doses of pirenzepine, occurring at doses below $100\,nmol\,kg^{-1}$, when the postjunctional receptors were either unaffected or partially blocked (Figure 1). As the dose of pirenzepine increased above $100\,nmol\,kg^{-1}$, vagally-induced bronchoconstriction gradually decreased and was finally abolished concurrently with inhibition of the response to ACh due to blockade of the postjunctional receptors.

One consequence of this enhancement of vagally-mediated bronchoconstriction by pirenzepine was that the ED_{50} for pirenzepine as an antagonist of vagally-induced bronchoconstriction was higher than that needed to inhibit ACh-induced bronchoconstriction (Table 1).

In a second group of animals, the effect of pirenzepine was studied after pretreatment with propranolol ($1\,mg\,kg^{-1}$, i.v.). The control bronchoconstrictor responses elicited by both ACh and vagal stimulation were increased by propranolol (see control responses in Figure 1). Despite this change, the potency of pirenzepine for the postjunctional receptors in the lung was unaltered by β-adrenoceptor blockade. There was no significant change in the potency of pirenzepine for vagally-mediated or ACh-induced responses in the heart (Table 1). However, pirenzepine no longer caused an increase in vagally-induced bronchoconstriction after propranolol pretreatment (Figure 1, Table 1).

Discussion

The results presented in this paper based on functional tests in the guinea-pig demonstrate that postjunctional muscarinic receptors in the heart and lung have similar affinity for pirenzepine *in vivo*. This is in agreement with previous work both *in vivo* and *in vitro* showing that pirenzepine can be used as a selective antagonist for M_1 receptors but does not distinguish between M_2 and M_3 muscarinic receptors (see Eglen & Whiting, 1986).

It is known from previous work that muscarinic receptors also exist on the parasympathetic nerve terminals innervating airway smooth muscle in several species including guinea-pig (Fryer & Maclagan, 1984), cat (Blaber *et al.*, 1985), dog (Ito & Yoshitomi, 1988) and man (Minette & Barnes, 1987). These muscarinic autoreceptors exert an inhibitory effect on pulmonary parasympathetic neurotransmission so that blockade with an antagonist potentiates vagally-mediated bronchoconstriction. Thus, as the effect of muscarinic antagonists on vagally-induced bronchoconstriction depends on the balance between their prejunctional and postjunctional antagonist action, the potency for the prejunctional receptors will be underestimated (Fryer & Maclagan, 1987a).

Gallamine is a potent antagonist for the pulmonary neuronal receptors in all of the above species suggesting that these receptors probably resemble the cardiac muscarinic receptors and differ from the receptors on airway smooth muscle where the potency of gallamine is low. Thus the neuronal receptors in pulmonary nerves appear to be similar to the muscarinic receptors mediating prejunctional inhibition in other autonomic nerves which are

known to be of the cardiac M_2 subtype (Ashe & Yarosh, 1984; Newberry *et al.*, 1985; Egan & North, 1986).

In view of these findings, it was surprising that the selective M_1 antagonist, pirenzepine, increased vagally-induced bronchoconstriction at low doses as described in this paper. In order to block the post-junctional M_3 muscarinic receptors on airway smooth muscle and the M_2 receptors on the atrial cell, the dose of pirenzepine had to be increased ten fold. This suggests that the pirenzepine-induced enhancement of bronchoconstriction due to vagal stimulation may be due to blockade of M_1 receptors, with a high affinity for pirenzepine, located in the pulmonary autonomic nerves.

The results with pirenzepine differed in one important respect from those previously obtained with gallamine in this species. Propranolol abolished the pirenzepine-induced enhancement of vagal stimulation, whereas such an effect of gallamine was unaltered by β-adrenoceptor blockade (Fryer & Maclagan, 1984). This suggests that these pirenzepine-sensitive M_1 receptors must be located in the sympathetic nervous pathway (see Figure 2), in contrast to the gallamine-sensitive receptors, which are located on the parasympathetic nerve terminals innervating airway smooth muscle (Faulkner *et al.*, 1986).

Stimulation of pulmonary sympathetic nerves causes bronchodilatation *via* activation of β-adrenoceptors on airway smooth muscle; there is also evidence that the sympathetic nerves may inhibit parasympathetic neurotransmission at the ganglia (Partanen *et al.*, 1982) and at the prejunctional cholinergic nerve terminals (Vermeire & Vanhoutte, 1979). Blockade by pirenzepine of M_1 receptors which facilitate neurotransmission in the sympathetic nerves would, therefore, reduce noradrenaline release and indirectly increase the effect of parasympathetic nerve stimulation. These M_1 receptors could be located either on the final terminals of the sympathetic postganglionic neurones or at the ganglia (see Figure 2). In the latter case, they would be comparable with the M_1 receptors with a facilitatory role that have been described in sympathetic ganglia in several species (Brown *et al.*, 1980; Mutschler & Lambrecht, 1984; Ashe & Yarosh, 1984; Newberry *et al.*, 1985). Activation of these receptors probably closes a specific subset of voltage-sensitive K^+ channels (Brown, 1983). It is interesting that this facilitatory mechanism is absent from parasympathetic ganglia (Brown, 1988).

These results also imply that the sympathetic nerves must have been activated whenever bronchoconstriction was elicited by vagal nerve stimulation or by injected ACh. This conclusion is supported by the finding described in this paper that the control

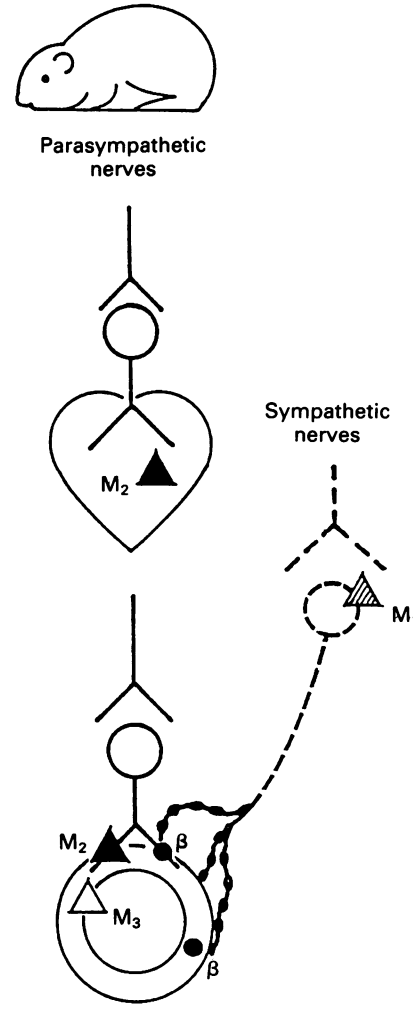


Figure 2 Proposed location of muscarinic receptors in autonomic nerves innervating heart and lung. M_1-pirenzepine-sensitive facilitory receptors: M_2-gallamine-sensitive inhibitory receptors: M_3 receptors on airway smooth muscle: β-adrenoceptors on cholinergic nerves and airway smooth muscle. The ganglionic nicotinic receptors have been omitted.

bronchoconstrictor responses to ACh and to vagal nerve stimulation were increased after propranolol pretreatment due to removal of the bronchodilator influence of the sympathetic nervous system.

Evidence for reflex activation of the pulmonary sympathetic nerves has been reported previously in the guinea-pig by McCulloch *et al.* (1967) using histamine as the bronchoconstrictor agent. It is not

clear whether this reflex is triggered by afferent nerve stimulation or by anoxia *per se* but the afferent limb of the reflex pathway must lie outside the vagal nerves as, in the experiments described in the present paper and in those of McCulloch *et al.* (1967), the animals were vagotomized.

The distribution of M_1 muscarinic receptors in the autonomic nerves supplying the lung appears to vary between different species. Although it was not possible to demonstrate M_1 receptors in the parasympathetic nerves innervating airway smooth muscle in the guinea-pig, such receptors have been demonstrated in these nerves in the dog (Ito & Yoshitomi, 1988). It has been suggested that M_1 receptors exist in the rabbit pulmonary parasympathetic nerves because pirenzepine was apparently a potent antagonist of vagally-induced bronchoconstriction in this species (Bloom *et al.*, 1987). However, the interpretation of the results in the rabbit is complicated by the fact that the influence of the sympathetic nervous system and blockade of postjunctional receptors were not directly excluded in the latter experiments. M_2 receptors, on the other hand, appear to exist in pulmonary parasympathetic nerves in many species. They have been demonstrated in parasympathetic nerves in the guinea-pig (Fryer & Maclagan, 1984), cat (Blaber *et al.*, 1985), dog (Ito & Yoshitomi, 1988) and human (Minette & Barnes, 1987). In each of these examples, the receptors exerted an inhibitory control on cholinergic neurotransmission to airway smooth muscle. This autoreceptor mechanism appears to be lacking in the rabbit (Maclagan & Faulkner, 1989) and in asthmatic patients (Minette *et al.*, 1988).

Figure 2 shows the mechanisms that underlie the results presented in this paper and in previous work from this laboratory showing that M_1 muscarinic receptors with a facilitatory function exist in the sympathetic nervous pathway in the guinea-pig lung (this paper) and M_2 receptors exist in the parasympathetic nerves in this species (Fryer & Maclagan, 1984). These M_2 receptors resemble the cardiac M_2 receptors as shown by the antagonist potency of gallamine (Fryer & Maclagan, 1987a).

As the sympathetic nervous system exerts an inhibitory effect on parasympathetic neurotransmission in the guinea-pig, any procedure that reduces the influence of the sympathetic nerves would be expected to increase the bronchoconstrictor effect of the cholinergic pathway. This explains why both M_1 antagonists and β-adrenoceptor blocking drugs (Maclagan & Ney, 1979) potentiate vagally-induced bronchoconstriction in this species.

In man, the existence of M_1 receptors in the sympathetic pathway to the lung has not been investigated but it is known that prejunctional β-adrenoceptors exist in human pulmonary parasympathetic nerves (Rhoden *et al.*, 1988). If the sympathetic nervous system exerts an inhibitory effect on parasympathetic neurotransmission in man, comparable to that shown in the guinea-pig, this could provide an explanation for the bronchospasm provoked by β-adrenoceptor blocking drugs in asthmatic patients, but not in normal subjects. Blockade of the prejunctional β-adrenoceptors should cause an increase in ACh release. In normal individuals this would activate the muscarinic autoreceptors to limit transmitter output to the airway smooth muscle. In asthmatic patients however, where there is an apparent absence of prejunctional M_2 receptors, β-adrenoceptor blockade might result in an increase in ACh release which would exacerbate bronchoconstriction.

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Effect of pirenzepine and gallamine on cardiac and pulmonary muscarinic receptors in the rabbit

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1 The effect of muscarinic antagonists considered to be selective for M_1 receptors (pirenzepine) and for M_2 receptors (gallamine) were studied on bronchoconstriction and bradycardia elicited by stimulation of the vagal nerves and by i.v. acetylcholine (ACh) in anaesthetized rabbits.

2 Pirenzepine was equipotent as an antagonist of ACh-induced responses at postjunctional muscarinic receptors in the heart, lung and blood vessels, whereas gallamine was at least ten times less potent at pulmonary and vascular muscarinic receptors. Thus, gallamine never caused complete inhibition of bronchoconstrictor or hypotensive responses to i.v. ACh, whereas doses of pirenzepine in excess of 1 μmol kg^{-1} abolished all muscarinic responses.

3 In the lung, both antagonists inhibited bronchoconstriction caused by vagal stimulation and ACh-induced bronchoconstriction to the same extent (pirenzepine, mean ED_{50} 65 ± 22 and 130 ± 28 nmol kg^{-1} respectively; gallamine, $ED_{50} > 10{,}000$ nmol kg^{-1} for both responses). Enhancement of vagally-induced bronchoconstriction was never observed.

4 In the heart, however, both pirenzepine and gallamine were ten times less potent as antagonists of vagally-induced bradycardia than of ACh-induced bradycardia. This differential blockade was unaltered by propranolol (1 mg kg^{-1}) pretreatment.

5 It is concluded that there is no evidence for M_1 or M_2 muscarinic receptors in the pulmonary innervation of the rabbit and the potency of the antagonists in abolishing vagally-induced bronchoconstriction was consistent with blockade of M_3 muscarinic receptors on airway smooth muscle.

6 The results suggest that M_2 muscarinic receptors may exert an inhibitory effect on transmission in the parasympathetic nerves innervating the heart in the rabbit. Blockade of such neuronal receptors would increase transmitter output to the atrial cells and explain the low potency of both antagonists in abolishing vagally-induced bradycardia in the rabbit.

Introduction

Muscarinic receptors have been shown to inhibit neurotransmission in pulmonary parasympathetic nerves in several species, including man. Blockade of these receptors with muscarinic antagonists causes potentiation of vagally-induced bronchoconstriction. The potency of gallamine for these prejunctional receptors in the cat (Blaber *et al.*, 1985), guinea-pig (Fryer & Maclagan, 1984), dog (Ito & Yoshitomi, 1988) and man (Minette & Barnes, 1987) suggests that these prejunctional receptors belong to the M_2 subtype. However, it has recently been claimed that, in the rabbit, muscarinic receptors of the M_1 subtype exist on the pulmonary parasympathetic nerves (Bloom *et al.*, 1987). This conclusion was based on the finding that pirenzepine was more effective as an antagonist of vagally-induced bronchoconstriction than of vagally-induced bradycardia. As the muscarinic receptors in the heart belong to the M_2 subtype, it was suggested that M_1 receptors with a high affinity for pirenzepine exist in the pulmonary vagal pathway in the rabbit. However, pirenzepine is known to act as a non-selective antagonist for M_2 and M_3 receptors at higher doses *in vivo* and blockade of postjunctional muscarinic receptors on airway smooth muscle and atrial cells was not measured directly in the experiments of Bloom *et al.* (1987). Other explanations of their results are possible.

The present experiments were designed to compare the effect of pirenzepine on pre- and postjunctional muscarinic receptors in the heart and lung in the rabbit *in vivo*. Inhibition of responses to i.v. acetylcholine (ACh) was used as a measure of blockade of the postjunctional muscarinic receptors. The effect of gallamine was also studied as the pulmonary

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effects of this M_2 antagonist have not previously been investigated in the rabbit.

Methods

Rabbits (1.5–2.0 kg, either sex) were used. They were anaesthetized with urethane (1.5 g kg^{-1}, Sigma Ltd) injected into an ear vein. The carotid artery was cannulated for the measurement of blood pressure and heart rate was derived electronically from the blood pressure signal. A cannula was placed in the left jugular vein for the injection of drugs. Both vagi were cut and the distal ends placed on shielded platinum electrodes immersed in a pool of liquid paraffin. The animal's body temperature was maintained at 37°C by use of a CFP 8185 homeothermic blanket control.

The anaesthetized animals were paralysed with suxamethonium chloride (100 μg kg^{-1}) injected every 10 min and respired with positive pressure, constant volume ventilation from a Harvard Rodent respirator. Bronchoconstriction was recorded (as described by Maclagan *et al.*, 1989) as an increase in pulmonary inflation pressure (Ppi) over the basal insufflation pressure change produced by the pump. Bronchoconstriction and bradycardia were elicited either by intravenous injection of ACh, or by preganglionic stimulation of both cervical vagus nerves (30 Hz, 0.2 ms, for 5 s). Pirenzepine or gallamine were injected i.v. in cumulative doses while the responses to nerve stimulation and ACh were measured alternately at 2 min intervals.

All results were expressed as the ratio of the response to agonist or nerve stimulation after the antagonist (B2) divided by the response before administration of the antagonist (B1). Potentiation and inhibition of the response is indicated by an increase or decrease, respectively, in the B2/B1 ratio.

The experiments were repeated in a second group of animals which received propranolol (1 mg kg^{-1}, i.v.) 30 min before administration of the first dose of pirenzepine.

Drugs

Urethane and suxamethonium chloride (Sigma), acetylcholine bromide (BDH), pirenzepine hydrochloride (Boehringer), gallamine triethiodide (May & Baker) and propranolol hydrochloride (ICI) were used. All drugs were dissolved in 0.9% NaCl solution (saline).

Results

Figure 1 shows records of bronchoconstriction and bradycardia elicited by preganglionic vagal nerve

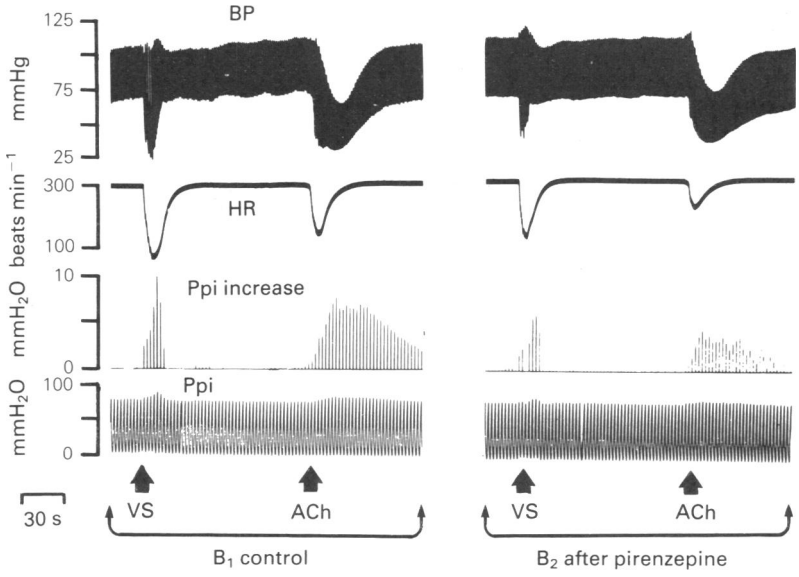


Figure 1 Stimulation of both vagus nerves (VS; 30 Hz, 0.2 ms, 5 s) and acetylcholine (ACh, 1 μg kg^{-1} i.v.) in an anaesthetized rabbit paralysed with suxamethonium caused bronchoconstriction (Ppi increase) and bradycardia. Pirenzepine (50 nmol kg^{-1}) reduced the responses to VS and ACh in heart and lung. In subsequent figures, the results are expressed as the ratio B2/B1 where B2 is the response after antagonist and B1 is the control response.

stimulation and by i.v. injection of ACh in an anaesthetized, paralysed rabbit. Administration of pirenzepine (50 nmol kg^{-1}) reduced the bronchoconstriction and bradycardia elicited by both types of stimuli. In Figures 2 and 3, the results are expressed as the ratio B2/B1 of the response obtained after the antagonist (B2) divided by the control response (B1). A decrease in the B2/B1 ratio indicates blockade of receptors in heart and lung.

Postjunctional muscarinic receptors in lung and heart

Administration of cumulative doses of either pirenzepine (Figure 2) or gallamine (Figure 3) reduced the bronchoconstriction and bradycardia induced by ACh. The mean ED_{50} values (the dose of antagonist which produced 50% inhibition of the control responses in each experiment) are summarised in Table 1. The results show that gallamine was much less potent as an antagonist for the pulmonary muscarinic receptors than for those in the heart. Even at the highest dose used (10 μmol kg^{-1}), gallamine did not produce more than 50% blockade of the bronchoconstrictor responses to ACh (Figure 3). This effect is also shown by the large ED_{50} value for gallamine as an antagonist of ACh-induced bronchoconstriction (> 10 μmol kg^{-1}; Table 1).

In contrast, pirenzepine was equipotent as an antagonist for the postjunctional muscarinic receptors on airway smooth muscle and on the atrial cells. (ED_{50} 130 ± 28 and 107 ± 21 nmol kg^{-1} for ACh-induced bronchoconstriction and bradycardia respectively).

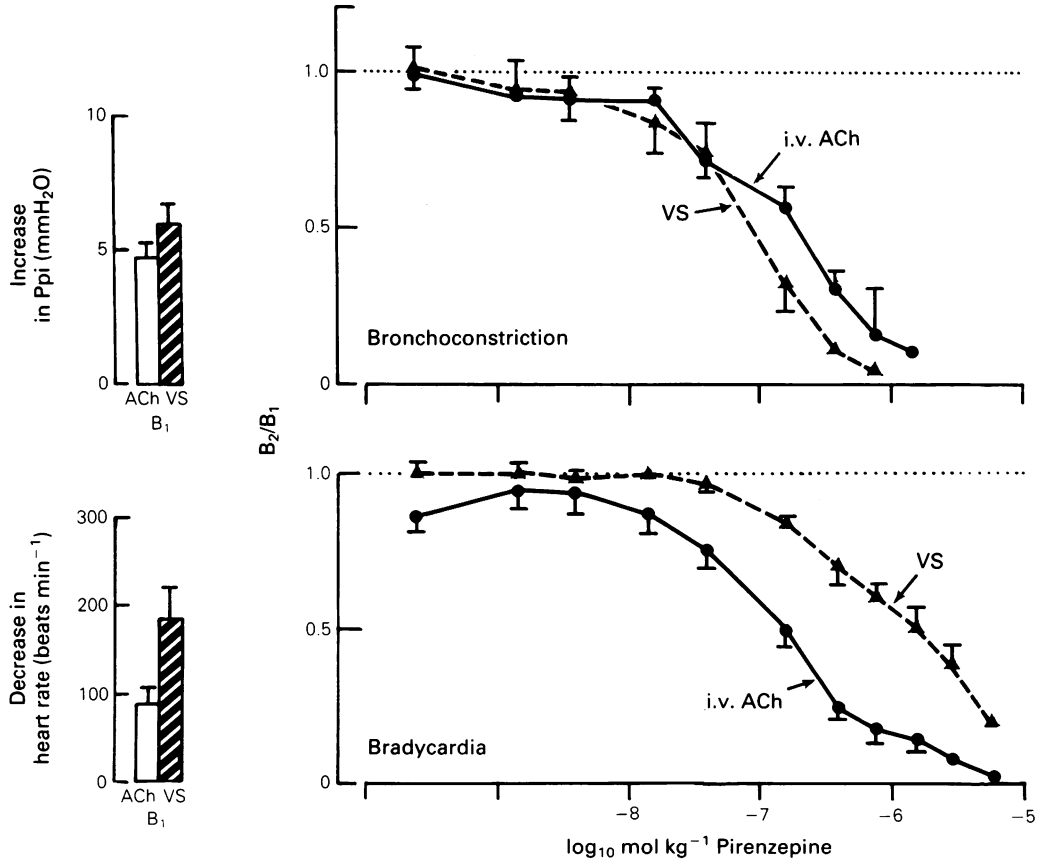


Figure 2 Summary of results showing the effect in the rabbit of pirenzepine on bronchoconstriction (measured as an increase in Ppi) and bradycardia induced by vagal nerve stimulation (VS; 30 Hz, 0.2 ms, 3 s, 10–20 V; dotted lines) or by intravenous acetylcholine (ACh, 1 μg kg^{-1}; solid lines). The control responses (B1) to VS (hatched columns) and ACh (open columns) before pirenzepine are shown on the left in absolute units. Results are expressed in the graphs as the ratio B2/B1 where B2 is the response after pirenzepine. Mean results with s.e.mean shown by vertical bars (n = 5).

Table 1 Doses of pirenzepine and gallamine which cause 50% inhibition (ED_{50}) of bronchoconstriction and bradycardia elicited by acetylcholine (ACh) compared with ED_{50} for vagally-induced responses in the rabbit

Response	ED_{50} (nmol kg^{-1}) Pirenzepine	Gallamine
Bronchoconstriction		
ACh-induced	130 ± 28	>10,000
Vagally-induced	65 ± 22	>10,000
Bradycardia		
ACh-induced	107 ± 21	1790 ± 761
Vagally-induced	1125 ± 428	14,250 ± 3940
Hypotension		
ACh-induced	691 ± 42	>10,000

ED_{50} values were calculated for each individual animal and the table shows mean values ± s.e.mean (n = 5). Control values for the two groups of animals are given in Figures 1 and 2 in absolute units.

Vascular muscarinic receptors

Figure 1 and Table 1 also show that pirenzepine was slightly less potent as an inhibitor of the hypotensive response to ACh compared with its effect on ACh-induced responses in the heart and lung. Gallamine, however, was almost inactive as an antagonist for the vascular muscarinic receptors *in vivo* and did not abolish the vascular or pulmonary responses to ACh even at the highest dose used (10 μmol kg^{-1}), in contrast to the cardiac responses which were inhibited by approximately 50% by a dose of 2 μmol kg^{-1}.

Effect of muscarinic antagonists on vagally-induced bronchoconstriction

The effect of the antagonists on vagally-induced bronchoconstriction was studied in the same animals by interposing periods of nerve stimulation between test injections of ACh (see Figure 1). The results of these experiments are shown for pirenzepine in Figures 1 and 2, for gallamine in Figure 3 and the effect of both antagonists is compared in Table 1. Both drugs inhibited bronchoconstriction induced by ACh and by nerve stimulation to the same extent. In these experiments, blockade of vagally-induced bronchoconstriction was consistent with blockade of postjunctional muscarinic receptors on airway smooth muscle. This contrasts with results previously obtained in the guinea-pig, where both antagonists caused potentiation of vagally-induced

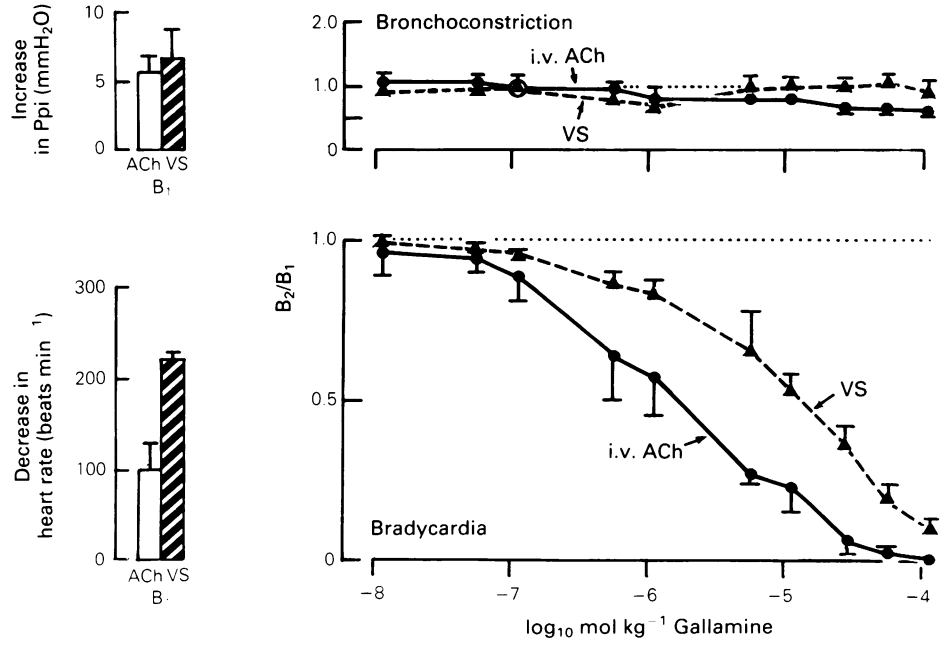


Figure 3 Summary of results showing the effect in the rabbit of gallamine on bronchoconstriction and bradycardia induced either by stimulation of the vagus nerves (dotted lines) or by intravenous injection of acetylcholine (ACh, solid lines). Results are expressed as in Figure 1 (mean with s.e.mean shown by vertical bars; n = 5).

bronchoconstriction due to blockade of neuronal receptors (Fryer & Maclagan, 1984; Maclagan *et al.*, 1989).

Effect of muscarinic antagonists on vagally-induced bradycardia

When the cardiac responses were studied, both antagonists were found to be more than ten times less potent in inhibiting bradycardia induced by vagal nerve stimulation than that induced by ACh (pirenzepine ED_{50} 1125 ± 428 for vagally-induced bradycardia compared with 107 ± 21 nmol kg^{-1} for ACh-induced bradycardia: gallamine ED_{50} $14{,}250 \pm 3940$ and 1790 ± 761 nmol kg^{-1} respectively).

In a second group of animals, propranolol (1 mg kg^{-1}) injected at the start of the experiment did not significantly alter the control responses to vagal nerve stimulation or ACh in heart or lung. In these experiments, the potency of pirenzepine as an antagonist of vagally-induced bradycardia was not significantly different from that seen in the untreated group (ED_{50} for pirenzepine to inhibit vagally-induced bradycardia; 1125 ± 428 before and 2267 ± 500 nmol kg^{-1} after propranolol $n = 5$, $P > 0.1$).

Discussion

The results presented in this paper show that in the anaesthetized rabbit, pirenzepine was approximately equiactive as an antagonist for the muscarinic receptors in the heart and airway smooth muscle, and slightly less potent for the muscarinic receptors in blood vessels. These results suggest that, at the doses used in these experiments, pirenzepine was acting as a non-selective antagonist for the muscarinic receptors in these tissues. This confirms previous work both *in vivo* and *in vitro* which show that, although pirenzepine is considered to be a selective antagonist for M_1 receptors, selectivity is lost at higher doses and the drug then acts as an antagonist at M_2 receptors in the heart and for M_3 receptors on smooth muscle (see review by Eglen & Whiting, 1986).

In our experiments in the rabbit, gallamine was a more potent antagonist for the muscarinic receptors on the atrial cells compared with those on vascular or airway smooth muscle. This is in agreement with published data for this antagonist in many other species obtained by a variety of methods, which has led to the concept that the cardiac receptors belong to a different subgroup of muscarinic receptors (M_2) from those in airway smooth muscle or blood vessels.

The possibility was considered that use of i.v. injections of ACh to elicit bradycardia as a measure of muscarinic receptor activation in the heart might also elicit reflex tachycardia due to the accompanying hypotension produced by the action of ACh on muscarinic receptors in blood vessels. The efferent pathway for such a reflex would be expected to lie in the cardiac sympathetic nerves, as the animals were vagotomised in the experiments described in this paper. This possibility is excluded, however, by the finding that propranolol pretreatment did not alter the apparent potency of the muscarinic antagonists as inhibitors of ACh-induced bradycardia. Thus, reflex tachycardia does not appear to have influenced the results obtained using i.v. ACh to measure blockade of post-junctional muscarinic receptors in the heart.

Propranolol pretreatment did not potentiate the control bronchoconstrictor responses to vagal nerve stimulation or to ACh. Thus, it is unlikely that sympathetic reflex responses to the lung were elicited by either stimulus. This contrasts with results obtained in the anaesthetized guinea-pig, where bronchoconstrictor stimuli are increased by propranolol due to blockade of a sympathetic bronchodilator reflex (McCulloch *et al.*, 1967; Maclagan *et al.*, 1989). The lack of a sympathetic bronchodilator reflex in the rabbit may be due to the effects of the general anaesthetic or to the absence of a sympathetic innervation of airway smooth muscle in that species (Mann, 1971).

Because of its low potency for the M_3 muscarinic receptor on airway smooth muscle, gallamine has previously been used as a convenient tool to demonstrate the existence of prejunctional M_2 muscarinic receptors in pulmonary parasympathetic nerves in several species. In the guinea-pig (Fryer & Maclagan, 1984), cat (Blaber *et al.*, 1985), dog (Ito & Yoshitomi, 1988) and man (Minette & Barnes, 1987) these prejunctional muscarinic receptors have been shown to exert an inhibitory effect on pulmonary parasympathetic neurotransmission. The present results show that gallamine did not cause enhancement of vagally-induced bronchoconstriction in the rabbit, indicating that neuronal muscarinic receptors do not exist in the pulmonary autonomic nerves in this species.

Although the rabbit does not have prejunctional receptors on the pulmonary vagal nerves, the present results suggest that neuronal muscarinic receptors exist on the vagal nerve fibres innervating the heart in this species. The dose of gallamine needed to block vagally-induced bradycardia was more than ten times greater than that needed to block ACh-induced bradycardia. Thus, vagally-induced bradycardia was unexpectedly resistant to the blocking action of gallamine in the rabbit. Involvement of the

sympathetic nervous system in this differential blockade was ruled out by the finding that propranolol pretreatment did not affect this result.

These findings can be explained by the presence of muscarinic receptors in the cardiac parasympathetic nerves in the rabbit which inhibit neurotransmission. Such receptors have previously been described in the cardiac nerves in the rat (Wetzel & Brown, 1985) and in the guinea-pig (Jeck *et al.*, 1988). Blockade of these receptors by gallamine would increase transmitter output from the vagus nerves and increase the concentration of antagonist required to inhibit vagally-induced bradycardia. The high potency of gallamine for these receptors suggests that they may be M_2 receptors.

Pirenzepine also showed greater potency for ACh-induced bradycardia compared with vagally-induced bradycardia suggesting that, at the doses used, this drug was also acting as an antagonist for the prejunctional M_2 receptors in cardiac parasympathetic nerves.

The conclusion that there are no prejunctional muscarinic receptors on the parasympathetic nerves innervating the lung in the rabbit differs from the conclusions of Bloom *et al.* (1987) who reported that M_1 receptors with an excitatory function existed in the pulmonary parasympathetic nerves in this species. Their conclusion was based on a comparison of the potency of pirenzepine in the heart and the lung. They reported that pirenzepine was more potent as an antagonist of vagally-mediated bronchoconstriction than of vagally-mediated bradycardia. This result has been confirmed in the present experiments. However, Bloom *et al.* did not test the degree of blockade of postjunctional muscarinic receptors in the heart and lung. When this test was added in our experiments, it could be seen that pirenzepine was equipotent as an antagonist for the postjunctional receptors in heart and lung and that the bronchoconstrictor responses induced by ACh and by vagal stimulation were inhibited to the same extent.

This changes the interpretation of the results. Pirenzepine no longer appears to be a potent antagonist for vagally-induced bronchoconstriction; in contrast, it is now seen to be much less potent as an antagonist of vagally-induced bradycardia than would be expected from the degree of blockade of the postjunctional receptors in the heart. Therefore, these results do not provide evidence for prejunctional M_1 receptors in the pulmonary parasympathetic nerves in the rabbit, as suggested by Bloom *et al.* (1987); on the contrary, their results and the results of the present experiments can be explained by the presence of prejunctional M_2 muscarinic receptors with an inhibitory function on the parasympathetic nerves innervating the heart. Blockade of these neuronal receptors would increase the amount of transmitter reaching the postjunctional M_2 receptors on the atrial cells and explain the low potency of pirenzepine and gallamine in abolishing vagally-induced bradycardia.

In conclusion, in most of the animal species studied, the muscarinic receptors located postjunctionally on the smooth muscle cells of the lung, the intestine and in blood vessels appear to be M_3 receptors, whereas the receptors in the heart are M_2 receptors. Considerable species differences occur, however, in the location of the different receptor subtypes in the autonomic nerves innervating heart and lung. The neuronal receptors appear to belong either to the M_1 or M_2 receptor subtype whose locations can be summarised as follows.

In the lung in the guinea-pig, the sympathetic nerves innervating airway smooth muscle contain M_1 receptors that facilitate transmission (Maclagan *et al.*, 1989) but this mechanism has not yet been demonstrated in the lung in any other species. On the other hand, the parasympathetic nerves innervating the lung have been demonstrated to contain M_2 receptors in several species. Activation of these receptors exerts an inhibitory effect on ACh release. This mechanism has been demonstrated in guinea-pig (Fryer & Maclagan, 1984), cat (Blaber *et al.*, 1985), dog (Ito & Yoshitomi, 1988) and man (Minette & Barnes, 1987). However, in the rabbit (this paper) neither M_1 nor M_2 receptors could be demonstrated in either branch of the autonomic nervous system innervating the lung.

The distribution of muscarinic receptors in the cardiac branches of the vagal parasympathetic nerves does not necessarily correspond to that seen in the pulmonary nerves in the same species. At present, the guinea-pig is the only species in which M_2 receptors have been demonstrated in both pulmonary (Fryer & Maclagan, 1984) and cardiac (Jeck *et al.*, 1988) parasympathetic nerves.

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Beneficial effect of amosulalol and phentolamine on post-hypoxic recovery of contractile force and energy metabolism in rabbit hearts

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1 The effects of phentolamine, an α-adrenoceptor blocking agent and amosulalol, an α_1 and β-adrenoceptor antagonist on hypoxia-induced impairment in cardiac function and metabolism were examined using the isolated heart Langendorff preparation of the rabbit.

2 Hypoxia induced cessation of cardiac contractile force, a rise in resting tension, a decrease in myocardial high-energy phosphates, an increase in tissue calcium content and the release of ATP metabolites from the heart. Subsequent reoxygenation resulted in little recovery of cardiac contractile force, and there were further increases in tissue calcium content and in the release of creatine kinase from the heart.

3 Treatment of hypoxic hearts with either 83 μM phentolamine or 45 μM amosulalol resulted in a suppression of the rise in resting tension, the tissue calcium accumulation and the release of creatine kinase and ATP metabolites during hypoxia. This treatment also elicited significant recovery of cardiac contractile force, restoration of myocardial high-energy phosphates, suppression of the release of creatine kinase and the accumulation of tissue calcium during reoxygenation. Both 83 μM phentolamine and 45 μM amosulalol exerted a significant prolongation of the effective refractory period of rabbit isolated atria.

4 Lower concentrations of phentolamine (16 μM) and amosulalol (9 μM), which are sufficient to exert an α-adrenoceptor blocking action, did not elicit an appreciable effect on the post-hypoxic recovery of cardiac contractile force.

5 These results suggest that phentolamine and amosulalol are capable of protecting the myocardium from hypoxia-induced derangements in cardiac function and metabolism. This effect is probably attributable to their membrane stabilizing effect, rather than to their α-adrenoceptor blocking action.

Introduction

Oxygen deficiency primarily induces functional and metabolic impairments in cardiac cells, which are believed to be dependent on the period and the severity of oxygen deficiency used (Hearse & Humphrey, 1975; Hearse *et al.*, 1977; Nayler *et al.*, 1979; Nayler, 1983; Murry *et al.*, 1986; Allen & Orchard, 1987). Several investigators have demonstrated a cardioprotective action of both calcium antagonists (Fleckenstein, 1971; Aschraf & Kahamathra, 1984) and β-adrenoceptor blocking agents in ischaemic hearts (Nayler *et al.*, 1978b; Wrangler *et al.* 1984). We have also shown that experimentally-induced hypoxic insults produce cardiac contractile failure, impairments in myocardial energy metabolism and changes in cell membrane permeability, which can be prevented by diltiazem, verapamil (Takeo *et al.*, 1988c) and coenzyme Q_{10} (Takeo *et al.*, 1987). α-Adrenoceptor blocking agents such as phentolamine and prazosin have been shown to be effective in protecting against post-ischaemic reperfusion-induced calcium accumulation in cat and rat hearts (Sharma *et al.*, 1983; Nayler *et al.*, 1985). This suggests that α- and/or β-adrenoceptor antagonists are beneficial for protection of the ischaemic or hypoxic myocardium, although the exact mechanism for the protection by these agents and the significance of the involvement of α- and β-adrenoceptors in the genesis and the development of the ischaemic injury are not fully understood. Amosulalol, 5-{1-hydroxy-2-(o-metho-

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xyphenoxy)ethyl)amino) ethyl}-2-methylbenzenesulphonamide hydrochloride, has been shown to elicit α_1- and β-adrenoceptor blocking effects (Takenaka *et al.*, 1982a; 1984; Honda *et al.*, 1986) but the effect of this agent on the ischaemic myocardium has not been studied in detail. The present study was undertaken to determine whether amosulalol has a protective action against hypoxia-induced alterations in cardiac function and metabolism. A representative α-adrenoceptor blocking agent, phentolamine, has also been examined in order to elucidate the possible contribution of α-adrenoceptors to this effect.

Methods

Perfusion of hearts

Male albino rabbits, weighing 1.8 to 2.2 kg, were anaesthetized with sodium pentobarbitone (35 mg kg^{-1}). Heparin (1000 u kg^{-1}) was also administered. Isolation and perfusion of hearts was carried out as described previously (Takeo *et al.*, 1987). After thoracotomy, the heart was quickly excised and chilled in a Krebs-Henseleit solution of the following composition (mmol l^{-1}): NaCl 120, KCl 4.8, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 1.25, $NaHCO_3$ 25, glucose 11. The heart was transferred to a non-recirculating Langendorff apparatus and perfused at 37°C with Krebs-Henseleit solution at a fixed flow rate of 16 ml min^{-1}. The perfusate was previously equilibrated with a gas mixture of 95% O_2 + 5% CO_2. The pH of the solution was 7.40 to 7.42. After a 10 min stabilizing period, the heart was paced at 180 beats min^{-1} under the stimulating conditions (duration, 1 ms; strength, 0.4V) by means of an electric stimulator (Nihon Kohden SEN-3201). The hearts were preloaded with a resting tension of 1.50 g. Cardiac contractile force was estimated by monitoring isometric tension development generated through a hook attached to the apex of the heart by means of a force displacement transducer (Nihon Kohden, TB-621T). Changes in resting tension and contractile force were displayed on an oscillographic thermal pen recorder (Nihon Kohden, WT-647G). After 25 min of perfusion (equilibration period), hearts were subjected to hypoxic perfusion with the buffer as described above, but with 11 mM mannitol replacing glucose to reduce anaerobic glycolysis rapidly. This perfusing solution was previously equilibrated with a gas mixture of 95% N_2 + 5% CO_2 (hypoxic conditions). After 25 min of hypoxic perfusion, the rabbit heart was perfused for 45 min with the Krebs-Henseleit solution containing glucose saturated with a gas mixture of 95% O_2 + 5% CO_2 (reoxygenated conditions). Po_2 values of the perfusing solutions for hypoxia and reoxygenation, when measured by means of a blood gas analyser (Denmark Radiometer, BMS-MK2), were 22 to 28 mmHg, and 620 to 645 mmHg, respectively. After perfusion under hypoxic or subsequent reoxygenated conditions, hearts were removed from the perfusion apparatus and analysed for cellular metabolites. Control hearts were perfused for 25 min under normoxic conditions in the absence of glucose (glucose-free normoxia), and then perfused for 45 min under normoxic conditions in the presence of glucose. Phentolamine (500 μg min^{-1}) and amosulalol (300 μg min^{-1}) were infused into the perfusate just before the aortic cannula at an infusion rate of 0.1 ml min^{-1} (83 μM phentolamine and 45 μM of amosulalol as the final concentration) during the hypoxic period, with an infusion pump (Termo STC-521). In a preliminary study, we examined the concentrations of the agents capable of eliciting a beneficial recovery of cardiac contractile force after post-hypoxic reoxygenation. Cardiac contractile force of the heart after 45 min reoxygenation under the present experimental conditions was 24% and 41% of the initial value when the heart had been treated with 9 and 18 μM amosulalol during the hypoxic perfusion, respectively, and 5% and 6% when the heart had been treated with 16 and 50 μM phentolamine (the mean values of two experiments). These recovery values for contractile force were much lower than those observed after treatment with 45 μM amosulalol or 83 μM phentolamine. These results motivated us to use 45 μM amosulalol or 83 μM phentolamine for the main study.

Myocardial high-energy phosphates

After perfusion of the hearts, a small apical portion of the left ventricle was rapidly frozen in liquid nitrogen. The frozen tissue was weighed, pulverized in a stainless steel tube with a stainless steel plunger under liquid nitrogen-cooling, and high-energy phosphate compounds extracted with 0.3 M $HClO_4$ and 0.25 mM ethylenediaminetetraacetate. The extract was neutralized with 2.5 M K_2CO_3, then centrifuged at 1,000 *g* for 20 min. The resulting supernatant fluid was employed as a sample for determination of adenosine triphosphate (ATP) and creatine phosphate (CP). Measurements of myocardial ATP content were performed according to the methods of Bücher (1947). For determination of myocardial CP content, the converting reaction of CP to ATP was employed according to the methods of Lowry & Passoneau (1972). After the conversion of CP to ATP, total ATP content of the myocardium in the reaction mixture was analysed according to the methods described above. Myocardial CP content

was calculated by subtracting the initial ATP content of the extract from the total ATP content. To determine the ratio of dry tissue weight to frozen tissue weight, a piece of frozen tissue was weighed. After drying at 120°C for 15 h, the tissue was weighed and the ratio was estimated. The mean value for dry tissue weight was 11.5 ± 0.7% of the frozen tissue.

Determination of calcium and water contents of the myocardium

After perfusion, about 100 mg of the left ventricle was sampled for determination of calcium and water contents of the tissue. The tissue was cut into four pieces, weighed and dried at 120°C for 15 h, and then tissue dry weight was estimated. Determination of tissue calcium content was performed according to the modified methods of Grochowski *et al.* (1976). The dried myocardium was digested for 4 h with 2.5 ml of 0.75 N HNO_3 at 25°C. After centrifugation of the digest for 10 min at 1,000 *g*, the supernatant fluid was diluted twice with 20 mM $LaCl_3$ – 0.1 N HCl. The calcium content was analysed in triplicate using an atomic absorption spectrometer (Shimazu AA-646, Kyoto).

Analysis of the perfusate eluted from the heart

During the perfusion, the perfusate eluted from the heart was collected at each minute of the perfusion. Ultraviolet absorbance of the perfusate, which is a rough estimate of the release of ATP metabolites from the perfused heart (Takeo *et al.*, 1988a) was monitored at 250 nm with a spectrophotometer (Hitachi 150-20). Changes in absorbance were expressed as the absorbance × 1000 g^{-1} wet weight tissue min^{-1}. All the perfusate from the heart during hypoxia and subsequent reoxygenation was also collected and analysed by means of a high-performance liquid chromatographic (h.p.l.c.) analyser (Hitachi 655), to determine net substances in the perfusate responsible for the absorbance at 250 nm. ATP metabolites were separated through a column of ODS-cellulose acetate, 4.6 mm in diameter and 15 cm in length (Cosmosil $5C_{18}$; Nakarai Chem. Co., Kyoto), by elution with 0.25 M $NH_4H_2PO_4$ and 5% CH_3CN at a flow rate of 1 ml min^{-1}. Absorbance of the eluate was monitored at 254 nm using a uv-detector (Uvilog 7). Retention times for ATP metabolites such as hypoxanthine, inosine and adenosine were 2.18, 2.85 and 6.34 min, respectively.

Creatine kinase activity of the perfusate

Creatine kinase (CK) activity of the perfusate eluted from the hypoxic and reoxygenated heart was measured according to the method of Bergmeyer *et al.* (1970).

Effect on maximal driving frequency of rabbit isolated atria

Male albino rabbits, weighing 1.8 to 2.2 kg were used and the hearts were set up in the Langendorff mode. The atria were rapidly dissected and suspended in a glass organ bath filled with a Krebs-Henseleit solution as described above. The medium was equilibrated with a gas mixture of 95% O_2 and 5% CO_2. An initial resting tension of 1 g was applied and the preparation was allowed to equilibrate for 30 min at 30°C. The isolated atria were electrically stimulated at 30°C by a 2 fold threshold stimulus with a duration of 1 ms. The maximal driving frequency was determined by identifying the frequency of the stimulus which the atria would no longer follow. After control maximal driving frequency was obtained, agents were applied to the organ bath. After 10 min equilibration, the maximal driving frequency was determined again in the presence of test drug. The maximal driving frequency was expressed as an effective refractory period.

Statistics

Results are expressed as the mean ± s.e.mean. Statistical signficance was calculated by use of Student's *t* test, paired *t* test or by analysis of variance followed by Dunnett's *t* test. Results with a probability of 5% or less were considered to be statistically significant ($P < 0.05$).

Results

Haemodynamic effects of the perfused heart

Rabbit isolated hearts were perfused for 25 min under hypoxic conditions in the presence and absence of either phentolamine or amosulalol, followed by 45 min reoxygenation in the absence of test drug. Changes in cardiac contractile force and resting tension are shown in Figure 1a and b, respectively. The initial developed tension, generated at an initial resting tension of 1.5 g, was 5.2 ± 0.4 g ($n = 59$). Contractile force of control hearts decreased to about 80% of the initial value after 25 min of normoxic perfusion. The contractile force of the heart markedly decreased immediately after the onset of hypoxia and reached nearly zero after 10 to 12 min of hypoxic perfusion. Reoxygenation of hearts pre-exposed to hypoxia for 25 min resulted in less than 10% recovery of the contractile force ($n = 6$). The rise in resting tension occurred about 5 min after the onset of hypoxia, and reached

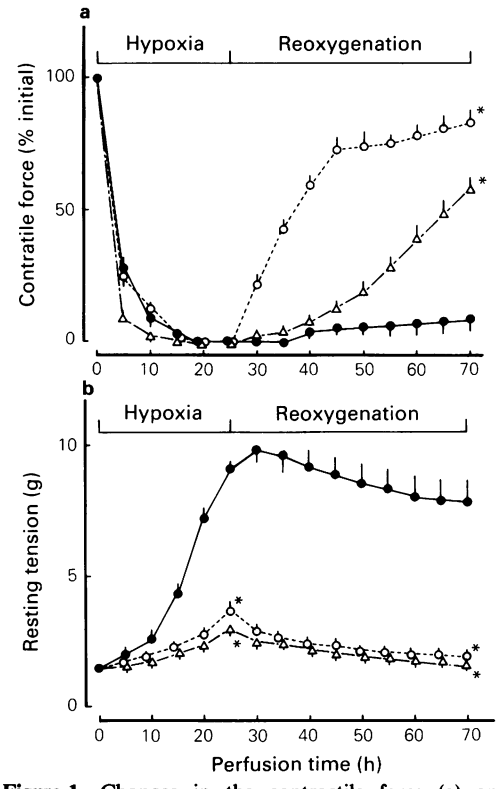


Figure 1 Changes in the contractile force (a) and resting tension (b) of the heart perfused for 25 min under hypoxic conditions in the absence (●) and presence of either phentolamine (△) or amosulalol (○), followed by 45 min of reoxygenation. Each value represents the mean of 6 experiments; vertical lines indicate s.e.mean. The results after 25 min of hypoxia and 45 min of reoxygenation were evaluated statistically. * Significantly different from the value for hearts without treatment with the agents ($P < 0.05$).

9.1 ± 0.3 g after 25 min of hypoxia ($n = 6$). A further small rise in resting tension was observed after 5 min of reoxygenation (9.8 ± 0.5 g; $n = 6$). The resting tension then gradually declined, but still remained at a level much higher than the initial level throughout reoxygenation.

When the heart was perfused under hypoxic conditions in the presence of either 45 μM amosulalol or 83 μM phentolamine, a significant suppression of the hypoxia-induced rise in resting tension was seen, although the hypoxia-induced decrease in the contractile force was not completely prevented by treatment with either agent. However, hearts which had received either 45 μM amosulalol or 83 μM phentolamine during hypoxia showed a marked recovery in contractile force after 45 min of reoxygenation; recovery of the contractile force of hearts treated with amosulalol (82.9 ± 4.4% of the initial value; $n = 6$) was greater than that with phentolamine (57.2 ± 3.8% of the initial value; $n = 6$). Perfusion of the heart for 25 min under normoxic conditions with glucose-free medium in the presence of either 45 μM amosulalol or 83 μM phentolamine, resulted in a decline in cardiac contractile force, which was followed by an appreciable recovery of the contractile force in the presence of glucose after a subsequent 45 min of normoxic perfusion (99.4 ± 2.4 or 69.2 ± 2.5% of the initial value, respectively, $n = 5$). There were no significant changes in resting tension of hearts perfused under normoxic conditions in the presence of either amosulalol or phentolamine.

High-energy phosphate content of the myocardium

After perfusion of the heart, myocardial high-energy phosphate content was measured and the results are shown in Figure 2. The initial values of ATP and CP contents were 24.6 ± 1.0 and 33.8 ± 3.0 μmol g^{-1} dry wt tissue, respectively ($n = 5$). When the heart was perfused for 25 min under normoxic conditions in the absence of glucose, myocardial ATP and CP contents decreased to 17.7 ± 1.2 and 15.7 ± 1.0 μmol g^{-1} dry wt tissue, respectively ($n = 4$). After reoxygenated perfusion with the medium containing glucose, these values tended to return toward the initial levels. The ATP content was almost completely restored, while the CP content recovered to approximately 85% of the initial level ($n = 4$). Perfusion of the heart for 25 min under hypoxic conditions resulted in a marked decrease in myocardial ATP and CP contents (7.4 ± 0.3 and 7.3 ± 0.3 μmol g^{-1} dry wt tissue, respectively; $n = 6$). Myocardial ATP and CP contents after 45 min of reoxygenation were 8.2 ± 0.2 and 13.6 ± 2.2 μmol g^{-1} dry wt tissue, respectively ($n = 6$), indicating little recovery of myocardial ATP and CP content upon reoxygenation. When the heart was subjected to hypoxic perfusion for 25 min in the presence of 45 μM amosulalol, myocardial ATP and CP contents (9.4 ± 0.4 and 9.7 ± 0.5 μmol g^{-1} dry wt tissue; $n = 6$) were slightly but significantly higher than those of hearts without the treatment. Tissue ATP content after 25 min of hypoxia in the presence of 83 μM phentolamine (11.6 ± 0.5 μmol g^{-1} dry wt tissue; $n = 6$) was higher than that not treated with phentolamine, whereas there were no significant differences in tissue CP contents of the hearts between the control and treated preparations. Treatment of hypoxic hearts with amosulalol significantly restored myocardial high-energy phosphates upon reoxygenation; myocardial ATP and CP after 45 min of reoxygenation were 22.6 ± 1.5 and 31.9 ± 1.1 μmol g^{-1} dry wt tissue, respectively

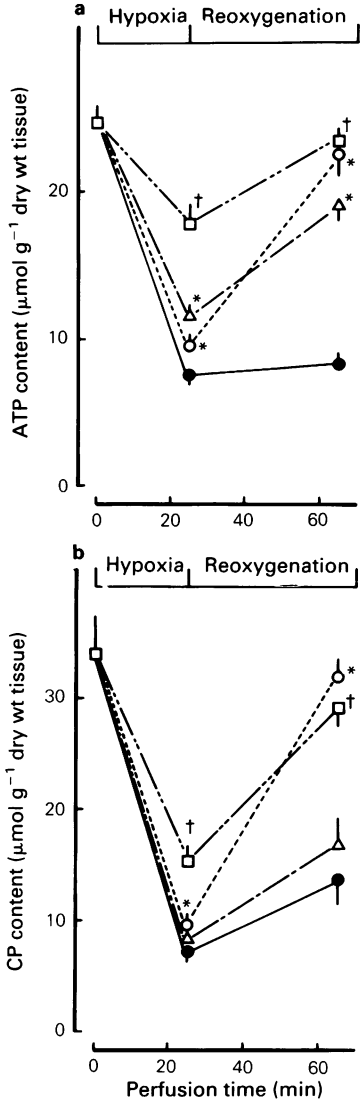


Figure 2 Myocardial ATP (a) and creatine phosphate (CP) (b) contents at 0, 25 min-hypoxia and 45 min-reoxygenation with or without (●) treatment of hypoxic hearts with either phentolamine (△) or amosulalol (○). Each value represents the mean of 4 to 6 experiments; vertical lines indicate s.e.mean. Statistical significance was evaluated between the hearts perfused under hypoxic and reoxygenated conditions in the absence and, the presence of the agents (*) or the hearts perfused under normoxic conditions (□) (†).

($n = 6$), which were almost the same as the control values. Similarly, treatment of hypoxic hearts with phentolamine significantly restored myocardial ATP and CP contents upon reoxygenation (80 and 50% of the initial value, respectively; $n = 6$). There was no significant difference in myocardial ATP and CP contents after 25 min and 65 min of normoxic perfusion between the treated and untreated preparations.

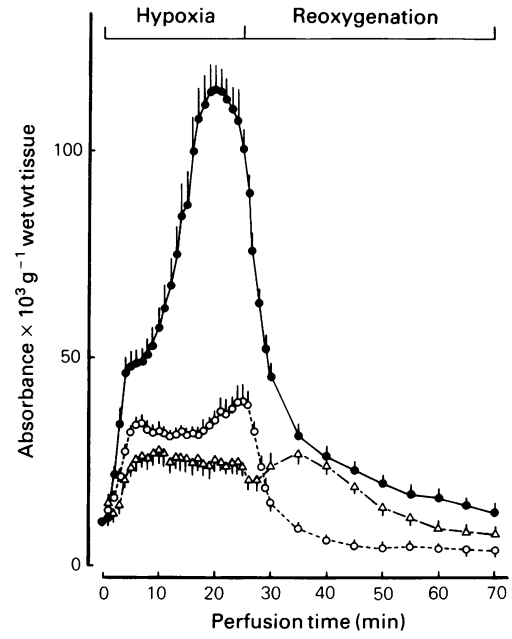


Figure 3 Changes in uv absorbance at 250 nm of the perfusate eluted from the perfused heart. (○) Represent values of the heart receiving amosulalol during hypoxia and (△) those of the heart receiving phentolamine. (●) Represent values obtained in the absence of treatment with the agents.

Changes in uv absorbance of the perfusate from perfused hearts

When the heart was exposed to hypoxia, an immediate increase in the absorbance at 250 nm of the perfusate eluted from the heart was seen, as shown in Figure 3. The absorbance was attenuated to a greater extent upon reoxygenation. Treatment of hypoxic hearts with either 83 μM phentolamine or 45 μM amosulalol markedly suppressed the hypoxia-induced increase in the absorbance of the perfusate.

H.p.l.c. analysis of the perfusate

To determine the substances in the perfusate responsible for an increase in the absorbance at 250 nm, samples of the perfusate during hypoxia and reoxygenation were analysed by h.p.l.c. The results are shown in Table 1. The control perfusate con-

Table 1 ATP metabolites released into the perfusate during hypoxia and subsequent reoxygenation

		Hypoxanthine	*Inosine*	*Adenosine*	*Total*
25 min-perfusion					
Normoxia without agents	(5)	ND	49 ± 4	ND	49 ± 4
Without treatment	(6)	221 ± 56*	1116 ± 214*	829 ± 185*	2166 ± 185*
Treatment with amosulalol	(6)	271 ± 49	18 ± 2†	13 ± 2†	302 ± 51†
Treatment with phentolamine	(6)	29 ± 9†	282 ± 55†	99 ± 25†	414 ± 88†
45 min-perfusion					
Normoxia without agents	(5)	ND	18 ± 10	ND	18 ± 10
Without treatment	(6)	362 ± 86*	1694 ± 302*	161 ± 2*	2217 ± 251*
Treatment with amosulalol	(6)	214 ± 90	16 ± 2†	2 ± 1†	232 ± 92†
Treatment with phentolamine	(6)	25 ± 10†	366 ± 40†	14 ± 7†	405 ± 45†

The heart was perfused for 25 min under hypoxic conditions in the presence and absence of phentolamine or amosulalol, followed by 45 min of reoxygenation. Values are expressed as nmol g^{-1} wet wt heart. Numbers in parentheses indicate number of experiments. ND indicates 'not detectable'. Each value represents the mean ± s.e.mean. * Significantly different from the value for the heart perfused under normoxic conditions ($P < 0.05$). † Significantly different from the value for the heart without treatment ($P < 0.05$).

tained mainly hypoxanthine and inosine and a minimal amount of adenosine. The sum of the three substances after 25 min of glucose-free normoxic and subsequently 45 min of normoxic perfusion was less than 70 nmol g^{-1} wet wt tissue. Release of hypoxanthine, inosine and adenosine was markedly increased under hypoxic conditions, which is consistent with previous results (Takeo & Sakanashi, 1983; Takew *et al.*, 1987; 1988a). The release of these compounds from the heart was also observed to a lesser extent during subsequent reoxygenation. Adenine nucleotides such as ATP, ADP and AMP or nicotinamide derivatives such as nicotine adenine dinucleotide (NAD), NAD reduced form (NADH), NAD phosphate (NADP) and NADPH were undetectable or negligible in the perfusate. Thus, the increase in absorbance was considered to be mainly due to the release of ATP metabolites from the perfused heart. Administration of 83 μM phentolamine during the hypoxic period significantly reduced the increase in ATP metabolites from the hypoxic and reoxygenated heart (81 and 82% reduction, respectively, $n = 6$). Suppression of the release of inosine and adenosine during hypoxia and reoxygenation was seen in hearts treated with amosulalol; more than 90% suppression was observed during the perfusion. No appreciable difference in ATP metabolite content of the perfusate was seen during normoxic perfusion in the presence of either phentolamine or amosulalol, as compared with that of the control heart.

Creatine kinase activity of the perfusate

Creatine kinase (CK) activity of the effluent from the perfused heart was measured. The results are shown in Figure 4. The initial value of the activity released from the perfused heart was 49 ± 5 μmol NADPH $min^{-1} g^{-1}$ wet wt tissue ($n = 59$). The CK activity of the perfusate after 25 min of glucose-free normoxic perfusion was slightly lower than the initial value. Hypoxia induced a significant increase in CK activity of the perfusate (151 ± 21 μmol NADPH $min^{-1} g^{-1}$ wet wt tissue after 25 min of hypoxia). Subsequent reoxygenation resulted in a further increase in CK release (1245 ± 383 μmol NADPH $min^{-1} g^{-1}$ wet wt tissue after 45 min of reoxygenation, $n = 6$). Perfusion of the heart under hypoxic conditions in the presence of either amosulalol or

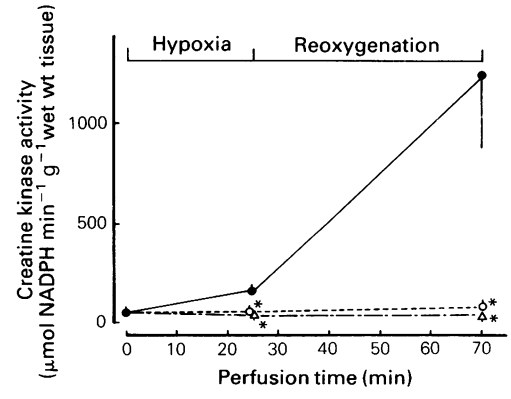


Figure 4 Creatine kinase activity of the perfusate at 0 min, 25 min-hypoxia and 45 min-reoxygenation in the absence (●) and presence of either phentolamine (△) or amosulalol (○). Each value represents the mean of 6 to 59 experiments; vertical lines indicate s.e.mean. * Significantly different from the value without treatment with the agents ($P < 0.05$).

phentolamine resulted in a marked suppression of the increase in CK release after 25 min of hypoxia (35 ± 10, and 13 ± 4 μmol NADPH $min^{-1} g^{-1}$ wet wt tissue, respectively, $n = 6$). Suppression of the release by these agents was also observed after 45 min of reoxygenation (amosulalol; 78 ± 24, and phentolamine; 81 ± 10 μmol NADPH $min^{-1} g^{-1}$ wet wt tissue, $n = 6$).

Calcium and water contents of the myocardium

The calcium and water contents in the left ventricle were measured after hypoxic and reoxygenated perfusion in the presence and absence of phentolamine and amosulalol. The results are shown in Figure 5. Calcium content of the heart perfused for 25 min under hypoxic conditions (3.7 ± 0.2 μmol g^{-1} dry wt tissue, $n = 6$) as well as for 45 min under subsequent reoxygenated conditions (4.4 ± 0.3 μmol g^{-1} dry wt tissue, $n = 6$) were significantly higher than the control (2.8 ± 0.3 μmol g^{-1} dry wt tissue, $n = 6$). The calcium content of the heart perfused under hypoxic conditions in the presence of either amosulalol or phentolamine (2.8 ± 0.1 and 2.6 ± 0.2 μmol g^{-1} dry wt tissue, respectively, $n = 6$) were significantly lower than those in the corresponding hearts in the absence of these agents. There was no significant difference in water content in the left ventricle, regardless of the conditions employed. Furthermore, under normoxic conditions, neither phentolamine nor amosulalol altered the calcium or water content of the perfused heart; for example, myocardial calcium content after 45 min of normoxic perfusion was 2.8 ± 0.2, and 2.9 ± 0.2 μmol g^{-1} dry wt tissue ($n = 5$), when hearts had been treated during 20 min glucose-free, normoxic perfusion with either phentolamine or amosulalol, respectively.

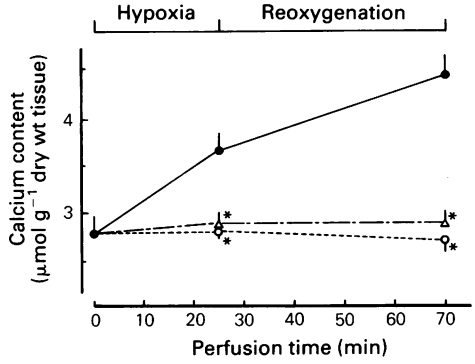


Figure 5 Myocardial calcium content at 0 min, 25 min-hypoxia and 45 min-reoxygenation with or without (●) treatment of hypoxic hearts with either phentolamine (△) or amosulalol (○). Each value represents the mean of 5 to 6 experiments; vertical lines indicate s.e.mean. * Significantly different from the value without the treatment ($P < 0.05$).

Maximal driving frequency of rabbit isolated atria

The maximal driving frequency was determined in the presence and absence of different concentrations of either phentolamine or amosulalol. The results are shown in Table 2. Amosulalol (45 μM) and phentolamine (83 μM) significantly prolonged the effective refractory period by 30 to 40%. In contrast, 30 μM or lower concentrations of phentolamine or less than 30 μM amosulalol did not elicit significant prolongation of the effective refractory period of the isolated atria.

Discussion

In the present study, rabbit isolated hearts were perfused under hypoxic and reoxygenated conditions. Under these conditions, we observed hypoxia-induced derangements in cardiac function such as cardiac contractile depression and rise in resting tension. It should be emphasized that the contractile force and the resting tension recovered little during subsequent reoxygenation. The hypoxic insults in the present study also induced changes in cardiac metabolism. Myocardial high-energy phosphates such as ATP and CP were significantly decreased to 30% of the initial value after 25 min of hypoxia, and

Table 2 Effects of phentolamine and amosulalol on the effective refractory period in rabbit isolated atria

		Control (ms)	*Effective refractory period* (% control) 1 μM	10 μM	30 μM	45 μM	83 μM
Phentolamine	(4)	158 ± 8	102 ± 3	109 ± 5	121 ± 12		139 ± 12*
Amosulalol	(5)	154 ± 8	100 ± 0	104 ± 2	116 ± 2*	133 ± 6*	

Effective refractory period was determined by measuring the maximal driving frequency of rabbit isolated atria. Effect of effective refractory period was expressed as % control. The initial resting sinus rate of the isolated atria used in the experiment for phentolamine and amosulalol was 115 ± 5 and 103 ± 6 beats min^{-1}, respectively. Numbers in parentheses indicate numbers of experiment.

* Significantly different from the control ($P < 0.05$).

were little restored after 45 min of reoxygenation. A significant accumulation of tissue calcium and release of CK from hearts was observed during hypoxic perfusion. These changes were further enhanced when the heart was reoxygenated. A release of ATP metabolites such as adenosine, inosine and hypoxanthine was also observed during hypoxic perfusion. These results are consistent with those described elsewhere (Hearse *et al.*, 1973; Schrader *et al.*, 1977; Nayler *et al.*, 1978a; Vary *et al.*, 1979; Poole-Wilson, 1980), suggesting that the experimental conditions are sufficient to produce oxygen-deficiency in most myocardial cells.

Brief periods of ischaemia are known to cause prolonged cardiac contractile dysfunction, so-called 'stunned myocardium' (Braunwald & Kloner, 1982). In a previous study (Nakahara & Takeo, 1986), it was observed that cardiac mitochondria were swollen or disrupted and the necrotic bands of myofibrils were clearly formed under the conditions similar to the hypoxic insults used in the present study. The present study also showed a release of CK from the perfused heart and an accumulation of tissue calcium during hypoxia and subsequent reoxygenation. According to Braunwald & Kloner (1982), a release of CK from the heart is a marker of irreversible damage in the myocardial cells. Furthermore, the perfusate in the present study does not contain humoral factors, hormones or nutrients except glucose, so that appreciable recovery in cardiac function and metabolism would not be expected to occur as long as myocardial cells are substantially damaged, even when oxygen is replenished. In fact, we observed in a preliminary study, that cardiac contractile force after 8 h of reoxygenation under the present experimental conditions was less than 10% of the initial value, which is similar to the contractile force after 45 min of reoxygenation. Thus, hypoxic conditions employed in the present study seem to be different from those producing stunned myocardium, and would seem to be more severe.

Treatment of hearts with a high concentration of either phentolamine (83 μM) or amosulalol (45 μM) during the hypoxic perfusion significantly suppressed the hypoxia-induced rise in resting tension, and release of CK and ATP metabolites from the heart, while hypoxia-induced decreases in cardiac contractile force and myocardial high-energy phosphates were influenced to a lesser degree. A marked effect of these agents was observed in the recovery of the contractile force upon reoxygenation; the contractile force of hearts treated with either amosulalol or phentolamine was 83 or 57% of the initial value, respectively. The recovery of the contractile force was found to be associated with the restoration of myocardial high-energy phosphates. Several investigators have demonstrated a close relationship between myocardial high-energy phosphates and recovery of cardiac contractile force after oxygen deficiency (Jennings *et al.*, 1978; Reibel & Rovetto, 1978; Hearse, 1979; Vary *et al.*, 1979; Takeo & Sakanashi, 1983). This is in agreement with our findings. Treatment of hypoxic hearts with amosulalol or phentolamine also prevented hypoxia- and reoxygenation-induced tissue calcium accumulation and reoxygenation-induced release of CK from the heart. These results indicate that treatment with these agents is capable of recovering cardiac function and metabolism after hypoxia. Sharma *et al.* (1983) have shown that treatment of anaesthetized cats with α-adrenoceptor blocking agents, 5 mg kg^{-1} phentolamine or 100 μg kg^{-1} prazosin, 2 min before reperfusion suppressed ischaemia/reperfusion-induced calcium overload. Also, Nayler *et al.* (1985) have demonstrated that pretreatment of the rat perfused heart with 1 μM prazosin before total ischaemia suppressed a gain in calcium caused by post-ischaemic reperfusion. These and the present findings suggest that some agents which possess α-adrenoceptor blocking actions are capable of protecting the myocardium from ischaemia-induced impairment of cardiac cells.

The protective action of α-adrenoceptor antagonists described above and phentolamine and amosulalol in the present study required the presence of high concentrations. In contrast, α-adrenoceptor blocking effects of phentolamine and amosulalol have been demonstrated at much lower concentrations of 0.1 to 1 μM (Shoji, 1981; Takenaka *et al.*, 1982b). If suppression of hypoxia-induced accumulation of tissue calcium was due to antagonism of α-adrenoceptors, treatment with lower concentrations might be expected to exert similar effects. We did not observe an appreciable effect of these agents on the hypoxic myocardium when lower concentrations of either amosulalol (9 μM) or phentolamine (16 μM) were used, suggesting that the protective effect seems not to be mediated through an α-adrenoceptor blocking action. This is supported by the postulation of Nayler *et al.* (1985) that prazosin-induced protection of the myocardium from ischaemic injury is due to a non-specific effect, and not antagonisms of α-adrenoceptors.

Amosulalol has been shown to exert selective α_1-adrenoceptor and non-selective β-adrenoceptor blockade with an α_1/β_1 ratio of one to one (Honda *et al.*, 1986). Its antagonistic potency at α_1 and β_1-adrenoceptors was respectively 4 times higher than phentolamine and 3 times less than propranolol (Takenaka *et al.*, 1982a). Since the results in the present study substantially failed to demonstrate an involvement of α-adrenoceptor blocking action in the beneficial recovery of post-hypoxic recovery of

cardiac function and metabolism, a β-adrenoceptor blocking action might be another possible mechanism in the case of amosulalol.

Apart from mechanisms relating to adrenoceptors, our results suggest several possible mechanisms underlying the action of amosulalol and phentolamine on hypoxic hearts. First, an energy sparing effect should be considered, like that attributed to the cardioprotective action of calcium antagonists (Fleckenstein, 1971). It should be noted that treatment with either amosulalol or phentolamine slightly but significantly preserved myocardial high-energy phosphates after 25 min of hypoxia. If this does play an important role in the recovery of cardiac contractile force, then an energy sparing effect may be relevant.

Secondly, preservation of ATP metabolites during hypoxic perfusion should be considered (Goldhaber *et al.*, 1982; Harmsen *et al.*, 1984). We have shown in a previous study that the release of ATP metabolites during hypoxic perfusion is related to the recovery of cardiac mechanical function (Takeo *et al.*, 1988a); that is, the greater the release of ATP metabolites during hypoxic perfusion, the lesser the recovery of cardiac contractile force during subsequent reoxygenation. Furthermore, in another set of experiments, we demonstrated that treatment with exogenous ATP metabolites such as adenosine, inosine and hypoxanthine during hypoxic perfusion suppresses hypoxia-induced derangements in cardiac function and metabolism, and facilitates the cardiac contractile force on replenishment of oxygen (Takeo *et al.*, 1988b). In the present study, treatment of hypoxic hearts with either amosulalol or phentolamine significantly suppressed the release of ATP metabolites from the heart during hypoxia and subsequent reoxygenation. This implies that ATP metabolites are preserved by the treatment with these agents, so that ATP synthesis is enhanced during subsequent reoxygenation.

Thirdly, protection of cardiac cells from hypoxia-induced increases in the cell membrane permeability should be considered. In the present study, treatment with either amosulalol or phentolamine during hypoxic perfusion significantly suppressed the release of CK from the perfused heart. The release of this enzyme is accepted to be a marker of an increase in cell membrane permeability (Hearse *et al.*, 1973; Hearse & Humphrey, 1975, Jennings, 1976; Ganote & Kaltenbach, 1979). Furthermore, we also observed a significant suppression in the accumulation of tissue calcium on treatment with these agents. It has been postulated that hypoxia-induced accumulation of myocardial calcium is caused by changes in cell membrane permeability (Poole-Wilson, 1980; Nayler, 1983; Nayler *et al.*, 1984). A similar conclusion was reached by Jarmakani *et al.* (1979) and Jennings *et al.* (1985, 1986). Thus, it is likely that treatment with these agents during hypoxic perfusion protects the myocardial cell membrane from hypoxia-induced increases in cell membrane permeability.

Another possible mechanism responsible for the cardioprotection of amosulalol and phentolamine may be a membrane stabilizing action. It is conceivable that this effect is related to the protective action of these agents against hypoxia- and reoxygenation-induced changes in cell membrane permeability, although, so far, there is no evidence that these two factors are correlated. In the present study, we have determined whether phentolamine and amosulalol are able to exert a membrane stabilizing action in concentrations used in the Langendorff preparation. For this purpose, effects of these agents on the maximal driving frequency of rabbit isolated atria were examined, since this action is believed to be a convenient indicator of the membrane stabilizing action of drugs (Benfey & Varma, 1966). It was found that both 83 μM phentolamine and 45 μM amosulalol produced a significant prolongation of the effective refractory period in rabbit isolated atria, suggesting a membrane stabilizing action of these agents at concentrations which elicited a beneficial effect on post-hypoxic recovery of cardiac function and metabolism. It should be mentioned that lower concentrations of these agents, such as 9 μM amosulalol and 16 μM phentolamine, which did not exert an appreciable effect on the post-hypoxic recovery of cardiac function and metabolism, appeared to produce little effect on the effective refractory period. Furthermore, phentolamine has been shown to elicit an inhibition of $\dot{V}_{max}$ of the action potential in rat ventricular muscle at concentrations ranging from 1 to 20 μM (Northover, 1983). These findings suggest that the beneficial effect of amosulalol and phentolamine observed in the present study is, at least in part, attributable to their membrane stabilizing action, rather than to antagonism of α-adrenoceptors.

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Generation of histamine-releasing activity from serum albumin by medium derived from stimulated neutrophils of rat

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1 Medium conditioned by rat neutrophils stimulated by N-formyl-methionyl-leucyl-phenylalanine (FMLP) has been found to generate mast cell histamine-releasing activity (HRA) when incubated with bovine serum albumin (BSA).

2 Histamine release increased as the concentration of BSA used to generate HRA was increased from 0.25 to 10 mg ml^{-1}, as the concentration of neurotrophil conditioned medium was increased and as the concentration of FMLP used to stimulate the neutrophils was increased. Histamine release was non-cytotoxic as it was inhibited by energy deprivation or by removal of calcium and it was accompanied by degranulation.

3 HRA was detectable after 30 min of incubation with BSA and its generation continued to increase over the 18 h of our measurements.

4 Generation of HRA was dependent upon the presence of medium from stimulated neutrophils and on the presence of BSA, although plasma could substitute for BSA. Likewise, HRA could be generated from γ-globulin although to a lesser extent than with albumin.

5 Generation was optimum at acid pH and was inhibited by prior boiling of the neutrophil conditioned medium or by the addition of pepstatin.

6 It is suggested that an enzyme(s) released from the neutrophil during stimulation acts on an albumin-like substrate to generate HRA. It is proposed that HRA is peptide in nature and may be generated during an inflammatory response.

Introduction

The local inflammatory response and the late phase reaction of the allergic response are accompanied by the emigration of neutrophils a few hours after the initial insult (Weissman *et al.*, 1980; Lemanske & Kaliner, 1982; Emes & Novak, 1983). Once in the tissue space phagocytic activity of the neutrophil ensues and their lysosomal enzymes are secreted to the extracellular environment and into intracellular phagosomes (Weissman *et al.*, 1980). In addition, there is evidence at this time for an accompanying secretion of histamine which is believed to be released from the tissue mast cell (Lemanske & Kaliner, 1982; Emes & Novak, 1983; Durham *et al.*, 1984). While the agent(s) responsible for eliciting this second phase of mast cell secretion is unknown, the infiltrating leukocytes have been implicated (Lemanske & Kaliner, 1982; Fantozzi *et al.*, 1983; Coble *et al.*, 1984; Durham *et al.*, 1984). Results from Ranadine & Cochrane (1971), Stendahl *et al.* (1983), and White & Kaliner (1987) as well as our laboratory (Cochrane *et al.*, 1988a) have suggested that neutrophils contain preformed products capable of stimulating mast cell histamine secretion. However, cell signalling can also be accomplished by the local generation of biologically active molecules via secreted enzymes acting on circulating precursors (Mains *et al.*, 1983; Carraway & Reinecke, 1984). The renin-angiotensin and the kallikrein-bradykinin systems are perhaps the best known examples of such signalling processes (Douglas, 1975). Recently Carraway *et al.* (1986, 1987) demonstrated the ability of the acid protease, pepsin, to generate a biologically active, neurotensin-related peptide from an albumin-like precursor. The generated nonapeptide

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was shown to have histamine-releasing activity similar to that of neurotensin and to bind to neurotensin receptors (Carraway *et al.*, 1986; 1987). With the array of proteases contained within the neutrophil granules (Woessner, 1979; Weissman *et al.*, 1980), we wondered if the stimulated neutrophil could act to generate biologically active molecules from serum albumin.

Here we describe the generation of mast cell histamine-releasing activity (HRA) from serum albumin by medium derived from stimulated rat neutrophils. Our results suggest that upon stimulation by chemotactic peptide, the neutrophil releases an enzyme(s) which can act on an albumin-like precursor to generate histamine-releasing activity. This activity is proposed to be peptide in nature. A preliminary account of this work has been presented previously (Cochrane *et al.*, 1988b).

Methods

Mast cells were isolated from the peritoneal and pleural cavities of male Sprague-Dawley rats (250–350 g) and purified to >90% homogeneity by centrifugation through a 22.5% metrizamide gradient as previously described (Cochrane & Douglas, 1974; Bibb *et al.*, 1986). Neutrophils were isolated from the peritoneal cavities of male Sprague-Dawley rats (300–400 g) following the induction of a sterile peritonitis. For this, 30 ml of a 0.9% NaCl solution containing 2 mg ml^{-1} shellfish glycogen Type II (Sigma) and 0.3 ml of streptomycin/penicillin (5 mg/5000 u ml^{-1}) were injected into the peritoneal cavities of anaesthetized (Nembutal, 50 mg kg^{-1}) rats. After 4 h, the animals were lightly anaesthetized with ether, killed by decapitation, and the peritoneal washings removed. Neutrophils were separated from mast cells by centrifugation through 22.5% metrizamide gradients. The interface cells were largely (>90%) neutrophils as judged by phase contrast microscopy (Cochrane & Douglas, 1974), specific staining and by their ability to secrete β-glucuronidase (Chandler *et al.*, 1983) and to generate leukotriene B_4 (Lewis & Austen, 1984) when stimulated by chemotactic peptide.

Hank's solution contained (mM): NaCl 138, KCl 5.4, CaCl 1.3, KH_2PO_4 0.5, $MgSO_4$ 1.2, Na_2HPO_4 2.5. The pH was adjusted to pH 7.2–7.4 unless otherwise noted. Sodium citrate buffer contained 0.1 M Na citrate: citric acid at pH 4.5. Before stimulation, neutrophils were incubated with cytochalasin B (5 μg ml^{-1}) for 15 min at 37°C, washed and resuspended in fresh Hank's solution. This causes the cytoplasmic (azorphil) granules to be extruded from the neutrophil when stimulated by chemotactic peptide (Weissman *et al.*, 1980).

Histamine was measured by fluorometric assay as previously described (Cochrane *et al.*, 1975). Histamine secretion is expressed as a % total (% total = supernatant histamine divided by (cell histamine plus supernatant histamine) × 100). Plasma histamine was determined by radioenzymatic assay as described previously (Carraway *et al.*, 1986). The chemotactic peptide, N-formylmethionyl-leucine-phenylalanine (FMLP, Sigma) was dissolved in dimethylsulphoxide and brought to 10^{-3} M with buffer. This solution served as a stock solution and was stored at −20°C until final dilution.

Results

Neutrophils (50–100 × 10^6 cells) were incubated at 37°C in Hank's solution and stimulated with FMLP (10^{-5} M). After 15 min, the cells were separated from the medium by centrifugation, the pH of the medium adjusted to 4.5 with acid (HCl) and bovine serum albumin (BSA, 10 mg ml^{-1}, Sigma, Fraction V) added. This mixture containing BSA was then incubated for 18 h at 37°C after which it was boiled for 15 min, centrifuged (11,000 *g* for 30 s), the supernatant fraction removed and its pH adjusted to 7.2. It was then assayed for HRA by its addition, in various dilutions, to suspensions of serosal mast cells incubated at 37°C in Hank's solution. Histamine release was determined after 10 min. The results of these experiments are shown in Figure 1. As the concentration of neutrophil incubate increased, the extent of histamine secretion increased up to a maximum of 57 ± 4%, $n = 5$. The inset in Figure 1 shows the time course for the generation of HRA from 10 mg ml^{-1} BSA incubated at 37°C, pH 4.5, with medium from FMLP-stimulated neutrophils (50 × 10^6 cells ml^{-1}) as described above. Note that significant HRA was evident after 2 h of incubation and its amount continued to increase up to 18 h. Longer periods of incubation were not used. The results shown are the mean (±s.e.mean) of three experiments assayed at a 50% dilution of generated HRA. An identical result was obtained when crystallized, lyophyllized BSA (Sigma) was used in place of Fraction V BSA. Thus a 50% dilution of generated HRA gave 54.4% histamine release when crystallized BSA was used. Again, BSA alone without neutrophil-conditioned medium produced no histamine release.

The secretory effect of the neutrophil-generated HRA on the isolated mast cell was inhibited by pretreatment of the mast cells with antimycin A (10^{-6} M) and deoxyglucose (10^{-4} M) in glucose-free medium for 10 min (this deprives the cells of a source of metabolic energy and therefore prevents exo-

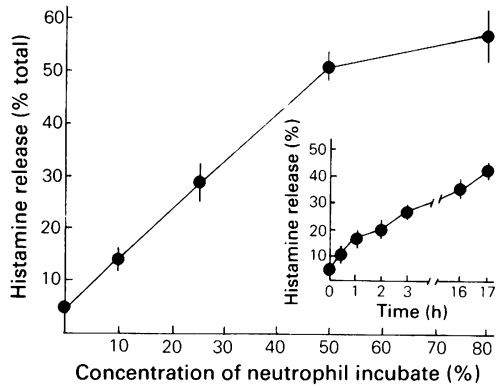


Figure 1 Histamine release from mast cells in response to various dilutions of histamine-releasing activity (HRA) generated from bovine serum albumin (BSA) by medium derived from stimulated rat neutrophils. Neutrophils ($50–100 \times 10^6$ cells ml^{-1}) were stimulated with FMLP (10^{-5} M), the medium removed and incubated with BSA (10 mg ml^{-1}) at pH 4.5 for 18 h at 37°C. It was then boiled, centrifuged ($11,000 \times g$ for 30 s), the supernatant fraction removed, the pH adjusted to 7.2 and added, at various dilutions, to suspensions of mast cells. Histamine release was then measured after 10 min. As the amount of generated HRA was increased histamine release increased to a maximum at 57 ± 4%. Each point represents the mean and vertical lines show s.e.mean, $n = 5$. Inset: time-course of generation of HRA as assayed by histamine release from isolated mast cells. HRA was generated as before using 50×10^6 neutrophils ml^{-1}. Aliquots were removed at the indicated times and assayed (at 50% dilution) for HRA. Note there is a significant generation of HRA by 2 h. Each point represents mean, $n = 3$.

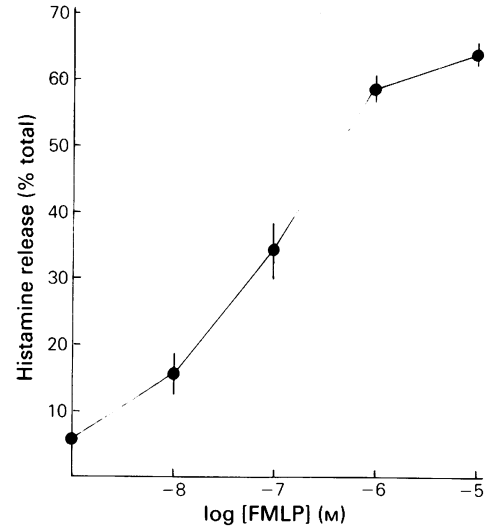


Figure 2 Histamine release from mast cells in response to histamine-releasing activity (HRA) generated from BSA (10 mg ml^{-1}) by medium derived from neutrophils (10×10^7 cells) stimulated by various concentrations of FMLP. Same procedure as in Figure 1 except that FMLP was added at the indicated concentrations. The HRA was assayed at a 50% dilution. Each point represents the mean and vertical lines show s.e.mean, $n = 3$.

cytotic histamine secretion (Foreman, 1981)). Thus mast cells that were not metabolically inhibited released 45.6 ± 3% histamine ($n = 3$) while those that were metabolically inhibited released only 15.0 ± 2% histamine ($n = 3$), when stimulated by a 50% dilution of HRA generated by our standard conditions described above. Similarly, the stimulant effect of the generated HRA on the mast cell histamine secretion was prevented by incubation of the mast cells for 3 h at 37°C in Ca-free medium containing 2 mM EGTA (Cochrane & Douglas, 1974; Cochrane *et al.*, 1982). Mast cells treated in this way and stimulated by a 25% dilution of HRA generated by our standard procedure released only 7.1 ± 1% histamine ($n = 7$), while those incubated in Ca-containing medium for an equivalent amount of time released 26.4 ± 3% histamine ($n = 3$).

HRA varied as the concentration of FMLP used to stimulate the neutrophils was varied. For these experiments, neutrophils (7×10^7) were stimulated with various concentrations of FMLP, the medium removed, the pH adjusted to 4.5 and incubated for 18 h at 37°C with BSA (10 mg ml^{-1}). HRA was then assayed at a 50% dilution as before. The results of these experiments are shown in Figure 2. Note that as the concentration of FMLP increased from 10^{-8} M to 10^{-5} M, the amount of HRA generated increased.

We next examined the effect of the concentration of BSA on the generation of HRA by medium derived from stimulated neutrophils. For these experiments, neutrophils (1×10^8) were again stimulated by FMLP (10^{-5} M), the medium removed, the pH adjusted as before, and incubated at 37°C for 18 h with various concentrations of BSA. After boiling and readjusting the pH of the medium to 7.4, the medium was assayed for HRA at a 50% dilution as above. The results of these experiments are shown in Figure 3. Note that histamine release sharply increased as the concentration of BSA was increased and reached a plateau at 2 mg BSA ml^{-1}. Further increases in the concentration of BSA, while holding the time of incubation and number of neutrophils constant, produced no further increase in HRA. A similar result was obtained when the number of stimulated neutrophils per ml used to produce the conditioned medium for incubation with BSA was increased.

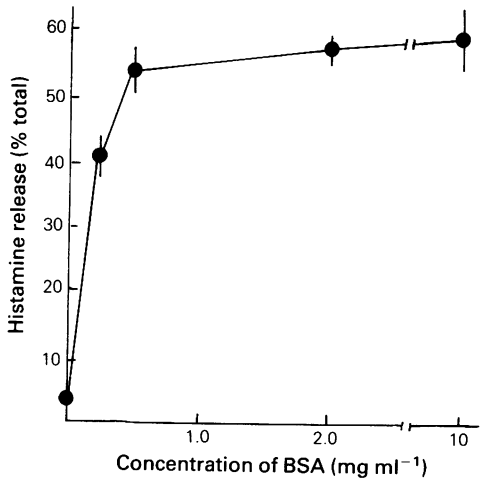


Figure 3 Histamine release from mast cells in response to histamine-releasing activity (HRA) generated from various concentrations of BSA by medium derived from stimulated neutrophils (1×10^8 cells). Same procedure as Figure 1 except that BSA was added at the indicated concentrations. Incubation was for 18 h at 37°C and HRA was assayed at a 50% dilution. Note that the generated HRA reached a plateau between 1–2 mg BSA ml^{-1}. Each point represents the mean and vertical lines show s.e.mean, $n = 3$.

In order to determine if the HRA we have measured required the presence of the stimulated neutrophil, we incubated BSA (2 mg ml^{-1}) for 18 h at 37°C, pH 4.5, in the presence and absence of medium from stimulated neutrophils. We then assayed the incubate for HRA at a 50% dilution as before. The results of this experiment are shown in Figure 4a. In the absence of medium from stimulated neutrophils, no HRA was produced. We next tested whether stimulation of the neutrophil by FMLP was required for the generation of HRA from BSA. For this, BSA (10 mg ml^{-1}) was incubated as before with medium derived from neutrophils to which either FMLP (10^{-5} M) or buffer had been added. After 18 h, the incubates were tested for HRA as before. As seen in Figure 4b, stimulation of the neutrophils by FMLP was essential for the generation of HRA. Simply incubating medium from resting neutrophils with BSA produced no significant HRA. Likewise, FMLP alone had no effect on mast cell histamine secretion and omitting BSA from the incubation medium produced no generation of HRA even after 18 h (Figure 4c). In these experiments, medium from stimulated neutrophils was incubated in the presence and absence of BSA (10 mg ml^{-1}) and assayed for HRA as before.

The above results suggested to us that an enzyme(s) was being released from the stimulated neutrophil and acting on BSA to generate HRA. To test this hypothesis we examined the effect of boiling the medium derived from stimulated neutrophils *before* it was incubated with BSA. Thus, medium from FMLP (10^{-5} M)-stimulated neutrophils was either boiled or kept at 37°C for 15 min, then incubated with BSA (10 mg ml^{-1}), processed and assayed for HRA as before. As shown in Figure 4d, boiling of the neutrophil conditioned medium before incubation with BSA completely prevented the generation of HRA.

When the pH of the incubate containing BSA and medium from FMLP-stimulated neutrophils was adjusted upwards toward more alkaline conditions, the generation of HRA was reduced (Figure 5). In these experiments, BSA (10 mg ml^{-1}) in the appropriate buffer (sodium citrate for low pH; Hank's for higher pH), was incubated at 37°C with medium from FMLP (10^{-5} M)-stimulated neutrophils (90×10^6 cells ml^{-1}). Before the start of the incubation, the pH of the medium was adjusted to those shown in Figure 5 and checked periodically throughout the incubation period. At the end of 18 h the medium was boiled and assayed for HRA at a 50% dilution as before. As the results in Figure 5 show, acidic conditions are more favourable for generating HRA with pH 4.5 being optimum. Figure 5a is the result of three or more experiments.

We next tested whether the generation of HRA was specific for albumin by substituting γ-globulin for BSA. For these experiments, incubates from stimulated neutrophils (100×10^6 cells) were incubated at 37°C, pH 4.5, as before with BSA or γ-globulin (each at 10 mg ml^{-1}) for 18 h then processed and assayed for HRA as before. The HRA generated with γ-globulin was $39.5 \pm 3\%$ at a 50% dilution, $31.5 \pm 4\%$ at a 25% dilution, and $10.5 \pm 2\%$ at a 10% dilution ($n = 3$). The corresponding values for BSA were $59.4 \pm 4\%$, $52.5 \pm 9\%$, and $22.1 \pm 5\%$, respectively ($n = 3$).

Recent experiments (Carraway *et al.*, 1986; 1987) have demonstrated an ability of the acid protease, pepsin, to generate biologically active neurotensin-related peptides. Like neurotensin, these peptides have histamine-releasing activity. In the present experiments we tested for the possibility that a pepsin-like, acid protease was being released from the stimulated neutrophil to generate HRA from BSA by using the acid protease inhibitor, pepstatin (Woessner, 1979). For these experiments, neutrophils were stimulated with FMLP as before and incubated at 37°C for 18 h with BSA (2 mg ml^{-1}) in the presence or absence of pepstatin (10^{-3} M). The incubate was then prepared and assayed for HRA as before. The results of this experiment (Figure 5b) indicate that in the presence of pepstatin, there was no generation of HRA. Pepstatin alone had no effect on the

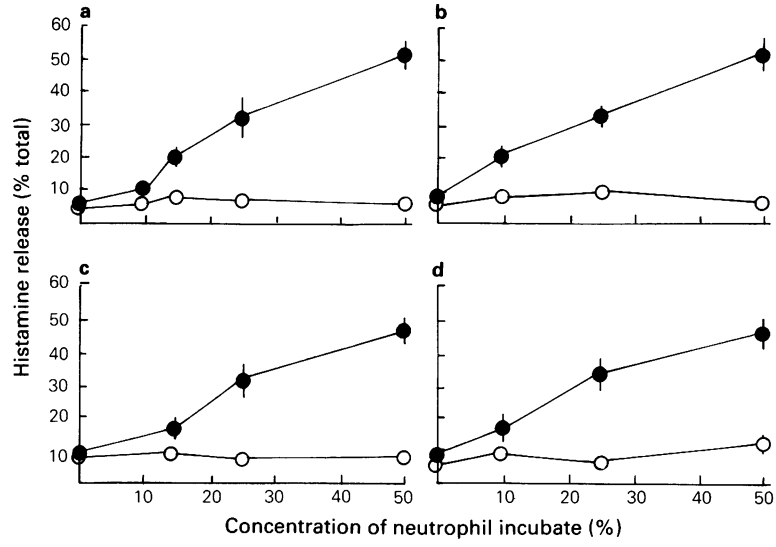


Figure 4 Histamine release from mast cells showing the effect of the presence or absence of neutrophils (a), chemotactic peptide stimulation (b), BSA (c), or boiling of the medium derived from stimulated neutrophils (d), on the generation of histamine-releasing activity (HRA). (a) BSA (10 mg ml^{-1}) was incubated for 18 h at 37°C pH 4.5 in the presence (●) or absence (○) of medium from stimulated neutrophils. The incubate was then assayed for HRA as before. Note that in the absence of neutrophils there was no generation of HRA. (b) Neutrophils were incubated at 37°C in the presence (●) or absence (○) of FMLP for 20 min, the medium removed and incubated with BSA (10 mg ml^{-1}) for 18 h at 37°C, pH 4.5. The incubate was then assayed for HRA as before. In the absence of FMLP, there was no generation of HRA. (c) Medium derived from stimulated neutrophils was incubated for 18 h at 37°C pH 4.5 in the presence (●) or absence (○) of BSA (10 mg ml^{-1}). The incubate was then assayed for HRA. The presence of BSA was necessary for the generation of HRA. (d) Medium derived from stimulated neutrophils was either incubated at 37°C for 20 min (●) or boiled for 20 min (○). Each was then incubated with BSA (10 mg ml^{-1}) for 18 h at 37°C, pH 4.5 and assayed for HRA as before. Note that boiling of the medium derived from stimulated neutrophils before its incubation with BSA prevented the generation of HRA. In each graph, each point represents the mean, and vertical lines s.e.mean, of three experiments.

mast cell nor did it inhibit the secretory activity elicited by compound 48/80 or neurotensin (data not shown). In other experiments, treatment of diluted HRA (1:4 dilution with buffer) generated by our standard procedure, with carboxypeptidase A (20 μg ml^{-1} for 1 h at 37°C pH 7.5) resulted in a 50% loss in the ability of HRA to release histamine from mast cells (mean of 3 experiments). A similar treatment of the peptide, neurotensin (10^{-5} M), resulted in a 63% loss of its histamine-releasing activity (28.3 $\pm$ 5.7% release to 10.5 $\pm$ 1.5% release, $n = 4$).

In each case, boiling (20 min) of the carboxypeptidase before incubation with HRA or neurotensin prevented the observed loss in histamine releasing ability. We next tested whether fresh plasma could substitute for BSA in the generation of HRA by medium from stimulated neutrophils. Medium from FMLP-stimulated neutrophils was incubated at 37°C for 18 h with fresh rat plasma diluted either 1:1 or 1:10 with Hank's buffer. The incubate was then processed and assayed for HRA as before. As shown in Figure 6, incubation of plasma with the medium from stimulated neutrophils resulted in a concentration-dependent generation of HRA. Plasma alone when added to isolated mast cells caused no histamine secretion.

Finally, we tested the ability of the generated HRA to increase cutaneous vascular permeability and to elevate plasma histamine levels (Carraway *et al.*, 1986). For the first set of experiments, rats were anaesthetized with sodium pentabarbitone (Nembutal, 50 mg kg^{-1}) and Evans Blue dye (0.4 ml of 1% in saline) injected intravenously. The abdominal area was then shaved and after 5 min, saline, HRA (a 50% dilution of a 2 h incubation of 0.1, 0.5, 1.5 or 6.0 mg ml^{-1} with stimulated neutrophil medium), neurotensin (0.1 nmol), compound 48/80 (1 μg ml^{-1}), each in a 100 μl volume, were injected intradermally. The animals were killed 10 min later and the inner surface of the skin exposed and exam-

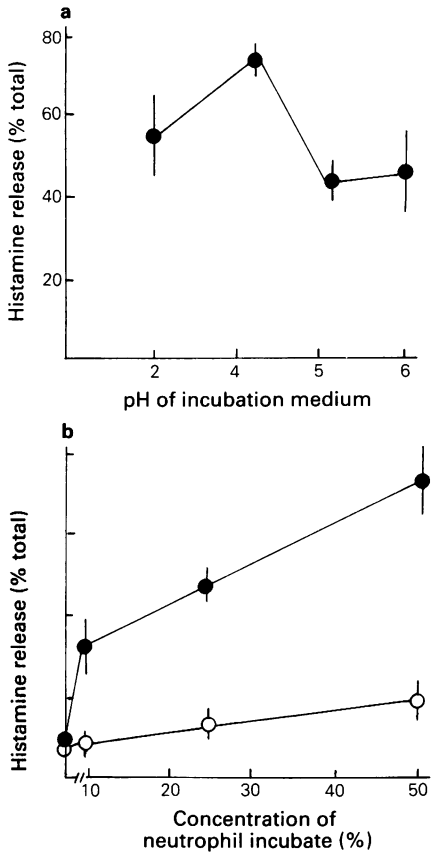


Figure 5 Histamine release from mast cells showing the effect of (a) pH and (b) pepstatin on the generation of histamine-releasing activity (HRA) from BSA by medium derived from stimulated neutrophils. (a) Neutrophils were stimulated with FMLP (10^{-5} M) as before and the medium incubated with BSA (10 mg ml^{-1}) for 18 h at 37°C at the indicated pH. The incubate was then assayed for HRA as before. (b) Neutrophils were stimulated as in (a) and the medium incubated with BSA (10 mg ml^{-1}) for 18 h at 37°C in the presence (●) or absence (○) of pepstatin (1×10^{-3} M). The incubate was then assayed for HRA as before. In the presence of the protease inhibitor pepstatin, no HRA was generated. Each point represents the mean and vertical lines show s.e.mean; (a) $n \geqslant 3$, (b) $n = 3$.

ined for blueing around the site of the injection. As shown in Figure 7, HRA elicited a pronounced increase in vascular permeability that was related to the amount of HRA injected. The intensity of the response was similar to those produced by neurotensin and compound 48/80 (Carraway *et al.*, 1982). Saline alone produced little increase in permeability.

In the second set of experiments, the level of plasma histamine was measured in anaesthetized

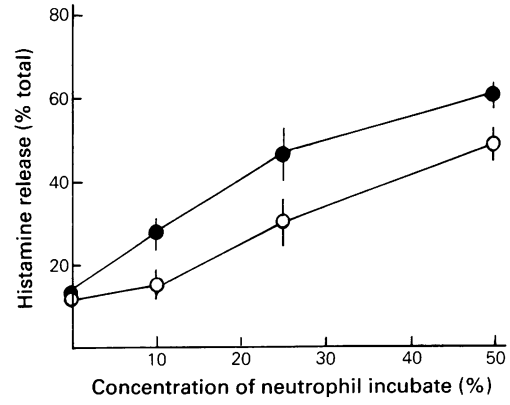


Figure 6 Histamine release from mast cells in response to histamine-releasing activity (HRA) generated from plasma by medium derived from stimulated neutrophils. Same procedure as Figure 1 except that fresh rat plasma (at a 1:1 (●) or 1:10 (○) dilution) was used in place of BSA. Each point represents the mean and vertical lines show s.e.mean, $n = 3$.

(sodium pentabarbitone, 50 mg kg^{-1}) rats following a single intravenous injection (0.4 ml) of either saline (0.9%) or a 50% dilution of pH adjusted HRA (2 mg ml^{-1} BSA, pH 4.0, at 37°C for 18 h with neutrophil conditioned medium). Samples of venous blood were then collected into ice-cold heparinized plastic tubes at 2, 4, 6, 9, 15 and 30 min after the injection. The plasma was then separated and assayed for histamine by radioenzymatic assay. The injection of HRA produced a sharp increase in plasma histamine that was evident at 2 min (13.0 ng per 100 μl plasma) and then declined back to control levels (1.0 ng per 100 μl plasma) at 30 min. The values of plasma histamine were: 8.0 ng per 100 μl at 4 min, 6.2 ng at 6 min, 4.5 ng at 9 min and 1.8 at 15 min after the injection.

Discussion

The results presented here demonstrate the ability of medium derived from stimulated rat neutrophils to generate, in a time-dependent manner, histamine-releasing activity from bovine serum albumin (Figure 1) or from fresh rat plasma (Figure 6). When added to isolated mast cells this HRA elicited a dose-dependent, non-cytoxic release of histamine, when injected intradermally it produced a marked increase in vascular permeability, and when injected intravenously it produced a prompt increase in the level of plasma histamine. We suggest that HRA is being generated from albumin (or an albumin-like precursor) by enzymes, possibly acid proteases, released from neutrophil lysosomal granules during

stimulation by chemotacic peptide. This hypothesis is supported by the following observations: (1) the generation of HRA required the presence of stimulated neutrophils together with BSA (Figure 4a, b, c); (2) HRA was produced in a time-dependent fashion (Figure 1, inset) and increased as the concentrations of BSA or FMLP were increased (Figures 2 and 3); (3) HRA generation showed a dependency on pH (Figure 5a), was inhibited by boiling the neutrophil conditioned medium before its incubation with BSA (Figure 4d), and was inhibited by the enzyme inhibitor, pepstatin (Figure 5b).

The secretory response of the mast cell elicited by HRA was non-cytotoxic as it was inhibited by metabolic deprivation or by Ca depletion. HRA is therefore similar to compound 48/80 (Cochrane & Douglas, 1974; Carraway *et al.*, 1982; Cochrane *et al.*, 1982), neurotensin (Carraway *et al.*, 1982), somatostatin (Theoharides & Douglas, 1978), and many other agents in eliciting a secretory response from the isolated mast cell (Cochrane, 1985). Thus it is likely that HRA stimulates the release of histamine by the common mechanism of exocytosis (Cochrane & Douglas, 1974; Foreman, 1981; Cochrane, 1985).

The nature of the HRA generated from BSA by stimulated neutrophils is at present unknown although it seems likely to us that it is a small peptide(s) cleaved from a larger precursor molecule such as albumin. Crude extracts of whole human neutrophils have been shown to generate a small, neutral peptide that was capable of stimulating contraction of the guinea-pig ileum (Wintroub *et al.*, 1974). Recently, studies by Carraway *et al.* (1986, 1987) have shown that small, neurotensin-related peptides, with a range of biological activities, can be generated from albumin-like precursors by the action of the enzyme, pepsin. Moreover, in the present experiment the biological activity of HRA was partially destroyed by treatment with carboxypeptidase A; a procedure that destroys the histamine-releasing activity of neurotensin as well as its receptor binding activity (Carraway *et al.*, 1986; 1987). In addition, we have found that HRA is recoverable from acid: acetone extracts of the generated material and that HRA is not able to be precipitated by 5% trichloroacetic acid (Cochrane & Boucher, unpublished results).

Whether the HRA we have described is generated *in vivo* is at the present time unknown. However, its ability to stimulate a secretory response from the isolated mast cell (Figure 1) and to elicit an increase in vascular permeability when injected intradermally (Figure 7), together with its generation by stimulated neutrophils (Figures 2, 3, 4 and 5), suggest a possible

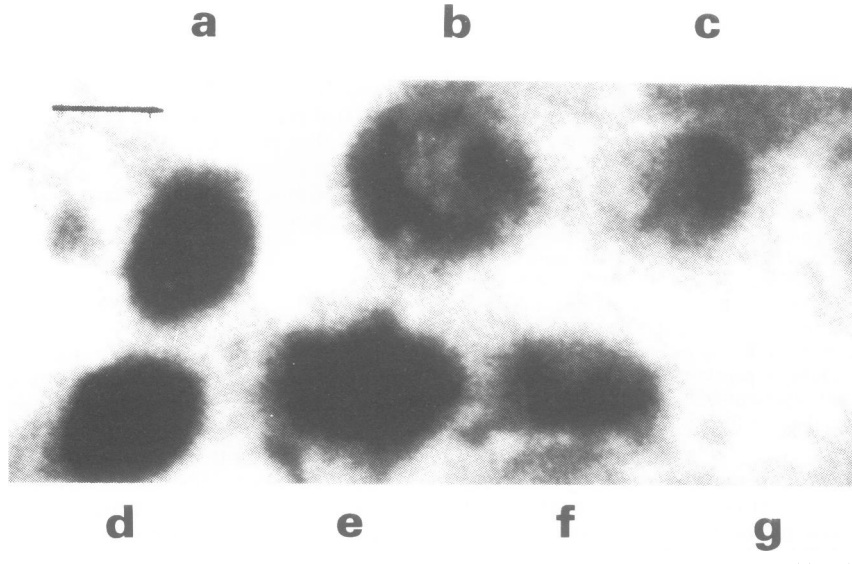


Figure 7 Effect of generated histamine-releasing activity (HRA) on cutaneous vascular permeability. Anaesthetized rats were injected intravenously with Evans Blue dye (0.4 ml of 1%). Five minutes later neurotensin, 0.1 nmol (a), a 50% dilution of HRA generated b incubation of 0.1 (c), 0.5 (f), 1.5 (b), or 6.0 (e) mg ml^{-1} BSA with medium from stimulated neutrophils, compound 48/80 1.0 $\mu g\,ml^{-1}$ (d), or saline (g) were injected intradermally. The animal was killed and the abdominal skin removed after 10 min. Note that HRA produced a marked increase in vascular permeability (blueing) around the injection site. Horizontal bar represents 10 mm.

role in the inflammatory response. During inflammation, there is a delayed invasion by neutrophils into the affected area where they phagocytose foreign particles (e.g. bacteria) and secrete their lysosomal granule enzymes. Among the various enzymes secreted are cathepsins (Woessner, 1979) which are known to cleave biologically active fragments from larger molecules (Perez *et al.*, 1983; Zull & Chuang, 1985; Pillai & Zull, 1986) and to be inhibited by pepstatin (Woessner, 1979). Thus it may be that a cathepsin released from the stimulated neutrophil during inflammation generates HRA from albumin which has entered the tissue space as a consequence of the initial increase in vascular permeability (Wasserman, 1979). This HRA could then initiate a second wave of mast cell secretion (Wasserman, 1979; Lemanske & Kaliner, 1982; Durham *et al.*, 1984) and possibly have other biological activities for example on monocytes or lymphocytes. Experiments are currently in progress to test this hypothesis.

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Effects of amiloride in guinea-pig and rat left atrial contraction as affected by frequency of stimulation and $[Ca^{2+}]_0$–$[Na^+]_0$ ratio: role of Na^+/Ca^{2+} exchange

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1 The effect of amiloride (0.5 mM) on guinea-pig and rat left atria driven at various rates of stimulation and different $[Ca^{2+}]_0$–$[Na^+]_0$ ratios has been studied.

2 Amiloride elicited a positive inotropic response in guinea-pig left atria driven at 0.1 Hz, 0.5 Hz and 1 Hz when $[Ca^{2+}]_0$ was 3.6 mM, 1.8 mM and 0.9 mM respectively but not when $[Ca^{2+}]_0$ was 2.7 mM at 0.1 Hz, 0.9 mM at 0.5 Hz and 0.45 mM at 1 Hz.

3 A positive inotropic response was obtained in guinea-pig left atria driven at 0.1 Hz and 1 Hz when $[Ca^{2+}]_0$–$[Na^+]_0^2$ was increased respectively from 8×10^{-5} to 16×10^{-5} and from 2×10^{-5} to 8×10^{-5}. The positive inotropic effect was evident only when the ratio was increased by increasing $[Ca^{2+}]_0$ and not by decreasing $[Na^+]_0$.

4 In the presence of amiloride, the force of contraction of guinea-pig left atria decreased instead of increasing, when the rate of stimulation was lowered from 1 Hz to 0.01 Hz. Amiloride inhibited the post-rest potentiation.

5 In rat left atria amiloride was devoid of any effect in all the above-mentioned experimental conditions.

6 It is suggested that the pattern of cardiac actions of amiloride can be explained by the inhibition of the Na^+/Ca^{2+} exchange system.

Introduction

Amiloride is a potassium-sparing diuretic that has been proposed as a useful pharmacological tool for elucidating the molecular basis and physiological role of Na transport systems in tissues and cells (Benos, 1982). In various epithelial tissues amiloride was shown to inhibit Na transport through two pathways, namely a conductive pathway and a Na^+/H^+ exchange system (Cuthbert & Fanelli, 1978; Frelin *et al.*, 1984). Recently, it has been demonstrated that amiloride inhibits Na^+/Ca^{2+} exchange in cardiac sarcolemmal vesicles (Floreani & Luciani, 1984; Siegl *et al.*, 1984; Debetto *et al.*, 1987) and prevents vascular smooth muscle contraction most probably through inhibition of Na^+/Ca^{2+} exchange (Bova *et al.*, 1988). *In vivo* and *in vitro* studies have shown that amiloride affected the inotropic and toxic responses of the cardiac muscle to digitalis glycosides (Seller *et al.*, 1975a,b; Waldorff *et al.*, 1981; Floreani & Luciani, 1984; Kennedy *et al.*, 1985). In electrophysiological studies amiloride induced lengthening of the action potential duration and reduction of the amplitude and the maximum rate of rise in ventricular muscle, sino-atrial node and Purkinje fibres (Marchese *et al.*, 1985; Satoh & Hashimoto, 1986). Furthermore, it has been shown that amiloride induced positive inotropic and negative chronotropic effects in isolated atrial muscle (Pousti & Khoyi, 1979). These effects have been confirmed (Floreani & Luciani, 1984; Kennedy *et al.*, 1986; Barret & Kau, 1986) and the hypothesis that the positive inotropic effect of amiloride is due to the inhibition of Na^+/Ca^{2+} exchange has been put forward (Floreani & Luciani, 1984; Luciani & Floreani, 1985; Kennedy *et al.*, 1986). A negative inotropic effect in cultured chick heart cells has been reported for amiloride (Kim & Smith, 1986) and a negative (Siegl *et al.*, 1984) or positive inotropic response (Floreani *et al.*, 1987) in guinea-pig papillary muscle has been observed for the amiloride derivative, 3'-4'dichlorobenzamil.

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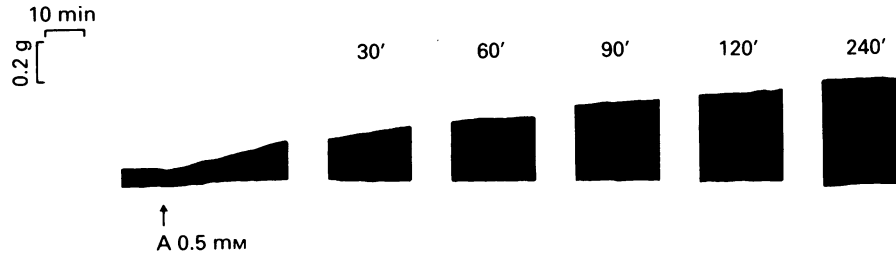


Figure 1 Record showing the time course of the positive inotropic effect of amiloride (A) in guinea-pig left atria driven at 1 Hz in normal $[Ca^{2+}]_o$ (1.8 mM) and $[Na^+]_o$ (149.2 mM). The mean values (±s.e.mean) of control contraction and of the contraction in the presence of amiloride were 90 ± 27 mg and 650 ± 140 mg respectively (5 preparations).

Since it is known that the Na^+/Ca^{2+} exchange system can move calcium in either direction across the cardiac sarcolemma (Sheu & Blaustein, 1986), the discrepancies in the effects of amiloride on cardiac contractility may be explained by postulating that the direction of Ca^{2+} movements through the Na^+/Ca^{2+} exchange may have different relevance in cardiac muscle of different species and under different experimental conditions.

In addition to the inhibition of the Na^+/Ca^{2+} exchange, several mechanisms have been proposed to account for the cardiac effects of amiloride, namely changes in ionic currents through membrane channels, inhibition of Na^+/K^+ ATPase and of Na^+/H^+ exchange (Frelin *et al.*, 1984; Marchese *et al.*, 1984; Kennedy *et al.*, 1985; Kim & Smith, 1986; Satoh & Hashimoto, 1986).

It has been hypothesized that Na^+/Ca^{2+} exchange may play a role in the influence exerted on cardiac contractility by the pattern of stimulation and by the $[Ca^{2+}]_o$–$[Na^+]_o^2$ ratio, which is believed to determine the amount of intracellular calcium available for binding to the contractile proteins (Orkand, 1968; Allen *et al.*, 1976; Lee *et al.*, 1985; Sutko *et al.*, 1986; Gibbons, 1986).

The effect of amiloride in guinea-pig and rat atrial myocardium driven at various frequencies of stimulation and in ionic conditions that alter the transmembrane distribution of either sodium or calcium has been investigated. Results show that the positive inotropic effect of amiloride is species-dependent (the drug is not active in rat atria) and is strictly related to the frequency of stimulation and to the $[Ca^{2+}]_o$–$[Na^+]_o$ ratio.

Methods

The experiments were performed on guinea-pig (250–350 g) and rat (200–300 g) left atria. The tissues were attached to two platinum hooks mounted on one end of a plexiglas holder, and placed vertically in water-jacketed 16 ml organ baths containing physiological salt solution continuously bubbled with 95% O_2: 5% CO_2 at 35°C and pH 7.36–7.40. The composition of the medium was (mM): NaCl 137, KCl 5.4, $CaCl_2 \cdot 2H_2O$ 1.8, $MgCl_2 \cdot 6H_2O$ 1, $NaH_2PO_4 \cdot 2H_2O$ 0.4, $NaHCO_3$ 12 and glucose 5.4.

The preparations were stimulated through the platinum hooks, which were connected to a GRASS S44 stimulator, by square wave pulses at a strength of twice the threshold voltage and a duration of 0.8–1.2 ms. Force generation was monitored by connecting one end of the tissue to an isometric force transducer (Battaglia Rangoni KV 200). A rough length-tension relationship was mapped out and the tissues were kept at the length at which submaximal tension had developed. An equilibration time of 1–1.5 h was allowed before the start of the experiments. The low-Na^+ solution was made by equiosmolar substitution of sucrose for NaCl.

In the trials designed to explore the force-frequency relationship, the frequencies ranged from 1 Hz to 0.01 Hz or from 1 Hz to 3 Hz and the steady force at each stimulation rate was considered. The amplitude-interval relationship of the first contraction elicited after different times of rest was determined by driving the atria under steady-state conditions at 1 Hz. The amplitude measured was that of the first contraction elicited 1 min, 2 min, 4 min, 8 min, 16 min after the end of the periods of steady-state condition. Since the effect of amiloride was very slow to reach equilibrium, in all the experiments in which the effect of the drug was observed in relation to the changes of the parameters of stimulation, we used one atrium as a control, driving it at 1 Hz for the same time as the atrium exposed to the drug.

In this study only one concentration of amiloride was used (0.5 mM), a concentration which is very close to the EC_{50} of the inotropic effect (Floreani & Luciani, 1984).

Results

Effect of frequency of stimulation and $[Ca^{2+}]_o$ on inotropic response to amiloride

Amiloride (0.5 mM) elicited a very slowly developing positive inotropic response (peak effect within about 4 h, increase from the control 659 ± 129%) in guinea-pig left atria bathed in normal physiological solution and stimulated at 1 Hz (Figure 1). Rat left atria in the same experimental conditions did not respond to amiloride.

When amiloride was applied to guinea-pig left atria driven at 0.1 Hz, the positive inotropic response did not occur (Figure 2a) unless Ca^{2+} was increased to 3.6 mM (Figure 2b). Since a direct relation has been found between intracellular Ca^{2+} activity and extracellular $[Ca^{2+}]$ or frequency of stimulation (Chapman, 1983), the positive inotropic effect of amiloride appeared to be strictly related to intracellular $[Ca^{2+}]$. To analyse this effect, we exposed guinea-pig left atria to amiloride which were driven at different frequencies of stimulation in different extracellular $[Ca^{2+}]$. As shown in Figure 3, a positive inotropic effect was not observed at 0.1 Hz,

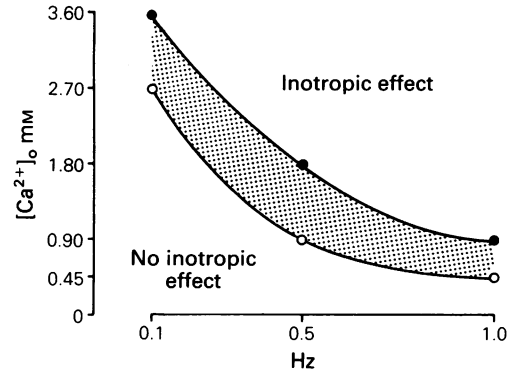


Figure 3 In guinea-pig left atria driven at 0.1 Hz, 0.5 Hz and 1 Hz two critical extracellular Ca^{2+} concentrations for each frequency of stimulation were determined: the lowest concentration (●) at which amiloride elicited positive inotropic response and the highest concentration (○) at which amiloride had no positive inotropic effect. The observations were made on separate atria, and at least four atria were used for each combination of $[Ca^{2+}]_o$ and frequency.

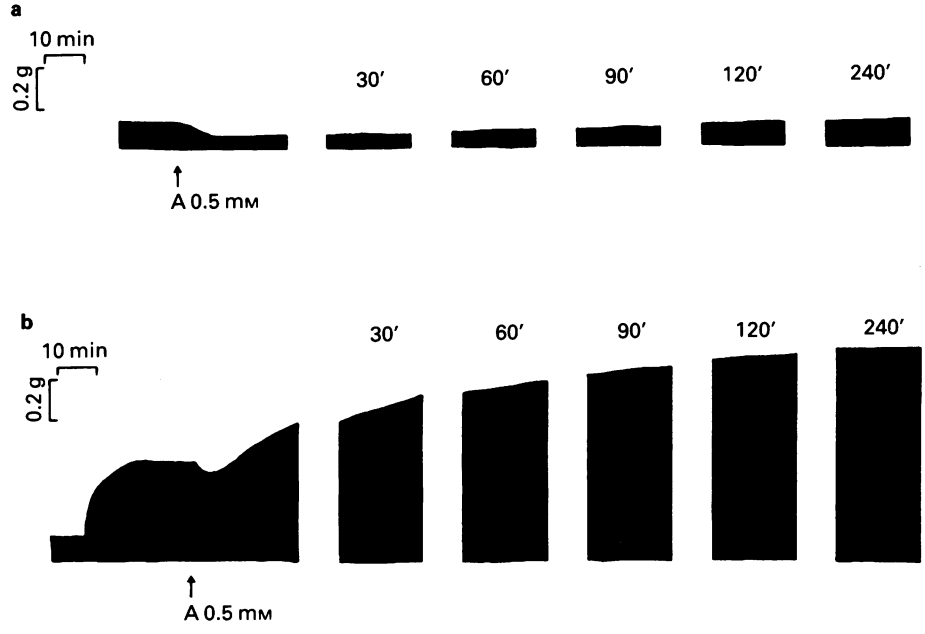


Figure 2 Guinea-pig left atria driven at 0.1 Hz. (a) Amiloride (A) had no positive inotropic effect in the atria bathed in normal Ca^{2+} ($CaCl_2$ 1.8 mM). The mean value (±s.e.mean) of contraction was 110 ± 35 mg. Five preparations were used. (b) Amiloride elicited positive inotropic response in left atria bathed in high Ca^{2+} ($CaCl_2$ 3.6 mM). The mean values (±s.e.mean) of control contraction and of contraction in the presence of amiloride were 519 ± 76 mg and 1170 ± 136 mg respectively. $[Na^+]_o$ was 149.2 mM in (a) and (b).

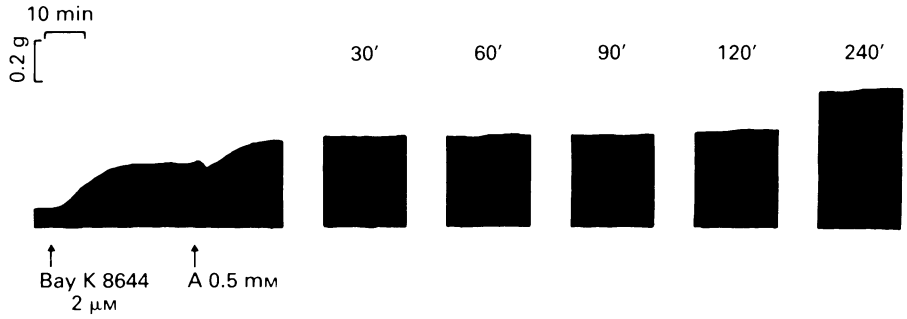


Figure 4 Record showing the positive inotropic effect of amiloride (A) in guinea-pig left atria driven at 0.1 Hz previously exposed to Bay K8644 (2 μM) ($CaCl_2$ 1.8 mM). The mean value ($\pm$s.e.mean) of the contraction in the presence of Bay K8644 was 333 mg $\pm$ 45; after the addition of amiloride the contraction was 593 mg $\pm$ 75. Three preparations were used.

0.5 Hz or 1 Hz in extracellular $[Ca^{2+}]$ below 2.7 mM, 0.9 mM or 0.45 mM respectively. The positive inotropic response was elicited by amiloride at 0.1 Hz in $[Ca^{2+}]_0$ 3.6 mM, at 0.5 Hz in $[Ca^{2+}]_0$ 1.8 mM and at 1 Hz in $[Ca^{2+}]_0$ 0.9 mM.

Therefore for each rate of stimulation a certain extracellular Ca^{2+} concentration existed below which amiloride was not able to induce the positive inotropic response. Further support for the relationship between intracellular $[Ca^{2+}]$ and the effect of amiloride was given by the experiment illustrated in Figure 4 in which positive inotropic response to the drug was observed in guinea-pig left atria driven at 0.1 Hz in $[Ca^{2+}]_0$ 1.8 mM provided that the tissue was previously exposed to Bay K 8644, which is known to enhance Ca^{2+} entry into cardiac cells (Brown *et al.*, 1984). An initial negative inotropic effect was observed, the duration and magnitude of which varied in different experimental conditions (Figures 1, 2, 4). Although no clear explanation of this effect can be given at present, we suggest that the transient negative inotropic response is due either to the known inhibitory effect of amiloride on Na^+/H^+ exchange (Frelin *et al.*, 1984) or to a blockade of T-type Ca^{2+} channels (Tang *et al.*, 1988).

Guinea-pig left atria driven at 1 Hz or 0.1 Hz in different $[Ca^{2+}]_0/[Na^+]_0^2$

Contractility of isolated heart preparations is strongly influenced not only by extracellular $[Ca^{2+}]$ but also by extracellular $[Na^+]$ (Blaustein, 1974). It has been found that intracellular Ca^{2+} and force of contraction are approximately related to the ratio $[Ca^{2+}]_0/[Na^+]_0^2$ (Chapman, 1983). The pattern of the cardiac action of amiloride with respect to the

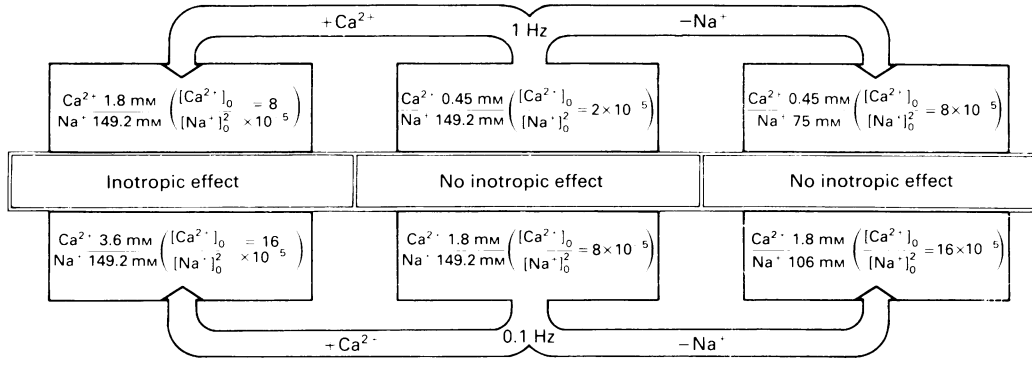


Figure 5 Influence of $[Ca^{2+}]_0/[Na^+]_0^2$ ratio at 0.1 Hz and 1 Hz on the ability of amiloride to induce positive inotropic responses in guinea-pig left atria. The effect of amiloride was tested at two different $[Ca^{2+}]_0/[Na^+]_0^2$ ratios for each frequency of stimulation. The $[Ca^{2+}]_0/[Na^+]_0^2$ was changed either by increasing $[Ca^{2+}]_0$ or by decreasing $[Na^+]_0$. In each condition at least four preparations were used.

$[Ca^{2+}]_0/[Na^+]_0^2$ ratio respectively at 1 Hz and 0.1 Hz was therefore examined. A positive inotropic response was obtained at 0.1 Hz when the $[Ca^{2+}]_0/[Na^+]_0^2$ ratio was increased from 8×10^{-5} to 16×10^{-5} and at 1 Hz from 2×10^{-5} to 8×10^{-5}. However, as shown in Figure 5, a positive inotropic effect was elicited only when the ratio was increased by increasing $[Ca^{2+}]_0$ and not by decreasing $[Na^+]_0$.

Thus the drug did or did not induce positive inotropic response at equivalent $[Ca^{2+}]_0/[Na^+]_0^2$ ratios, according to how the value was reached.

Force-frequency relationship and post-rest contractions of guinea-pig and rat left atria

The force-frequency relationship and post-rest inotropy have been used as an indirect approach to study the mechanisms which determine at any time the amount of Ca^{2+} available for the contractile proteins (Blinks & Koch-Weser, 1961; Kruta, 1964; Lipsius *et al.*, 1982).

Amiloride did not modify the force-frequency relationship of guinea-pig left atria in the range of high frequencies, from 1 Hz to 3 Hz but strongly influenced the behaviour of the force of contraction in response to a decrease in the rate of stimulation, from 1 Hz to 0.01 Hz. In the presence of the drug, the contractility of guinea-pig left atria decreased, instead of increasing, when the frequency was lowered from 1 Hz to 0.01 Hz (Figure 6). Moreover, amiloride inhibited the potentiation of the first beat observed when the preparations driven at 1 Hz were left quiescent for 1 min, 2 min, 4 min, 8 min and 16 min (Figure 7). Amiloride did not influence rat left atria exposed to the same changes of the modalities of stimulation.

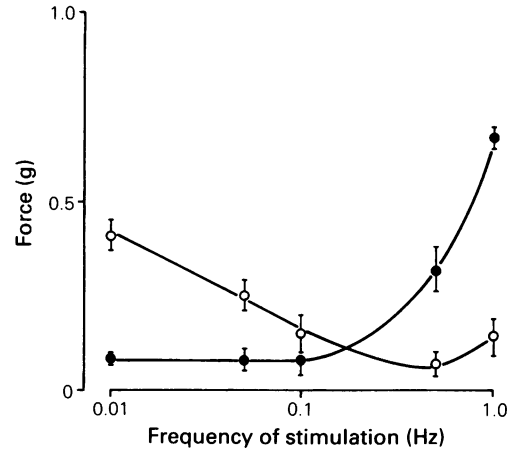


Figure 6 Force-frequency relationship of guinea-pig left atria as affected by amiloride. In the range of low frequencies, the contraction increases with decreasing frequency (○); in the presence of amiloride (●) a direct relation between force and frequency was observed. Since the effect of amiloride was very slow to reach equilibrium, two sets of four atria were used, one set serving as a control. The atria were driven at 1 Hz for at least 210 min and then the frequency was decreased. Each point represents the mean value of four observations, vertical bars showing s.e.mean.

Discussion

The results show that the positive inotropic effect of amiloride in guinea-pig left atria was markedly affected by frequency of stimulation and extracellular calcium and sodium concentrations, in that it was elicited only at certain combinations of values of these parameters. Rat left atria did not respond to amiloride.

The dependence of contractile behaviour on external $[Ca^{2+}]$ and stimulation frequency of isolated cardiac muscle preparations from most mammalian species is a well known phenomenon (Blinks & Koch-Weser, 1961; Gibbons, 1986). In ventricular muscle a relationship has been established between the strength of the twitch, intracellular calcium concentration and frequency of driving (Bers, 1983; Chapman, 1983; Allen *et al.*, 1984). The relation between the force of contraction and intracellular calcium, calculated from aequorin signals, was found to be unchanged when the contraction was increased either by raising the calcium concentration of the bathing solution or by increasing the frequency of stimulation (Allen & Kurihara, 1980). Measurement of intracellular Ca^{2+} activity with Ca^{2+}-sensitive microelectrodes has demonstrated that intracellular calcium increases with stimulation rate (Lado *et al.*, 1982) or with increasing extracellular calcium (Sheu & Fozzard, 1982).

Amiloride elicited positive inotropic responses in guinea-pig atria driven at 1 Hz and 0.5 Hz when bathed in external $[Ca^{2+}]$ 1.8 mM but did not when the external $[Ca^{2+}]$ was lowered to 0.45 mM and to 0.9 mM respectively. At 0.1 Hz, amiloride did not exert a positive inotropic effect unless $[Ca^{2+}]_0$ was increased from 1.8 mM to 3.6 mM or the atria were previously exposed to the calcium agonist Bay K 8644. This pattern of action suggests that intracellular $[Ca^{2+}]$ critically influenced the effect of amiloride on the force of contraction, since the appearance of the inotropic effect seemed to be closely linked to experimental conditions in which intracellular calcium was raised above a certain level by increasing the frequency, the bathing $[Ca^{2+}]$ or by adding Bay K 8644.

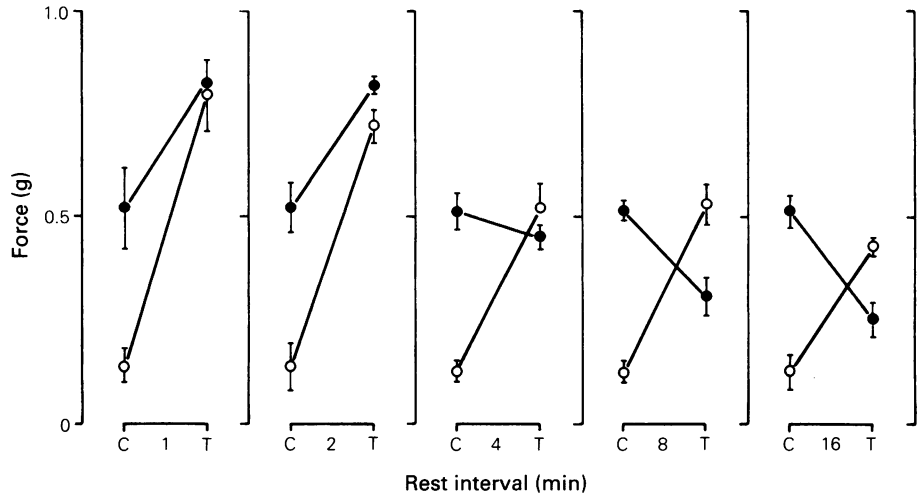


Figure 7 Inhibition of guinea-pig left atria post-rest potentiation by amiloride (●). The atria were subjected to rest intervals of 1, 2, 4, 8 and 16 min separated by 15 min periods of uninterrupted 1 Hz stimulation. The force of contraction before and of the first twitch after the rest periods were considered respectively as the steady-state (C) and the post-rest (T) response. To avoid bias due to the long time required for reaching the equilibrium effect of amiloride, two sets of 4 atria were used, one serving as a control (○). Each point is the mean of four observations, vertical bars showing s.e.mean.

However, two points have to be considered. (1) At the temperature used in our experiments, the frequency-strength relation of guinea-pig atria revealed that the steady level of the strength at 0.1 Hz and at 1 Hz were quite similar. Although intracellular $[Ca^{2+}]$ was not measured, assuming that the magnitude of developed tension gives an estimate of the Ca^{2+} involved in excitation-contraction coupling (Sheu & Fozzard, 1982; Chapman, 1983; Blinks, 1986), it is likely that the amount of Ca^{2+} available for the contraction was almost the same at 0.1 Hz and at 1 Hz. Moreover, in the range of frequencies we explored, the lowest contraction was observed at 0.5 Hz, a frequency at which amiloride was effective in eliciting positive inotropic response in normal external $[Ca^{2+}]$. (2) It has been established that the contractility of isolated heart preparations depends on the ratio of $[Ca^{2+}]$ to $[Na^+]$ in the bath (Sheu & Blaustein, 1986; Gibbons, 1986). Therefore intracellular $[Ca^{2+}]$ and developed tension can be increased not only by raising external $[Ca^{2+}]$ but also by lowering external $[Na^+]^2$ (Sheu & Fozzard, 1982; Allen *et al.*, 1983; Chapman, 1983). When $[Ca^{2+}]_0$ was too low to allow the inotropic effect of amiloride, raising external $[Ca^{2+}]$ or lowering external $[Na^+]$ should have increased the availability of intracellular Ca^{2+} to a similar extent, since these changes were designed to increase the value of the $[Ca^{2+}]_0/[Na^+]_0^2$ ratio by the same magnitude at each rate of stimulation. If the positive inotropic response to amiloride was determined by a certain level of intracellular $[Ca^{2+}]$, irrespective of the mechanism by which this level was achieved, the drug should have had an inotropic effect when intracellular $[Ca^{2+}]$ was increased, but this was not the case. Thus, the inotropic ability of amiloride appears to be critically correlated not only with intracellular $[Ca^{2+}]$ but also with the concentration of Na^+ in the medium.

In isolated vesicle studies, amiloride has been shown to inhibit the sarcolemmal Na^+/Ca^{2+} exchange (Floreani & Luciani, 1984; Siegl *et al.*, 1984; Debetto *et al.*, 1987). This exchange system and the ATP-driven Ca^{2+} pump are the two major mechanisms for Ca^{2+} efflux from cardiac cells (Sheu & Blaustein, 1986). Although the relative contribution of these two systems in extruding Ca^{2+} is not well determined, it has been established that the pump has high affinity and low capacity for internal Ca^{2+}, whereas the Na^+/Ca^{2+} exchange is a low affinity, high capacity system (Blaustein, 1977; Trumble *et al.*, 1980; Caroni & Carafoli, 1981; Wakabayashi & Goshima, 1981; Di Polo, 1983; Barry & Smith, 1984; Philipson, 1985). The direction and level of operation of Na^+/Ca^{2+} exchange are determined by the electrochemical gradient for Na^+ and the membrane potential (Mullins, 1979; Reeves & Hale, 1984; Eisner & Lederer, 1985; Philipson, 1985). It has been proposed that a decrease of external $[Na^+]$, lowering the $[Na^+]_0/[Na^+]_i$ ratio and

hence the Na^+ gradient across the sarcolemma, depresses the Ca^{2+} extrusion through the Na^+/Ca^{2+} exchange (Sheu & Fozzard, 1982; Lee *et al.*, 1985). Therefore in low external $[Na^+]$ the operational level of the exchange is expected to be low. This may be consistent with the lack of inotropic effect of amiloride in low external $[Na^+]$, since the inhibition of an already depressed exchange may have no functional consequences because of the contribution of the system in Ca^{2+} efflux is actually too low. If we hypothesize that the relative contribution of Na^+/Ca^{2+} exchange and ATP-driven Ca^{2+} pump in extruding Ca^{2+} is dependent on external $[Ca^{2+}]$ and rate of stimulation, we can also explain the dependence of the positive inotropic effect of amiloride on different $[Ca^{2+}]$ at different rates of stimulation. Amiloride had no inotropic effect on guinea-pig left atria driven at 1 Hz, 0.5 Hz, 0.1 Hz in external $[Ca^{2+}]$ of respectively 0.45 mM, 0.9 mM, 2.7 mM. At each of these external $[Ca^{2+}]$ and rate levels, the ATP-driven Ca^{2+} pump may have a more important role than Na^+/Ca^{2+} exchange in extruding Ca^{2+}, as the intervals between stimuli in relation to external $[Ca^{2+}]$ (and hence to $[Ca^{2+}]_i$) are long enough to enable Ca^{2+} efflux through a system with high affinity for Ca^{2+} such as the ATP-driven Ca^{2+} pump.

It has been suggested that differences in the level of operation of Na^+/Ca^{2+} exchange in removing Ca^{2+} from the cells are responsible for the differences in contractile behaviour, with respect to the parameters of stimulation, existing between both atrial and ventricular myocardium and cardiac muscle from rat and other mammalian species (Sutko *et al.*, 1986). Rat cardiac muscle has some anomalous features (Noble & Powell, 1985); its contractility is less dependent on external $[Ca^{2+}]$ than that of other mammalian species (Willerson *et al.*, 1978; Sutko & Willerson, 1980; Mitchell *et al.*, 1984); its force of contraction does not demonstrate positive staircase in the range of high rates, but increases as the rate of stimulation is decreased (Langer, 1983; Orchard & Lakatta, 1985; Stemmer & Akera, 1986). At the low-frequency end of the frequency-strength curve, guinea-pig left atria exhibits similar behaviour (Blinks & Koch-Weser, 1961; Gibbons, 1986). Although most of the Ca^{2+} directly involved in activating contraction comes from intracellular stores (mainly sarcoplasmic reticulum (SR)), the magnitude of the tension depends on the relative contribution of the sarcolemmal mechanisms which regulate the Ca^{2+} fluxes in and out the cells, since these mechanisms determine the filling of the SR with Ca^{2+} and its release (Gibbons & Fozzard, 1975; Lipsius *et al.*, 1982; Chapman, 1983; Fabiato, 1983; Winegrad, 1986). Since the amount of Ca^{2+} entering the cells during each cardiac cycle does not change appreciably with rate changes (Beeler & Reuter, 1970; New & Trautwein, 1972), it is likely that the progressive increase of contraction induced by the progressive slowing of stimulation rate may be correlated with the progressive decline of operation of a Ca^{2+} extruding system, caused by the fact that more time is allowed to SR for Ca^{2+} loading. A time-dependent recycling of Ca^{2+} by the SR has been proposed by Orchard & Lakatta (1985) as a mechanism for the inverse relationship between strength and frequency in rat heart muscle. Moreover it has been postulated that Ca^{2+} efflux via Na^+/Ca^{2+} exchange may be less favoured in rat cardiac muscle (Sutko *et al.*, 1986). In the low frequency range, a time-dependent recycling of Ca^{2+} may be responsible for the increase of contraction as the frequency is decreased in guinea-pig atria as well. If this is so, the lack of positive inotropic effect of amiloride both on guinea-pig atria driven at very low frequencies and normal external $[Ca^{2+}]$ and on rat atria driven at 1 Hz can be accounted for by the fact that the Na^+/Ca^+ exchange is not relevant in removing Ca^{2+} from the cells: in rat atria as a species feature, in guinea-pig atria as a consequence of the lengthening of intervals between the stimuli.

Amiloride induced profound changes in guinea-pig (but not in rat) atria force-frequency relationship in the range of low frequencies, since in the presence of the drug the guinea-pig atria responded to the decrease of frequency with a decrease rather than increase of developed tension. Moreover, amiloride abolished the post-rest potentiation of the first contraction elicited after different times of rest in guinea-pig but not in rat atria.

The Na^+/Ca^{2+} exchange system has been implicated not only as a Ca^{2+} efflux but also as a Ca^{2+} influx mechanism in cardiac cells (Philipson, 1985; Sheu & Blaustein, 1986). It has been proposed that the direction of the carrier operation changes in each cardiac cycle (Gibbons, 1986), Ca^{2+} influx occurring on depolarization and Ca^{2+} efflux on repolarization (Mullins, 1979; Eisner & Lederer, 1985; Kimura *et al.*, 1986; Mechmann & Pott, 1986). Although the relative magnitude of the contribution of the Na^+/Ca^{2+} exchange in determining respectively Ca^{2+} entrance and loss is not known, evidence has been presented indicating a significant contribution to Ca^{2+} entry during depolarization by Na^+/Ca^{2+} exchange (see Gibbons, 1986). The contractile behaviour of guinea-pig atria, in which the force of contraction increases after rest (very low frequencies of stimulation or first contraction after rest) suggests a progressive Ca^{2+} gain, most probably due to a decreased activity of Ca^{2+} extruding systems between one beat and the following (the time-dependent recycling of Ca^{2+} by SR mentioned

above). If we assume that in atrial tissue at each contraction after rest the contribution of the Na^+/Ca^{2+} exchange is mainly in Ca^{2+} entry and not in Ca^{2+} efflux, we can explain the response to amiloride: the net effect of the inhibition of Na^+/Ca^{2+} exchange by the drug is a decrease of Ca^{2+} influx. Moreover, the lack of effect of amiloride on the contractile response of rat atria to low frequencies and to rest can be ascribed to the peculiarity of the cardiac tissue of this species in which the sarcolemmal mechanisms of Ca^{2+} flux are probably less important in the control of contractility than in other mammalian species.

In conclusion, we propose that the pattern of cardiac action of amiloride may be correlated with the relative level and the prevailing direction of operation of Na^+/Ca^{2+} exchange.

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Mechanism of xanthine-induced relaxation of guinea-pig isolated trachealis muscle

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1 Four 3-alkylxanthines (3-methylxanthine, 3-n-propylxanthine (enprofylline), 3-n-butylxanthine and 3-iso-butylxanthine) and four 1-methyl-3-alkylxanthines (1-methyl-3-methylxanthine (theophylline), 1-methyl-3-n-propylxanthine, 1-methyl-3-n-butylxanthine and 1-methyl-3-iso-butylxanthine (IBMX)), were compared in terms of cyclic AMP phosphodiesterase (PDE) inhibition and trachealis muscle relaxation. The relationship between xanthine structure and cyclic AMP PDE inhibition was also studied.

2 Xanthine induced relaxation of guinea-pig isolated trachealis muscle was measured against spontaneous tone.

3 The four 1-methyl-3-alkylxanthines were each significantly more potent than the corresponding 3-alkylxanthines in relaxing the isolated trachealis muscle. The 1-methyl-3-alkylxanthines were similarly more potent than the corresponding 3-alkyl derivatives in inhibiting low K_m cyclic AMP PDE. There was a strong positive correlation between low K_m cyclic AMP PDE inhibition and the tracheal smooth muscle relaxation evoked by the xanthine derivatives.

4 Since methylation of the 1-position of each 3-alkylxanthine increased the potency of the derivative in inhibiting low K_m cyclic AMP PDE and in relaxing trachealis muscle and since a strong positive correlation was observed between the relaxant EC_{50} and the K_i value of each xanthine derivative, it is suggested that low K_m cyclic AMP PDE inhibition by xanthines plays an important role in their tracheal relaxant effect.

Introduction

Xanthines such as theophylline are effective bronchodilators and various studies have been performed to elucidate their mechanism of action. Katsuki & Murad (1977) and Polson *et al.* (1982) studied the cyclic nucleotide phosphodiesterase (PDE) inhibitory effect of xanthine agents. Persson *et al.* (1982) and Fredholm & Persson (1982) examined the adenosine-antagonizing effect of xanthine agents. Peach (1972) studied the use of xanthine agents to control intracellular calcium. Farmer & Chick (1967) and Stubbs *et al.* (1984) observed the effect of xanthine agents on the release of catecholamines from the adrenal medulla. Horrobin *et al.* (1977) studied inhibition induced by xanthine of prostaglandin synthesis in vascular smooth muscle.

However, the pharmacological actions of xanthine agents have not been fully clarified by any of these studies for the following three reasons: (1) In the first three studies, the concentration of the xanthine inhibiting PDE or antagonizing adenosine did not correlate with the concentration effective in causing relaxation. (2) Xanthine agents are strong smooth muscle relaxants even where the maintenance of muscle tone is independent of prostaglandin synthesis. (3) Whether or not xanthine agents directly influence the release of catecholamines from the adrenal medulla remains uncertain. It has recently been reported that bronchodilators such as agonists at β-adrenoceptors, xanthine agents and dibutyryl cyclic AMP induce hyperpolarization of the cell membranes in guinea-pig isolated trachealis muscle with a muscle relaxant effect (Honda *et al.*, 1986). β-Adrenoceptor stimulants and xanthine agents tested in combination acted to relax the isolated trachealis muscle in guinea-pigs (Persson & Gustafsson, 1986). Also recently, a significant relationship was found between PDE inhibition and trachealis muscle relaxation although there was no obvious correlation between the increase in intracellular cyclic nucleotides and trachealis muscle relaxation (Bryson & Rodger, 1987).

Therefore, the cyclic nucleotide PDE system is once again receiving attention. We compared the cyclic AMP PDE inhibitory effect of various xanthine derivatives with the myorelaxant effect of these derivatives on tracheal preparations obtained from

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Table 1 K_i values for cyclic AMP phosphodiesterase and $-\log EC_{50}$ values for the relaxant effects of various xanthine derivatives on guinea-pig isolated trachealis muscle ($n = 6$)

Xanthine derivative	K_i *value* (μM) *Low* K_m cyclic AMP 0.1 ~ 0.2 (μM)	$-log\ EC_{50}$ (M) *Spontaneous tone*
3-Alkylxanthine		
3-Methylxanthine	122 ± 2	3.85 ± 0.05
3-n-Propylxanthine (Enprofylline)	42 ± 1	5.14 ± 0.25
3-n-Butylxanthine	32 ± 2	5.08 ± 0.11
3-iso-Butylxanthine	37 ± 2	4.92 ± 0.10
1-Methyl-3-alkylxanthine		
Theophylline	56 ± 2*	4.55 ± 0.13*
1-Methyl-3-n-propylxanthine	1.9 ± 0.2*	6.01 ± 0.05*
1-Methyl-3-n-butylxanthine	1.2 ± 0.3*	5.87 ± 0.11*
IBMX	5.0 ± 0.5*	6.05 ± 0.14*

Low K_m cyclic AMP PDE: $K_m = 0.61 \pm 0.04\ \mu M$, $V_{max} = 49.5 \pm 5\ pmol\ mg^{-1}\ min^{-1}$.
K_i values were determined by the method of Dixon (1953), and the EC_{50} values were calculated from log · probit plots of the individual relaxation responses.
* Significantly different ($P < 0.01$) from each 3-alkylxanthine corresponding to 1-methyl-3-alkylxanthine.
All data are presented as mean ± s.e.mean.

guinea-pigs, in order to clarify how the trachealis muscle relaxant effect of xanthine agents correlates with PDE inhibition. We also investigated the changes in tracheal relaxant potency and PDE inhibitory potency which resulted from methylation of the 1-position of 3-alkylxanthines.

Methods

Tissue preparation and trachealis muscle relaxation

Male Hartley guinea-pigs weighing 200–400 g were stunned and bled, and the trachea was removed. One tracheal ring was cut from the pharyngeal end of each trachea. The ring was opened by cutting through the cartilaginous region diametrically opposite the trachealis muscle, and the ends of the cartilaginous region were ligated. The preparation was placed in an organ bath of 1 ml volume. Tension was measured with an isometric transducer (Minebea Co., Ltd, UL 10GR), and tension changes were recorded with a pen recorder (Matsushita Communication Industrial Co., Ltd, VP-6621A). Using the method of Baba *et al.* (1985), the preparation was perfused at a constant flow of 1.5 ml min^{-1} at 37°C in Krebs solution of the following composition (mM): NaCl 137, $KHCO_3$ 5.9, $CaCl_2$ 2.4, $MgCl_2$ 1.2, and glucose 11.8. Isoprenaline (2×10^{-5} M) was added to produce complete relaxation, then tension of about 0.5 g was applied to the preparation. The preparation was again perfused at 37°C in Krebs solution and spontaneous tone was allowed to develop. After the tension had stabilized, the preparation was then perfused for 20 min in the presence of a xanthine derivative at various concentrations. Relaxation occurring on exposure of the tissue to a Ca^{2+}-free medium containing 0.01 mM EGTA was defined as 100%, and the relaxation caused by each xanthine was calculated in terms of this standard.

Cyclic AMP phosphodiesterase assay

Cyclic AMP PDE activity was measured by a method similar to that devised by Thompson & Appleman (1971). Trachealis muscle was homogenized in Tris HCl buffer (40 mM Tris HCl, 10 mM $MgCl_2$ and 4 mM 2-mercaptoethanol). The homogenate was cooled and centrifuged at 10,000 *g* in a refrigerated centrifuge. The supernatant fluid was collected. A mixture consisting of 50 μl of the test xanthine solution or vehicle solution, 25 μl of cyclic AMP (1×10^{-7}–4×10^{-5} M) and 25 μl of [^{3}H]-cyclic AMP (1 mCi ml^{-1}) was placed in a glass test tube incubated at 30°C for 5 min; 100 μl of the supernatant solution from the tissue homogenate was added and the mixture was incubated at 30°C for 10 min. The reaction mixture was placed in boiling water for 1 min to stop the reaction. After the mixture had been cooled with water, 50 μl of *Crotalus atrox* snake venom solution was added and the mixture was incubated at 30°C for 10 min. Following that, an ion exchange resin (Dowex 1-X2) was added, and the mixture was allowed to stand for 15 min. The mixture was centrifuged at 3,000 r.p.m. for 5 min in a bench centrifuge. The supernatant solution was collected. The radioactivity in 0.5 ml of the supernatant was measured with a liquid scintillation counter. The K_m value and the V_{max} value were obtained from Lineweaver-Burk plots. Since diphasic

graphs are observed for guinea-pig trachealis muscle, a high K_m enzyme value and a low K_m enzyme value can be obtained. The K_i value was obtained by the method of Dixon (1953).

Drugs used were isoprenaline (Sigma), 3-methylxanthine (Sigma), theophylline (Sigma), 1-methyl-3-iso-butylxanthine (IBMX, Sigma), 3-n-propylxanthine, 3-n-butylxanthine, 3-iso-butylxanthine, 1-methyl-3-n-propylxanthine and 1-methyl-3-n-butylxanthine (synthesized in our laboratories), EGTA (Sigma), cyclic AMP (Yamasa), [^{3}H]-cyclic AMP (Yamasa), Tris HCl (Sigma), 2-mercaptoethanol (Sigma), Dowex 1-X2 (Dow Chemical), *Crotalus atrox* snake venom (Sigma).

Statistical analysis

All the results are expressed as the mean ± s.e.mean, and the *t* test without correspondence was applied. *P* values less than or equal to 0.05 were considered to indicate a significant difference between means.

Results

Relaxation by xanthine derivatives

Table 1 shows the $-\log EC_{50}$ values for the trachealis muscle in the condition of spontaneous tone while Figure 1 shows the concentration-response curves for 1-methyl-3-alkylxanthines and the corresponding 3-alkylxanthines for trachealis in spontaneous tone. These results reveal that relaxant potency of xanthine derivatives is increased both when a 3-methyl substituent is replaced by a longer 3-alkyl chain and when a 3-alkyl derivative is additionally methylated in the 1-position.

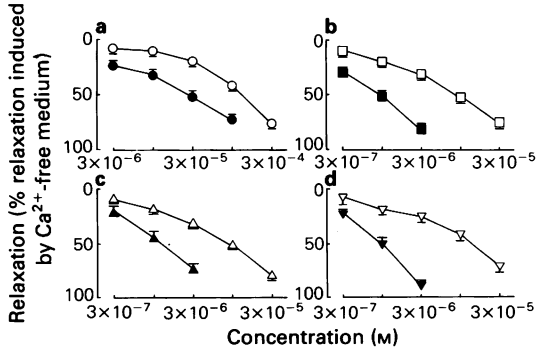


Figure 1 Log concentration-relaxation curves of the 3-alkyl xanthines and the 1-methyl-3-alkyl xanthines: (a) (○) 3-methylxanthine and (●) theophylline; (b) (□) 3-n-propylxanthine (enprofylline) and (■) 1-methyl-3-n-propylxanthine; (c) (△) 3-n-butylxanthine and (▲) 1-methyl-3-n-butylxanthine; (d) (▽) 3-iso-butylxanthine and (▼) 1-methyl-3-iso-butylxanthine. Each curve was derived from experiments on tracheal smooth muscle specimens from six guinea-pigs. Data points represent means with s.e.mean shown by vertical bars.

Cyclic AMP phosphodiesterase activity

For guinea-pig tracheal smooth muscle, low (high affinity) and high (low affinity) K_m cyclic AMP PDE activities were observed. Examination of the K_i values of individual xanthine derivatives revealed that for the low K_m enzyme, inhibitory potency of the xanthines is increased both when a 3-methyl substituent is replaced by a longer 3-alkyl chain and when a 3-alkyl derivative is additionally methylated in the 1-position (Table 1).

Discussion

Studies by Persson *et al.* (1982) revealed that alkyl-substitution in the 1-position of the xanthine nucleus yielded a xanthine with adenosine antagonist activity. This observation stimulated active discussion on the relationship between the bronchodilator action and adenosine-antagonizing action of xanthines. However, theophylline and related substituted methylxanthines such as IBMX, 8-phenyltheophylline, caffeine, and theobromine are effective inhibitors of adenosine at A_1- and A_2-purinoceptors in concentrations between 20 and 100 times lower than those required to inhibit phosphodiesterase (Fredholm & Persson, 1982). Furthermore, enprofylline, which has been shown to be almost entirely free of antagonism of the functional and biochemical effects of adenosine, has a greater bronchodilator potency than some xanthines alkylated in the 1-position (Lunell *et al.*, 1982., Persson, 1983). We have therefore come to believe that the adenosine-antagonizing action of xanthine does not represent the main mechanism of its bronchodilator effect.

A number of studies have also been conducted on the mechanism by which the PDE inhibitory action of xanthine and the related increase in intracellular cyclic AMP concentration may lead to the relaxation of trachealis muscle. Doubt has been cast on the operation of this mechanism by observation of a difference between the effective blood concentration of theophylline and its PDE inhibitory activity in the isolated airway (Polson *et al.*, 1978; Bergstrand, 1980). Furthermore, drugs such as papaverine and dipyridamole, which are strong PDE inhibitors, are not effective as bronchodilators *in vivo* (Ruffin & Newhouse, 1981).

However, in consideration of a recent finding that there was a supra-additive interaction between terbutaline and both theophylline and enprofylline, which are PDE inhibitors (Persson & Gustafsson,

1986), and also of a recent report that PDE inhibitors such as papaverine, IBMX, SKF 94120, etc., showed a strong relaxant effect on guinea-pig isolated trachealis muscle (Bryson & Rodger, 1987), a strong correlation seems to be suggested between the PDE inhibitory action and the bronchodilator action of xanthine. Furthermore, a significant correlation has been observed between the *in vitro* PDE inhibitory activity and the bronchodilator potency of xanthine (Newman *et al.*, 1978; Polson *et al.*, 1982).

We conducted experiments with 2 main objectives: one was to look for a possible structure-activity relationship by comparing the 3-alkylxanthine derivatives with their corresponding 1-methyl-3-alkylxanthine analogues in terms of their cyclic AMP PDE inhibitory action and their relaxant effect on guinea-pig isolated trachealis. The 1-methyl-3-alkyl derivatives were more potent in inhibiting high affinity cyclic AMP PDE than their 3-alkyl analogues and similar potency differences were obtained for relaxation of trachealis muscle. Furthermore, in both groups of analogues, an increase in the size of the 3-alkyl substituent from methyl to n-butyl increased potency as regards both enzyme inhibition and tracheal relaxation. A structure-activity relationship thus became obvious; that is, 1-methylation and a longer 3-alkyl chain not only elevate high affinity cyclic AMP PDE inhibitory potency, but also increase potency for relaxation of trachealis muscle. These findings usefully supplement the structure-activity relationship for the adenosine-antagonizing action of xanthines reported by Persson *et al.* (1982). There are reports that alkylation of the 1- and 3-position increases tracheal relaxant potency and that, within limits, an increase in the size of the 3-alkyl analogues also increases bronchodilator potency (Parker *et al.*, 1956; Persson *et al.* 1985; Takagi *et al.*, 1988). For tracheal smooth muscle, cyclic AMP PDE activity is of 2 types, i.e., low K_m (high-affinity) and high K_m (low-affinity). The tissue concentration of cyclic AMP is normally no greater than 1 μM. Therefore the high affinity enzyme of K_m; 0.61 ± 0.04 μM is considered to be the isozyme of greatest physiological importance.

For xanthine-induced inhibition of the low K_m enzyme, we found a clear, strong correlation with the relaxant effect on trachealis muscle ($r = 0.916$, $P < 0.01$) (Figure 2). This was the other main objective of our experiments. Polson *et al.* (1982) examined cyclic AMP PDE activity in canine trachea and detected high-affinity cyclic AMP PDE activity (peak V), the K_m of which was 0.63 ± 0.09 μM. They reported that, for 6 different methylxanthines (1-methylxanthine, 3-methylxanthine, 7-methylxanthine, caffeine, theophylline and IBMX) a strong correlation existed between xanthine-induced inhibition of high-affinity cyclic AMP PDE and of isolated canine trachealis muscle contracted by means of methacholine. Despite the different animal species used, the results of the present study are therefore consistent with those of earlier studies.

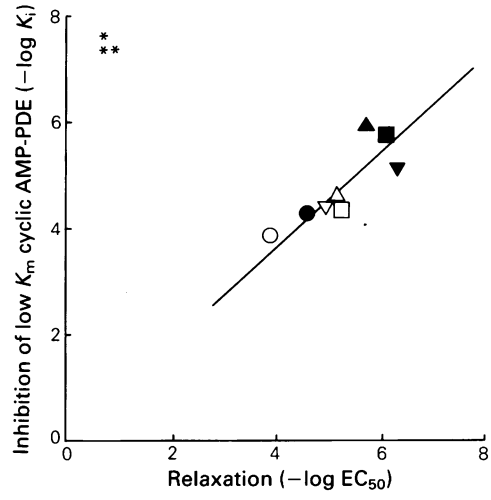


Figure 2 Correlation between inhibition of low K_m cyclic AMP phosphodiesterase and relaxation of guinea-pig isolated trachealis muscle. Data from Table 1: (○) 3-methylxanthine; (●) theophylline; (□) 3-n-propylxanthine (enprofylline); (■)1-methyl-3-n-propylxanthine; (△) 3-n-butylxanthine; (▲)1-methyl-3-n-butylxanthine; (▽) 3-iso-butylxanthine; (▼)1-methyl-3-iso-butylxanthine (IBMX). * $r = 0.916$, ** $P < 0.01$.

On the basis of all the above results, it can be concluded that in the guinea-pig the low K_m cyclic AMP PDE inhibitory activity of xanthine derivatives is closely related to their tracheal relaxant activities *in vitro*.

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Divalent cation dependence of the inhibition by phenothiazines of mediator release from mast cells

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1 The divalent cations calcium, strontium and barium – and in that order of decreasing effectiveness – were capable of supporting the stimulated release of histamine from rat peritoneal mast cells (RPMC).

2 The responsiveness of mast cells to stimulation in the presence of divalent cations was, in general, markedly enhanced when the cells were first depleted of their intracellular calcium stores.

3 The putative calmodulin antagonists, chlorpromazine, promethazine, thioridazine (phenothiazines) and W-7 (a naphthalene sulphonamide) all inhibited histamine release in the presence of divalent cations in both untreated cells and in RPMC depleted of their intracellular calcium.

4 Histamine release induced by antigen, compound 48/80 and ionophore A23187 was inhibited by this class of compounds most effectively in the presence of extracellular barium, less so in the presence of strontium and least so in calcium-containing media.

5 In the experimental situation where the extracellular calcium concentration was reduced (< 1 mM), the phenothiazines inhibited the stimulated release of histamine more effectively.

6 *In toto*, these results suggest that strontium and barium, as well as calcium, can support histamine release from RPMC by directly interacting with an intracellular divalent cation-binding site that may be calmodulin. As a consequence, one mechanism by which the phenothiazines and W-7 may modulate the secretory response could reflect an antagonism of a divalent cation interaction at that same site, although other additional potential sites of inhibitory action are indicated, dependent on the stimulus employed for secretion.

Introduction

The rat peritoneal mast cell (RPMC) is responsive to a wide variety of stimuli that include IgE-directed ligands, polybasic compounds and ionophores, all of which differ in the detailed mechanisms by which they evoke exocytosis (Pearce *et al.*, 1985). However, it is generally recognized that one property these stimuli do have in common is the ability to raise cytosolic calcium levels, either by promoting the influx of the cation from the extracellular milieu or by deploying intracellular calcium from sequestered stores (Pearce, 1982). This suggests that, although the processes involved in causing an intracellular rise in calcium may differ between secretagogues, once calcium has been recruited the ensuing biochemical steps may be similar if not identical for the majority of stimuli.

Once cytosolic calcium levels have been raised, the mechanism(s) by which the cation mediates its effects has not been determined although the calcium-binding protein calmodulin may be of importance (Pearce, 1982). This ubiquitous protein possesses four binding sites of differing affinity for calcium (Klee *et al.*, 1980; Brostrom & Wolff, 1981). The binding of calcium induces a conformational change in the protein (Burger *et al.*, 1984), leading to the exposure of a hydrophobic site(s) and this site is, presumably, essential for the association with and activation of a given enzyme or effector protein (Cox, 1984). Since calmodulin regulates the activity of a multitude of enzymes it would be particularly disadvantageous if after any permissive increases in

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calcium a promiscuous activation of enzymes occurred. One concept that has been put forward is that the specific activation of one enzyme, in preference to another, may be dependent on the number of calmodulin binding sites ($n = 1$–4) occupied by calcium and that this level of occupancy is determined by the extraneous concentration of calcium (Klee *et al.*, 1980).

In addition to calcium, other ions are able to bind to and activate calmodulin albeit less effectively (Levin & Weiss, 1977; Cheung, 1984). Calcium, strontium and barium, and in that potency order, bind to calmodulin and the degree of binding to the protein correlates with the activation of calmodulin-dependent phosphodiesterase. And herein lies the aim of the present study. These same ions can support the stimulated release of histamine from mast cells (Foreman & Mongar, 1972; Atkinson *et al.*, 1979). If calmodulin, or a related protein, were important in exocytosis then the phenothiazines might be expected to display preferential inhibitory activity in the presence of divalent cations following the series: barium > strontium > calcium. Since barium would be least likely to bind to and initiate an effect at a calcium-binding site, it follows that the phenothiazines would be best equipped to antagonize that divalent cation interaction rather than those due to strontium or calcium.

Methods

Buffers

Tyrode buffer contained (mM): NaCl 137, glucose 5.6, N-2-hydroxyethyl piperazine-N'-2-ethane sulphonic acid (HEPES) 10, KCl 2.7, NaH_2PO_4 0.4 $CaCl_2$ 1. CMF Tyrode contained the above constituents but $CaCl_2$ was omitted. EDTA Tyrode was CMF Tyrode with ethylenediamine tetraacetic acid (EDTA, 0.1 mM) included. The pH of all buffers was titrated to 7.3.

Mast cell isolation

Male Sprague-Dawley rats (250–350 g; Joint Animal House, University College London) were anaesthetized with diethyl ether, killed and exsanguinated. Mixed peritoneal cells were obtained by direct abdominal lavage by methods described elsewhere (Atkinson *et al.*, 1979). The resultant cell population contained 2–5% mast cells. For some experiments, rats were sensitized to the nematode *Nippostrongylus brasiliensis* and specific antigen prepared as described (Ogilvie, 1967; White & Pearce, 1982).

Depletion of intracellular calcium from mast cells

RPMC were incubated (25 min, 37°C) in CMF Tyrode with or without a combination of ionophore A23187 (10 nM) and EDTA (0.1 mM). Untreated and ionophore/EDTA treated cells were then sedimented (150 *g*, 3 min, RT) and washed three times in calcium-free buffer. Both treated and untreated cells were resuspended in CMF Tyrode, an aliquot of which, from either cell preparation, was challenged with an optimal releasing concentration (5 $\mu g\,ml^{-1}$) of compound 48/80 for secretion in a medium free of calcium. Treated cells were refractory to this stimulus, an indication that calcium stores had been depleted, whereas untreated cells responded normally releasing 30–35% of their total histamine. Cells treated in EDTA Tyrode alone over this period and challenged with the same concentration of compound 48/80 released 10–15% of their histamine suggesting an incomplete removal of intracellular calcium stores.

Histamine release protocol

Prewarmed aliquots (37°C, 250 μl) containing 2–4 $\times$ 10^4 treated or untreated mast cells in CMF Tyrode were added to conical polystyrene tubes (15 ml, Sarstedt) containing an equivalent volume of the appropriate concentration of divalent cation and/or phenothiazine (or W-7). After preincubation (5 min), secretion was initiated by the addition of secretagogue in a minimal volume (10 μl). After 10 min at 37°C the release reaction was terminated by the addition of an excess (2.5 ml) of ice-cold buffer. The cells were then sedimented (150 *g*, 3 min, 4°C) and the supernatants saved. The cell pellets were resuspended in buffer (3 ml) and placed in a water bath (100°C, 10 min) to release residual cell-associated histamine. The histamine contents of the supernatants and cell pellets were determined employing the fluorometric method of Shore *et al.* (1959). Histamine release in the absence of secretagogue served as a measure of spontaneous histamine secretion. Histamine release was thus expressed as a percentage of the amine present in the supernatant with respect to the total amount of histamine found in both the supernatant and cell pellet. All values were corrected for the appropriate spontaneous histamine release.

Materials

The following were purchased: A23187 (Calbiochem); EDTA (Fisons); sheep anti-rat IgE (Miles); compound 48/80, dimethylsulphoxide (DMSO), and HEPES (Sigma); W-7 (N-(6-aminohexyl)-5- chloro-

1-naphthalene-sulphonamide; Rikaken, Japan). The following were gifts: chlorpromazine and promethazine (Mrs W.J. Adams, May and Baker); thioridazine (Mr G.A. McCormick, Sandoz).

Compound 48/80, was prepared fresh daily in glass distilled water (GDW). Lyophilized anti-IgE was reconstituted in sterile GDW and stored as the neat solution at −20°C. Specific antigen was prepared in 0.9% sterile saline as described (Ogilvie, 1967) and stored frozen at a concentration of 1 worm equivalent (WE) μl^{-1}. The ionophore A23187 was made up as a stock solution (1 mM) in DMSO and stored in appropriate aliquots at −20°C. Stock solutions (1 mM) of the phenothiazines were prepared in CMF Tyrode and made fresh daily. W-7 was prepared as a 5 mM stock solution and stored at 4°C.

Statistics

The statistical significance of changes in response were analysed by comparing control and treated cells by use of Student's *t* test for paired data.

Results

Divalent cation dependence of histamine release from depleted and non-depleted mast cells

Mast cells were depleted of their intracellular calcium stores by incubating (25 min, 37°C) the cells with the ionophore A23187 (10 nM) in the presence of EDTA (0.1 mM). The divalent cation dependence of histamine release from these cells was compared to that seen in untreated cells.

The effect of calcium (0.01–4 mM), strontium (1–8 mM) and barium (2.5–10 mM) on IgE-mediated histamine release induced by antigen (20 WE ml^{-1}) from mast cells isolated from rats sensitized to the nematode *Nippostrongylus brasiliensis* is shown in Figure 1. Increasing the extracellular concentration of divalent cation, over the ranges cited, produced an increase in histamine release in both non-depleted (Figure 1a) and depleted (Figure 1b) mast cells. (Not shown in Figure 1 is the finding that 16 mM barium is capable of supporting ca. 30% histamine release from antigen-activated mast cells whose intracellular calcium has not been removed). The effect of depletion was to reduce the concentration of divalent cation required to support a given level of histamine release.

This point is more dramatically illustrated in Figure 2 where the effect of extracellular cations on histamine release induced by the pharmacological stimulus, compound 48/80 is shown. Some ten fold

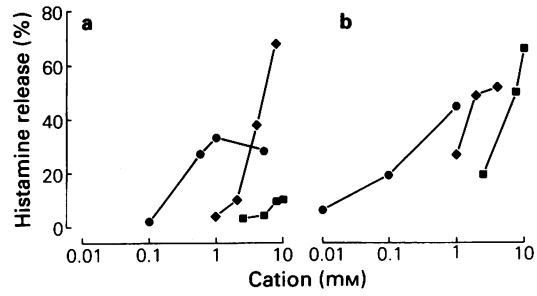


Figure 1 The effect of varying concentrations of calcium (●), strontium (◆) and barium (■) on histamine release induced by allergen (20 WE ml^{-1}) from non-depleted (a) and depleted (b) mast cells. Values are means, $n = 2$.

greater concentrations of barium and strontium were required to support matched levels of secretion in untreated (Figure 2a) RPMC than in cells depleted of their intracellular calcium (Figure 2b) – a property not shared by calcium where the same concentrations of the cation were capable of mediating similar levels of secretion, independent of the intracellular calcium content of the mast cell.

In data not shown, studies demonstrated that strontium (4–10 mM) and barium (4–16 mM) could substitute for calcium (0.5–5 mM) in histamine release induced by the ionophore A23187 (0.1–1 μM) from depleted mast cells. In order to obtain comparable levels of histamine release (*ca.* 30%) to that seen with calcium (0.5 mM), not only were higher concentrations of strontium (>2 mM) and barium (>6 mM) required, but a higher concentration of ionophore A23187 (1 μM cf 0.1 μM) was also necessary.

In total, these studies (and others not mentioned) were performed to establish the appropriate concentration of cation and secretagogue required to

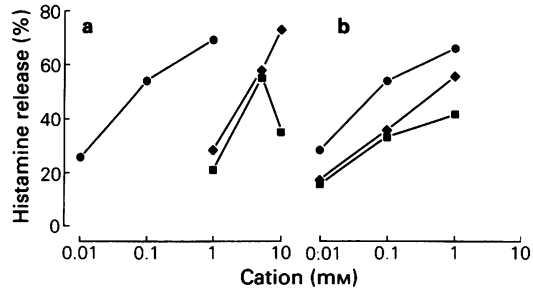


Figure 2 The effect of varying concentrations of calcium (●), strontium (◆) and barium (■) on histamine release induced by compound 48/80 (1 μg ml^{-1}) from non-depleted (a) and depleted (b) mast cells. Values are means, $n = 2$.

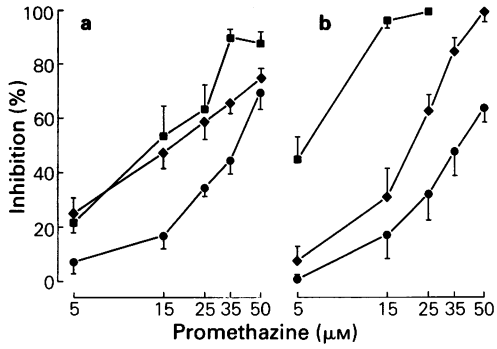


Figure 3 The inhibition by promethazine of histamine release induced by allergen (20 WE ml^{-1}) in the presence of calcium (●), strontium (◆) or barium (■) in both untreated cells (a) or cells depleted of their intracellular calcium (b). Concentrations (mM) of divalent cation and control releases (%) were for (a), (●) 1 mM; 44 ± 4%, (◆) 4 mM; 41 ± 6% and (■) 16 mM; 30 ± 4% and for (b), (●) 1 mM; 39 ± 6%, (◆) 1.5 mM; 33 ± 6% and (■) 7 mM; 27 ± 2%. Values are means and vertical lines indicate s.e.mean, n = 4–6.

produce matched levels of secretion for application in the ensuing series of investigations.

Effects of promethazine and W-7 on antigen-induced histamine release in the presence of divalent cations

Promethazine (5–50 μM) inhibited, in a dose-dependent manner, the secretion induced by antigen (20 WE ml^{-1}) from non-depleted (Figure 3a) and depleted (Figure 3b) mast cells isolated from rats sensitized to the nematode *Nippostrongylus brasiliensis*. For matched levels of release, promethazine inhibited antigen-induced secretion in the presence of barium most effectively, strontium rather less well, and calcium least effectively – a trend that held for treated and untreated mast cells alike. ID_{50} values (those concentrations of the phenothiazine required to inhibit secretion by 50%) are given in Table 1.

The naphthalene sulphonamide, W-7 (5–50 μM) also inhibited antigen-induced histamine release from depleted RPMC where the order of inhibitory activity in the presence of cation followed the series: barium (ID_{50}; 9 μM) > strontium (15 μM) > calcium (23 μM; data not shown, n = 5).

Effects of thioridazine on compound 48/80-induced histamine release in the presence of divalent cations

Thioridazine (5–25 μM) inhibited histamine release induced by the polybasic compound, 48/80 from non-depleted (Figure 4a) and depleted (Figure 4b) mast cells. The inhibition observed was dose-

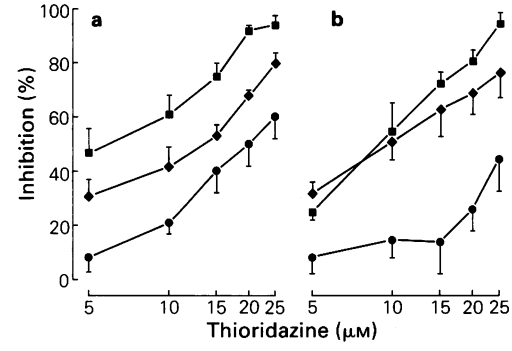


Figure 4 Effects of divalent cations (1 mM) on the inhibition by thioridazine of histamine release induced by compound 48/80 in the presence of calcium (●), strontium (◆) or barium (■). Inhibition was studied in non-depleted (a) and depleted (b) mast cells. Concentrations (μg ml^{-1}) of compound 48/80 and control releases (%) were for (a), (●) 0.15 μg ml^{-1}; 38 ± 5%, (◆) 5; 38 ± 4 and (■) 5; 29 ± 3 and for (b), (●) 0.15; 33 ± 2, (◆) 0.15; 32 ± 5 and (■) 1; 28 ± 2. Values are means and vertical lines indicate s.e.mean, n = 5–7.

Table 1 Effect of divalent cations on the inhibition by phenothiazines of histamine release induced by stimuli

	Antigen		*Compound 48/80*	
Cation	*Non-depleted* ID_{50}^{PMT*} (μM)	*Depleted* ID_{50}^{PMT} (μM)	*Non-depleted* ID_{50}^{TDZ*} (μM)	*Depleted* ID_{50}^{TDZ} (μM)
Barium	17 ± 5	5 ± 1	<5	8 ± 1
Strontium	18 ± 4	19 ± 3	12 ± 2	10 ± 1
Calcium	38 ± 3	35 ± 5	18 ± 2	25 ± 5

* ID_{50}^{PMT} (μM) ID_{50}^{TDZ} (μM) represent the concentrations of promethazine and thioridazine required to inhibit histamine release by antigen (20 WE ml^{-1}) and compound 48/80 by 50%. Control releases were ca. 35%. Values are means ± s.e.mean and are based on at least four experiments. Further particulars relevant to the table may be found in the legends to Figures 3 and 4.

Table 2 Effect of calcium on the inhibition by thioridazine of histamine release induced by compound 48/80

	Non-depleted		*Depleted*		
Calcium	*48/80* ($\mu g\,ml^{-1}$)	ID_{50}^{TDZ}* (μM)	*48/80* ($\mu g\,ml^{-1}$)	ID_{50}^{TDZ} (μM)	P
0	10	17 ± 1	—	—	
0.01	5	14 ± 1	5	7 ± 1	
0.1	0.25	7 ± 1	0.25	17 ± 2	<0.005
1	0.15	18 ± 2	0.15	25 ± 5	<0.1

* ID_{50}^{TDZ} (μM) represents the concentration of thioridazine required to inhibit histamine release induced by compound 48/80 by 50%. Control releases ranged from 30 ± 3% to 38 ± 5%. Values are means ± s.e.mean and are based on the individual inhibitory dose-response curves for thioridazine (5–25 μM) from at least five experiments.

dependent and thioridazine was most active in the presence of barium, least potent in the presence of calcium and displayed intermediary activity in the presence of strontium. This rank order of potency for inhibition was identical in depleted and non-depleted mast cells (IC_{50} values are given in Table 1).

The modulatory activity of thioridazine was also studied in the presence of various concentrations of calcium (0.01, 0.1 and 1 mM) and in the absence of the cation (data not shown). Since 48/80, in the absence of extracellular calcium, mediates secretion via the mobilization of intracellular calcium stores, only non-depleted mast cells responded to 48/80 under the latter conditions (ID_{50} values are given in Table 2).

In non-depleted mast cells activated with 48/80, the effect of lowering the extracellular concentration of calcium from 1 to 0.1 mM was to improve the inhibitory activity of thioridazine (it should be noted that, in order to produce comparable levels of mediator release under each calcium-containing condition, the concentration of secretagogue had to be adjusted accordingly). The concentrations of 48/80 (0.15 $\mu g\,ml^{-1}$ at 1 mM calcium and 0.25 $\mu g\,ml^{-1}$ at 0.1 mM) employed in the above-mentioned instances did not induce histamine release in the absence of extracellular calcium (<5% histamine release for either condition). Reducing the extracellular calcium (0.1 mM) still further led to a decrease in the inhibitory activity of thioridazine. The concentration (5 $\mu g\,ml^{-1}$) of 48/80 required to provoke release under this calcium condition, induced significant levels of histamine release (25 ± 4%) when the cation was excluded from the experimental medium, suggesting that a substantial proportion of the response was due to the mobilization of intracellular calcium stores. Inhibition of compound 48/80-induced release from non-depleted RPMC by thioridazine was essentially identical either in the presence of 0.01 mM calcium or in the absence of cation.

In depleted mast cells the complicating presence of intracellular calcium stores would not be expected to prevail. Under these conditions, lowering the concentration of extracellular calcium (1, 0.1 and 0.01 mM) progressively increased the ability of thioridazine (5–25 μM) to inhibit secretion (see Table 2).

Effect of chlorpromazine on ionophore A23187-induced histamine release in the presence of divalent cations

Chlorpromazine (10–50 μM) inhibited histamine release induced by the ionophore A23187, in the presence of calcium (0.1–5 mM), strontium (0.5–12 mM) and barium (4–20 mM; Figure 5). The inhibitory activity of chlorpromazine displayed the following preferential cation dependency for inhibition: barium > strontium > calcium (ID_{50} values calculated from inhibition curves for ca. 25% histamine release were 7, 11 and 23 μM, respectively). Whereas at low concentrations of calcium (0.1–0.5 mM) chloropromazine inhibited histamine release effectively, in the presence of higher concentrations (>1 mM) of the cation the inhibitory action of the phenothiazine was markedly impaired.

Discussion

The RPMC has evolved as a useful paradigm of stimulus-secretion coupling responses. Its utility lies in the ease with which it may be isolated and by virtue of the fact that it responds to a wide array of stimuli (Pearce *et al.*, 1985). Studies with radiolabelled calcium (Foreman *et al.*, 1977) and the fluorescent intracellular calcium probe quin 2 (Beaven *et al.*, 1984; White *et al.*, 1984) suggest that a cytosolic increase in calcium is the central event leading to exocytosis. Secretagogues promote entry of the cation into the cytosol either by increasing the permeability of the cell membrane to calcium (immunological and pharmacological ligands), mobilizing intracellular stores of the cation (polybasic

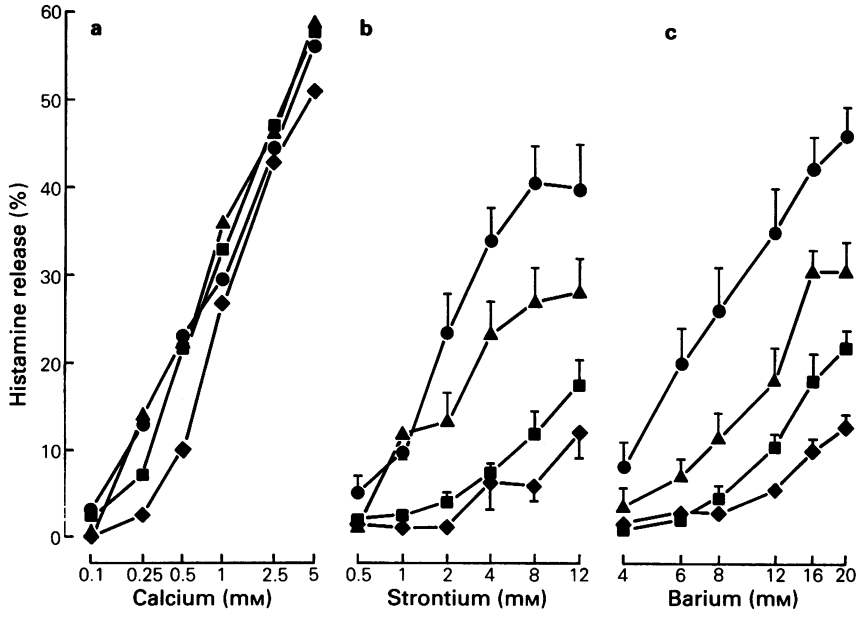


Figure 5 Effects of divalent cations on the inhibition by chlorpromazine of histamine release induced by ionophore A23187 from cells depleted of their intracellular calcium. The cells were resuspended in varying concentrations of calcium (a), strontium (b) or barium (c) before challenge with the ionophore; 0.5 μM for (a) and 1 μM for (b) and (c). Concentrations (μM) of chlorpromazine were (●) 0, (▲) 10, (■) 25 and (◆) 50. Values are means and vertical lines indicate s.e.mean (except in (a) where error bars have been omitted for reasons of clarity), $n = 4$.

compounds), or directly translocating the cation from the exterior to the interior of the cell (ionophores).

Once in the cell, the mechanism by which the cation exerts its effects is unknown. However, one possibility is an interaction with and activation of calmodulin, the intracellular calcium receptor (Means & Dedman, 1980). Calmodulin is known to regulate the activity of a number of enzymes some of which may be implicated in the processes leading to degranulation (Pearce, 1982). Indirect evidence supporting a role for calmodulin in mast cell function was suggested by the finding that the rank order of potency of a series of anti-calmodulin compounds (phenothiazianes and W-7) for the inhibition of mediator release from mast cells compared favourably with that for the inhibition of calmodulin (see Table 3; Douglas & Nemeth, 1982; Peachell &

Table 3 Effect of phenothiazines on histamine release induced by compound 48/80 and anti-IgE

Phenothiazine	*anti-IgE*	*ID_{50} (μM)* 48/80*	*CaM*	P**
Thioridazine	6.0 ± 0.3	16.5 ± 2.2	18	<0.005
Chlorpromazine	14.8 ± 2.0	36.5 ± 4.1	40	<0.005
Promethazine	21.8 ± 0.9	123.3 ± 3.5	340	<0.0005

* ID_{50} values represent the concentrations of phenothiazine required either to block histamine release induced by anti-IgE (1/400) or compound 48/80 (0.15 $\mu g\,ml^{-1}$) by 50% or to inhibit calmodulin (CaM) activity by 50%. Values for the inhibition of calmodulin have been taken from the literature (Levin & Weiss, 1976; Prozialeck & Weiss, 1982). Control histamine releases ranged from 29 ± 5% to 39 ± 5% and the values cited for inhibition of histamine release are means ± s.e.mean and are based on six experiments in each instance.

** The P value compares the inhibition by a phenothiazine of histamine release induced by anti-IgE with the inhibition by that same phenothiazine of secretion induced by compound 48/80.

Pearce, 1985). Moreover, the protein has been detected in mast cells and has also been found associated with the granule membrane (Chakravarty & Nielsen, 1985). By analogy with other systems, the specific phosphorylation of key proteins by calmodulin-dependent protein kinases may be important in the translocation of granules to the periphery and/or any fusional processes thereafter.

The present study was designed to try to establish potential sites of action of the phenothiazines in the inhibition of mast cell secretion. The use of different divalent cations proved particularly enlightening in this respect. Both strontium and barium are capable of taking the place of calcium in a number of smooth muscle preparations and secretory systems (Hagiwara & Byerly, 1981). In accord with previous findings (Foreman & Mongar, 1972; Atkinson *et al.*, 1979), strontium and barium could substitute for calcium in supporting histamine release induced by a variety of stimuli although, in general, higher concentrations of the former two cations were required to mediate secretion. The need for higher concentrations of strontium and barium may reflect either a relative inability of these ions to enter the cell and/or an impaired ability to interact with an intracellular calcium-binding site, since it has been shown that calmodulin activation by metal ions displays an order of dependency following the series: calcium > strontium > barium (Levin & Weiss, 1977; Cheung, 1984). That cells extensively depleted of their intracellular calcium were more responsive to a given concentration of cation suggests that the potency series does not solely reflect a selective impermeability of the cell membrane to a cation and that the increased electrochemical and concentration gradients promote a facilitated influx of the cation into the cytosol. This finding with depleted cells favours the alternative suggestion that barium and strontium may mimic the effects of calcium by binding to an intracellular calcium-binding site.

As a consequence of the above studies, it seemed reasonable to suppose that barium was less likely to bind to a cation 'receptor' site and that the phenothiazines would be better equipped to antagonize secretion induced by barium than that initiated by strontium which, in turn, would be more effectively modulated than the release induced by calcium. For the large part, this conjecture held up to experimental evaluation. Thus, the phenothiazines (and W-7) were found to inhibit secretion induced by a variety of stimuli in the presence of divalent cations preferentially in the order barium > strontium > calcium, a trend which held for depleted and non-depleted cells alike. The phenothiazines, therefore, by virtue of their cation-dependence for inhibition, appear to exert their inhibitory effects by a specific mechanism(s). This contrasts with the situation for a number of anti-allergic compounds which are thought to block secretion by a generalized membrane-stabilizing effect (Pearce & Clements, 1982). Although the results are consistent with an effect of the phenothiazines at an intracellular divalent cation binding site, it is equally feasible to account for the data as phenothiazine antagonism at an extracellular divalent cation binding site when either antigen or compound 48/80 is employed as the stimulus. Indeed, our previous studies (Peachell & Pearce, 1985) have shown that immunological release is more effectively inhibited by the phenothiazines than that evoked by polybasic compounds (see Table 3). This suggested an additional component of inhibitory action for the phenothiazines, perhaps an interference of some aspect of channel formation or competition by drug and cation for a binding site before internalization of the latter, in addition to effects distal to calcium influx. Thus, in view of the fact that calcium channels bind divalent cations in the following order of affinity, calcium > strontium > barium (Hagiwara & Byerly, 1981), the preferential activity displayed by the phenothiazines in the presence of these divalent cations may reflect an inhibitory action for this class of compound not unlike that found for calcium antagonists (e.g. dihydropyridines) in other biological systems (Karaki *et al.*, 1982).

Further evidence for an extracellular calcium antagonist-like effect for the phenothiazines was observed when the inhibition by thioridazine of compound 48/80-induced secretion from depleted mast cells incubated in varying extracellular calcium concentrations (1, 0.1 and 0.01 mM) was studied. Lowering the extracellular calcium concentration led to a progressive increase in the ability of thioridazine to prevent secretion, a finding that suggests that, the antagonistic effects of the phenothiazine at the level of calcium entry become increasingly prominent as the extracellular calcium concentration is reduced. This form of evidence has been used in the past to characterize calcium antagonist activity (Karaki *et al.*, 1982; Chand *et al.*, 1985). Studies along identical lines in non-depleted cells also suggested, although less definitively, a calcium-antagonist-like effect for thioridazine. That said, it seems unlikely that inhibition of calcium entry would be significantly operative at an extracellular concentration of 1 mM calcium for compound 48/80-induced secretion, since thioridazine was equipotent in the presence and absence (where no influx of the cation occurs) of extracellular calcium. The inhibitory effects of thioridazine under both of these conditions are likely to be exerted, predominantly, distal to calcium influx.

In the paired situation where 0.1 mM calcium is present extracellularly and compound 48/80 is used to induce secretion in both treated and untreated

cells, calcium entry blockade would be expected to be similar in both types of cell. Inhibitory effects distal to calcium entry would also be operative and if the sole additional blockade were exerted at an intracellular calcium binding site, then inhibition by thioridazine should be similar in depleted and non-depleted cells. Yet, the potency of thioridazine is significantly higher in preventing secretion from non-depleted cells. This suggests that some additional inhibitory component may be operative in non-depleted cells and as the most obvious difference between the two types of cell is their intracellular calcium content, it is possible that the phenothiazine exerts some inhibitory effect at this level. The pool of calcium that enters the non-depleted cell after activation may directly bind to a calcium effector site or else displace an intracellular pool of calcium which, in turn, interacts with the calcium effector site. The stabilization by thioridazine of this intracellular calcium pool may reflect an additional site of action for the phenothiazines.

Studies of the effects of thioridazine on secretion from non-depleted cells in the presence of barium, strontium and calcium indicate that the extracellular presence of cations influences which pool (i.e. either extracellular or intracellular) of cation is deployed for secretion. In order to induce secretion by compound 48/80 in the presence of strontium or barium in non-depleted cells, a concentration ($5\,\mu g\,ml^{-1}$) of the polyamine that can initiate secretion in the absence of divalent cations is required. If secretion in the presence of either barium or strontium were solely due to the mobilization of intracellular calcium, then thioridazine should be equipotent as an inhibitor of secretion under all conditions. Yet, the rank order of potency for the inhibition by thioridazine in the presence or absence of cations was, barium > strontium > calcium = no calcium. These results suggest that, in situations where a stimulus is sufficiently potent to draw on both intra- and extracellular sources of divalent cations, it may be disposed to facilitating influx of the cation in preference to mobilizing intracellular stores of calcium.

Perhaps, the single most important piece of evidence that indicates an effect of phenothiazines distal to divalent cation influx is the chlorpromazine inhibition of ionophore-induced release in depleted mast cells. Ionophores act by translocating divalent cations into the interior of the cell by-passing any calcium channels or receptor-mediated events at the cellular surface. An inhibitory effect in this experimental situation cannot be explained in terms of its possible antagonism of normal divalent cation entry nor, in depleted cells, to an effect on intracellular calcium pools. Instead, the inhibition by chlorpromazine of ionophore-induced release is most logically explained by an effect at an intracellular calcium receptor site, possibly calmodulin. As discussed, the rank order of inhibitory activity in the presence of different divalent cations (barium > strontium > calcium) is consistent with this view.

It should be noted that, in addition to anti-calmodulin activity, the phenothiazines and W-7 have also been found to inhibit protein kinase C (Adunyah *et al.*, 1982; Schatzman *et al.*, 1983). Because protein kinase C may be important in stimulus-secretion coupling in the mast cell (Katakami *et al.*, 1984), especially in situations where the extracellular concentration of calcium is reduced, some caution in the interpretation of results is warranted. These problems notwithstanding, a clear association of these compounds with a divalent cation-dependent process cannot be disputed.

In summary, the results presented herein illustrate some important points. Calcium, strontium and barium (and in that decreasing potency order) can support histamine release from activated mast cells. It is not entirely clear whether the incoming ion interacts directly with an intracellular cation site or displaces intracellular calcium which, in turn, constitutes the effector pool. However, a preponderance of evidence suggests that all three ions can bind directly to an internal site to initiate secretion. In accord with our initial hypothesis, the phenothiazines and W-7 block the stimulated release of histamine from RPMC displaying a preferential divalent cation dependency for inhibition of barium > strontium > calcium. A major component of the inhibitory activity of these compounds is probably exerted distal to calcium influx since ionophore/cation-induced secretion in depleted cells was inhibited by chlorpromazine. In conclusion, these studies suggest that the phenothiazines and W-7 mediate their effects at an intracellular calcium-binding site (calmodulin?), although additional inhibitory components may be operative dependent upon the stimulus employed and the experimental conditions.

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The role of $GABA_A$ receptor function in peristaltic activity of the guinea-pig ileum: a comparative study with bicuculline, SR 95531 and picrotoxinin

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1 The peristaltic activity of the guinea-pig ileum was studied in the absence and in the presence of the blockade of $GABA_A$ receptors.

2 Bicuculline (1–30 μM), improved at the highest concentrations the efficiency of peristalsis by enhancing the frequency of propulsive contractions and the amount of fluid ejected per unit of time.

3 Neither SR 95531 (0.3–10 μM), a novel $GABA_A$ receptor antagonist, which competitively antagonized 3-aminopropane sulphonic acid induced contractions in myenteric plexus-longitudinal muscle preparations (pA_2 value: 6.47), nor picrotoxinin (1–30 μM) modified peristaltic parameters or influenced the potentiating effect of bicuculline on peristaltic activity.

4 In myenteric plexus-longitudinal muscle preparations, bicuculline (1–30 μM) enhanced the amplitude of electrically-induced cholinergic contractions without modifying submaximal contractions to applied acetylcholine. SR 95531 and picrotoxinin had no effect on twitch amplitude. In the presence of each of these compounds, bicuculline retained its potentiating effect.

5 The results obtained with SR 95531 and picrotoxinin question the view that $GABA_A$ receptors may exert a critical role in intestinal propulsion by modulating the activity of nerve pathways subserving peristalsis. Bicuculline potentiates the peristaltic activity of the ileum probably via a facilitatory effect on enteric cholinergic transmission that is independent of $GABA_A$ receptor blockade.

Introduction

A large body of evidence indicates that in the ganglia of the myenteric plexus of the guinea-pig ileum there are neurones containing γ-aminobutyric acid (GABA) the fibres of which contribute to the interganglionic strands and the nervous ramifications within the circular muscle (Jessen *et al.*, 1983; Miki *et al.*, 1983; Tanaka, 1985; Hills *et al.*, 1987). This extensive neural distribution together with the presence of high affinity sites for GABA uptake and release (Kerr & Krantis, 1983) is consistent with GABA being an enteric neurotransmitter (Jessen *et al.*, 1979; Krantis *et al.*, 1986; Jessen *et al.*, 1987). The lack of direct effects of GABA on the intestinal musculature (Krantis *et al.*, 1980) and the observation that GABA may influence both excitatory and inhibitory enteric nerves (Krantis *et al.*, 1980; Krantis & Kerr, 1981a; Kaplita *et al.*, 1982; Giotti *et al.*, 1983; Kleinrok & Kilbinger, 1983; Taniyama *et al.*, 1983), suggest that GABA-releasing neurones could be involved in nerve pathways mediating peristalsis. Early work showed that exogenous GABA has inhibitory effects on ileal peristaltic activity (Hobbiger, 1958; Inouye *et al.*, 1960). An inhibitory role for endogenous GABA has recently been suggested, based on the increased efficiency of guinea-pig ileal peristalsis after exposure to bicuculline (Schwörer & Kilbinger, 1988), a competitive antagonist of $GABA_A$ receptors. In these experiments, however, the efficiency of peristalsis was not modified by applied GABA nor by a GABA desensitizing procedure.

In this study, several pharmacological approaches have been used to assess the influence of $GABA_A$ receptor function on propulsive activity of the

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guinea-pig ileum. Besides bicuculline, SR 95531 (Wermuth & Bizière, 1986), a competitive $GABA_A$ receptor antagonist in the central nervous system (not yet evaluated in the intestine) and picrotoxinin, a drug which blocks chloride ion (Cl^-) conductance at the ionophore associated with the $GABA_A$ receptor (Johnston, 1978; Krantis & Kerr, 1981a), have been tested.

Methods

Albino male guinea-pigs weighing 300–350 g were stunned and bled. Segments of distal ileum were taken between 10–30 cm proximal to the ileo-caecal junction and placed in a Petri dish. The lumen was washed out with warm (36°C) Tyrode solution.

Whole intestinal preparations and induction of peristalsis

Whole intestinal segments 10 cm long were set up horizontally in a bath containing 100 ml Tyrode solution of the following composition (mM): NaCl 137.9, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1.04, $NaHCO_3$ 11.9, NaH_2PO_4 0.4, glucose 5.5 and gassed with 95% O_2 and 5% CO_2 at 36°C. Longitudinal movements were recorded with an isotonic transducer loaded at 1–1.5 g. Peristalsis was induced according to the method described in detail by Tonini *et al.* (1981) with minor modifications concerning mainly the technique to detect 'threshold pressure' and the degree of radial distension of the gut wall. Two appropriately calibrated pressure transducers were connected to the aboral end of the preparation to detect the pressure at which peristalsis was triggered (threshold pressure) and the peak pressure (fluid ejection pressure) achieved during each peristaltic event respectively. Since 'threshold pressure' was consistently lower than peak pressure, a tenfold magnification of recorder sensitivity was usually required to detect this pressure value (see tracing P_2 of Figure 2). Peristalsis was achieved by delivering into the lumen a near-threshold amount of fluid (approximately 80–90% of that required to induce the peristaltic reflex) at a rate of 0.75 ml min^{-1} by a LKB Varioperpex II pump. This resulted in a few organized propulsive waves (approximately 8 every 30 min). The ejected fluid was replaced by an equivalent amount 10 s after completion of each peristalsis. Since this procedure does not induce a series of continuous repetitive propulsive contractions such as those evoked by the original procedure (approximately 12 in a 10 min cycle), any drug-induced facilitatory effect on peristaltic activity can be amplified. For example, under these conditions, caerulein increased by 320% the frequency of propulsive contractions at a concentration (3 nM) which causes only a 60% increase in the original procedure (unpublished data from our laboratory). Bicuculline (1–30 μM), SR 95531 (0.3–10 μM) and picrotoxinin (1–30 μM) were added to the bath at the beginning of a 30 min cycle. When bicuculline was administered in preparations pretreated with SR 95531 or picrotoxinin, these compounds were added to the bath 5 min before bicuculline. No more than two concentrations of the same drug were tested in each preparation. The effects on peristaltic activity, evaluated in terms of number of peristaltic waves and amount of fluid ejected per cycle (see Tonini *et al.*, 1981), were compared with those obtained in a previous control cycle. A 10 min rest period was allowed between two consecutive cycles. All experiments started after a 60 min equilibration period.

Myenteric plexus-longitudinal muscle preparations

Strips of longitudinal muscle of ileum of about 30 mm in length, with the myenteric plexus attached (Paton & Zar, 1968), were set up isometrically (tension 0.5 g) in 4 ml organ baths containing Tyrode solution kept at 36°C. Electrical field stimulation was delivered by means of two silver electrodes placed at the top and the bottom of the chamber. Stimulation parameters were: 0.1 Hz, pulse duration 0.1 ms, 25 V (a voltage causing a contraction equivalent to 80% of the maximal amplitude of cholinergic contractions). These preparations were used to assess the influence of $GABA_A$ receptor antagonists and picrotoxinin on the electrically-induced cholinergic twitch response. Any drug-induced change in twitch amplitude was evaluated as the percentage variation of the basal response taken as 100% response.

In separate experiments, unstimulated myenteric plexus-longitudinal muscle preparations were set up isotonically (load: 0.5 g) in 10 ml organ baths to quantify the antagonism of SR 95531 on the phasic cholinergic contractions caused by the $GABA_A$ receptor agonist 3-aminopropane sulphonic acid (3-APS). Concentration-response curves to 3-APS (1–300 μM) were constructed in the absence and in the presence of SR 95531 (0.3–3 μM) left in the bath 10 min before agonist administration. In each preparation, only one 3-APS concentration was tested, the drug being administered at 20 min intervals during which the preparations were repeatedly washed to avoid the potential occurrence of $GABA_A$ receptor desensitization. The response to each 3-APS concentration was expressed as a percentage of the maximum response induced by 300 μM 3-APS. Dose-ratios were calculated at the EC_{50} level with and without SR 95531. The apparent pA_2 value was calculated by the method of Arunlakshana & Schild (1959). The equilibration period was at least 30 min.

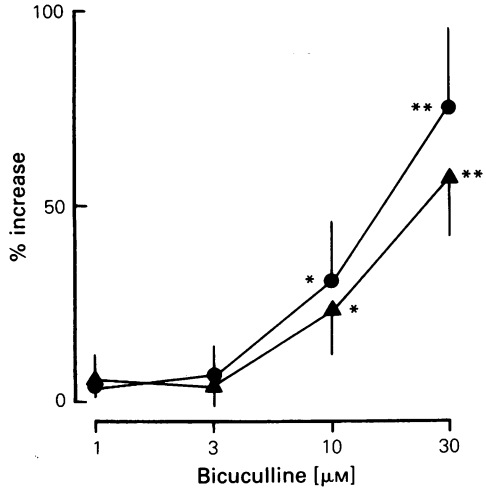


Figure 1 Enhancing effect of bicuculline on intestinal persistalsis. Data are given as percentage increase (vs control) of number of peristaltic waves (●) and amount of fluid ejected (▲) during a 30 min cycle. Points and bars represent means ± s.e.mean of 4–8 experiments (1 μM, $n = 4$; 3 and 10 μM, $n = 7$; 30 μM, $n = 8$). The asterisks indicate significant difference from the control value (*$P < 0.05$; **$P < 0.01$).

Data analysis

Results are expressed as means ± standard error of the mean (s.e.mean). Student's t test for paired and unpaired data was used for statistical comparisons.

Drugs

The following drugs were used: 3-amino-1-propane sulphonic acid (3-APS), (−)-bicuculline methiodide, picrotoxinin, hyoscine hydrobromide, acetylcholine chloride (ACh) (all from Sigma, St. Louis, MO, U.S.A.), 2-(3-carboxypropyl)-3-amino-6-(4-methoxyphenyl)-pyridazinium bromide (SR 95531) (RBI, Natick, MA, U.S.A.). All drugs were dissolved in distilled water except picrotoxinin which was dissolved in ethanol and further diluted in distilled water.

Results

Effects of $GABA_A$ receptor blockade on peristalsis

Peristalsis was elicited by maintaining a radial distension of the gut wall just below the threshold level which was approximately 2 cmH_2O. This was achieved by delivering into the lumen 0.5 ml (0.6 ml fluid is the amount usually required to trigger the peristaltic reflex) to avoid the occurrence of continuously repetitive propulsive contractions. By this procedure, the peristaltic activity in a 30 min control cycle was characterized by clusters of 2 or 3 propulsive waves occurring approximately 3 times per cycle. The number of peristaltic waves and the amount of fluid ejected were 7.7 ± 0.3 ($n = 47$) and 4.0 ± 0.3 ml ($n = 47$) per cycle, respectively.

After a control cycle, bicuculline (1–30 μM) was administered at the beginning of the subsequent cycle. No effects were observed up to 3 μM but 10 and 30 μM bicuculline dose-dependently increased

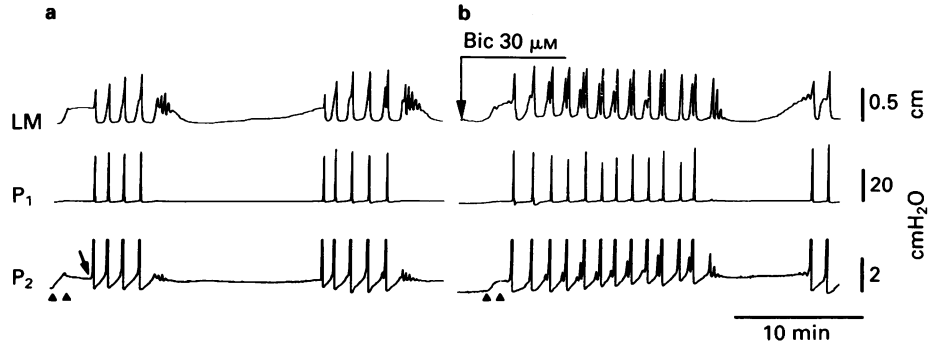


Figure 2 A representative tracing illustrating control peristaltic activity of isolated ileal segments (a) and the facilitatory effect of 30 μM bicuculline (Bic) on peristalsis (b). From top to bottom: Contraction of the longitudinal musculature (LM) associated with peristalsis; recording of maximal peak pressure (fluid ejection pressure) achieved during each peristaltic event (P_1); pressure values (the first indicated with an arrow) at which peristalsis developed (P_2). Note that the fluid delivered into the lumen [in (a) and (b) the first injection of fluid was performed during the interval between marks] did not trigger an immediate peristaltic reflex since the stimulus was maintained at a near-threshold level.

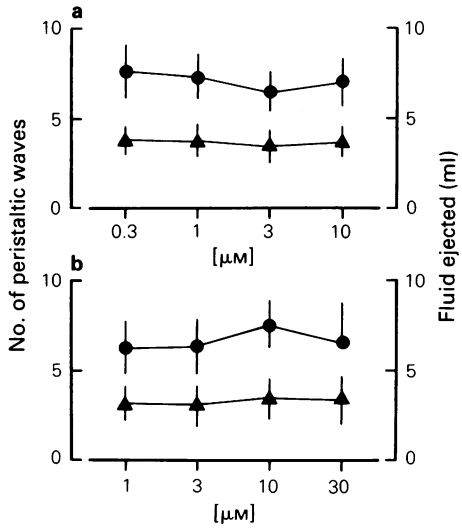


Figure 3 Failure of SR 95531 (a) and picrotoxinin (b) to modify intestinal peristalsis evaluated in terms of number of peristaltic waves (●) and amount of fluid ejected (▲) during a 30 min cycle. For clarity, control values are not indicated (they were virtually identical to those shown in the presence of the drugs). Points and bars represent means ± s.e.mean of 4–6 experiments.

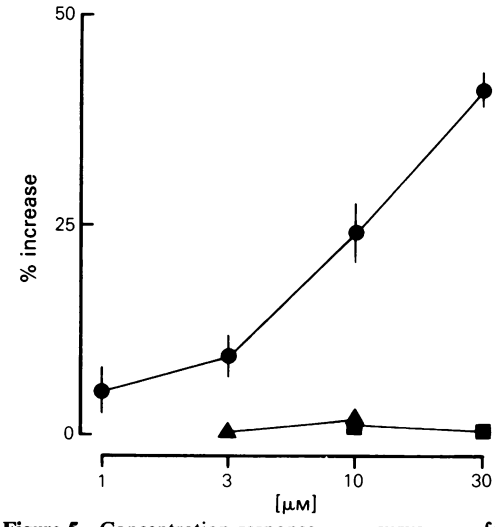


Figure 5 Concentration-response curve for bicuculline-induced (●) enhancement of the amplitude of electrically-induced submaximal (80%) cholinergic contractions in the myenteric plexus-longitudinal muscle preparations. Note the ineffectiveness of SR 95531 (▲) and picrotoxinin (■). Points and bars represent means ± s.e.mean of 3–7 experiments. The amplitude of contractions are significantly enhanced ($P < 0.05$) at concentrations of bicuculline $\geqslant 3\,\mu$M.

the number of peristaltic waves and the amount of fluid ejected per cycle (Figures 1 and 2). This bicuculline-induced facilitatory effect was not observed in all the preparations tested. The percentage of preparations responding to 10 and 30 μM bicuculline was 42.8% (3 out of 7) and 62.5% (5 out of 8), respectively. In the 5 preparations responding to 30 μM bicuculline, a slight but consistent increase of the activity of the longitudinal coat which at times persisted during the entire cycle was observed. Fluid ejection pressure in the presence of 10 or 30 μM bicuculline (20.9 ± 2.0 cmH_2O and 21.3 ± 20 cmH_2O, $n = 24$, respectively) was not different from that observed under control conditions (21.7 ± 1.9 cmH_2O, $n = 24$).

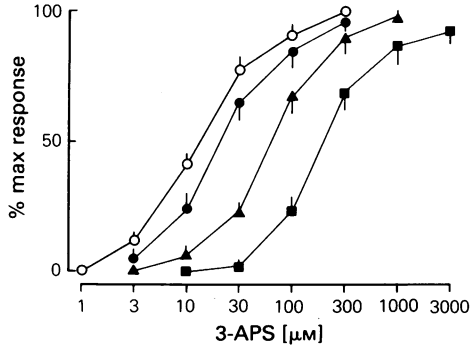


Figure 4 Concentration-response curves for 3-aminopropane sulphonic acid (3-APS)-induced contractions in the myenteric plexus-longitudinal muscle preparations in the absence (open circles) and in the presence (closed symbols) of varying concentrations of the GABA$_A$ receptor antagonist SR 95531 (0.3 μM: ●; 1 μM: ▲; 3 μM: ■). Note the parallel rightward shift of the 3-APS curve without depression of the maximum agonist response. Points and bars represent means ± s.e.mean of 4–8 experiments.

SR 95531 (0.3–10 μM), a competitive GABA$_A$ receptor antagonist approximately 4 times as potent as bicuculline in the intestine (see below), and picrotoxinin (1–30 μM) did not influence the peristaltic parameters (Figure 3). In preparations in which bicuculline had been shown to potentiate peristaltic activity, the potentiating effect of bicuculline was not affected by pretreatment with 10 μM SR 95531 ($n = 4$) and 30 μM picrotoxinin ($n = 4$).

Interaction experiments in myenteric plexus-longitudinal muscle preparations

Since apparently no data on SR 95531-induced antagonism of GABA$_A$ receptors are available for the intestine, experiments were performed in the

myenteric plexus-longitudinal muscle preparations with 3-APS as the $GABA_A$ receptor agonist. Figure 4 shows a parallel rightward shift caused by three concentrations of SR 95531 on the phasic cholinergic contractile response to 3-APS. Antagonism was rapidly and completely reversible upon washing. The slope of the Arunlakshana & Schild plot (-1.15 ± 0.25) was not significantly different from -1, the value expected for competitive antagonism. The apparent pA_2 value was 6.47.

In the electrically-stimulated preparations, bicuculline (1–30 μM) invariably caused a concentration-dependent increase of the amplitude of cholinergic twitch contractions (Figure 5). This effect was not persistent and at 10 and 30 μM bicuculline, a 50% decay was observed after 13 and 20 min, respectively. In contrast, submaximal contractions to applied ACh (0.1 μM), mimicking in amplitude those evoked by electrical stimulation, were not affected by bicuculline at the highest concentration (30 μM; $n = 5$).

SR 95531 (3, 10 μM; $n = 6$) and picrotoxinin (10, 30 μM; $n = 6$) (Figure 5) did not modify the amplitude of electrically-induced contractions. Neither of these drugs interfered with the potentiating effect of bicuculline on these preparations ($n = 8$) (Figure 6).

Discussion

The influence of $GABA_A$ receptor blockade on the peristaltic activity of the guinea-pig isolated ileum was characterized by use of different antagonists. Bicuculline enhanced propulsive efficiency as indicated by the increase in number of peristaltic waves and amount of fluid ejected. The bicuculline-induced facilitatory effect probably reflects an enhanced excitability of the preparation to radial distension which in this study was maintained at a near-threshold level. In other words, it appears that this near-threshold radial distension represented the 'new' threshold level for peristalsis in the presence of bicuculline. In contrast, peristalsis was not influenced by SR 95531, a competitive $GABA_A$ receptor antagonist (Wermuth & Bizière, 1986; Mienville & Vicini, 1987) which in the myenteric plexus-longitudinal muscle preparation is approximately 4 times as potent as bicuculline in antagonizing 3-APS-induced contraction (bicuculline pA_2: 5.7–5.91, Kaplita *et al.*, 1982 and Giotti *et al.*, 1983; SR 95531 pA_2: 6.47, present results). Peristalsis was also unaffected by picrotoxinin, a drug that blocks Cl^- conductance at the ionophore associated with the $GABA_A$ receptor (Johnston, 1978; Krantis & Kerr, 1981a). These observations, together with the finding that the facilitatory effect of bicuculline on peristalsis was not influenced by pretreatment with either SR 95531 or picrotoxinin, suggest that the observed effect of bicuculline is probably unrelated to $GABA_A$ receptor blockade. Bicuculline, in spite of being not completely devoid of non-specific effects (Bartolini *et al.*, 1985), has been commonly (and often solely) employed to evaluate the role of $GABA_A$ receptor function on intestinal peristalsis. In the guinea-pig isolated colon, bicuculline has been shown either to increase (Frigo *et al.*, 1987) or to inhibit (Krantis & Kerr, 1981b) peristaltic activity and to be ineffective in modifying both the excitatory and inhibitory reflexes subserving peristalsis (Krantis *et al.*, 1980).

Recently, the facilitatory effect of bicuculline on ileal propulsive activity has been explained by assuming an involvement of endogenous GABA in the inhibition of the peristaltic reflex, possibly via $GABA_A$ receptors located on inhibitory non-adrenergic, non-cholinergic (NANC) nerve pathways (Schwörer & Kilbinger, 1988). However, the latter

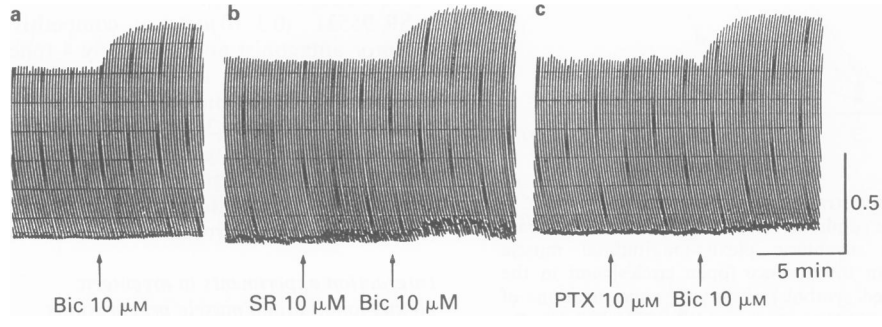


Figure 6 Bicuculline (Bic)-induced potentiation of electrically-induced cholinergic contractions in the myenteric plexus-longutidinal muscle preparations (a). Failure of SR 95531 (SR: in b) and picrotoxinin (PTX: in c) to modify the amplitude of twitch contractions. Note that bicuculline fully retains its potentiating effect in the presence of SR 95531 or picrotoxinin.

hypothesis is questionable because in the guinea-pig ileum activation of $GABA_A$ receptors is associated with excitation of cholinergic nerves (Krantis & Kerr, 1981a; Kaplita *et al.*, 1982; Giotti *et al.*, 1983; Kleinrok & Kilbinger, 1983; Taniyama *et al.*, 1983; Tonini *et al.*, 1987) without any apparent effect on NANC nerves. Based on these findings, bicuculline would be expected to inhibit rather than to facilitate propulsive motility. Moreover, if GABA-releasing neurones were functionally involved in the peristaltic event, the blockade of $GABA_A$ receptors by bicuculline would, at least in theory, unmask (or potentiate) a further inhibitory effect of GABA via $GABA_B$ receptors (Bowery *et al.*, 1981) located on the excitatory nerve pathways subserving peristalsis (Kaplita *et al.*, 1982; Giotti *et al.*, 1983; Kleinrok & Kilbinger, 1983; Cherubini & North, 1984a; Ong *et al.*, 1987). This is probably the mechanism through which applied GABA inhibits propulsive activity in the guinea-pig ileum (Hobbiger, 1958; Inouye *et al.*, 1960). The failure of SR 95531 and picrotoxinin to potentiate peristaltic activity in our preparations contrasts with the view that $GABA_A$ receptors may modulate peristalsis. As mentioned above, the persistence of the effect of bicuculline in the presence of SR 95531 or picrotoxinin is in agreement with an action of bicuculline unrelated to $GABA_A$ receptor blockade.

In an attempt to understand the facilitatory effect of bicuculline on peristalsis, we have re-assessed the effect of this compound on cholinergic transmission since bicuculline, at least in the central nervous system, is endowed with some 'cholinergic activity' partly related to acetylcholinesterase inhibition (Gardner & Webster, 1973; Svenneby & Roberts, 1974; Breuker & Johnston, 1975). In the intestine, a bicuculline-induced facilitatory effect on ACh release (at concentrations having no anticholinesterase activity) has been demonstrated only in the guinea-pig colon (Frigo *et al.*, 1987). In contrast, a number of studies have indicated that bicuculline (10 μM) fails to modify ACh release (Kleinrok & Kilbinger, 1983) and electrically-induced cholinergic contractions (Krantis & Kerr, 1981a; Allan & Dickenson, 1986) in the small intestine of the same animal species. We were surprised, therefore, to find that bicuculline invariably and dose-dependently increased the amplitude of cholinergic twitch responses in the myenteric plexus-longitudinal muscle preparation at concentrations as low as 3 μM, while SR 95531 and picrotoxinin were ineffective. Less unexpected, however, was the persistence of this effect in the presence of SR 95531 or picrotoxinin, a finding similar to that obtained in experiments on peristalsis. Based on the inability of bicuculline to modify the response to applied ACh (see also Krantis & Kerr, 1981a; Ong & Kerr, 1983), it is likely that the potentiating effect of bicuculline on electrically-evoked cholinergic contractions is due to enhanced ACh release. This action might underlie the facilitatory effect of bicuculline on propulsion, which is especially prominent at a concentration (30 μM) also found to depolarize the membrane of myenteric neurones in the guinea-pig ileum (Cherubini & North, 1984b). There are no plausible reasons which explain the discrepancy with previous results concerning the effect of bicuculline on ileal cholinergic transmission. We wish to point out, however, that our findings were obtained in submaximally-stimulated preparations in which facilitatory effects on cholinergic transmission could be detected more easily. Nevertheless, further experiments on ACh release are required to clarify this point.

Our findings indicate that the choice of $GABA_A$ receptor antagonists is critical when the functional role of $GABA_A$ receptors is examined. SR 95531 may represent a suitable alternative to bicuculline as a competitive $GABA_A$ receptor antagonist in studies on intestinal motility.

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Evidence for prazosin-resistant, rauwolscine-sensitive α-adrenoceptors mediating contractions in the isolated vascular bed of the rat tail

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1 The postjunctional α-adrenoceptors mediating contractions in the isolated vascular bed of the perfused rat tail have been investigated, in the presence and absence of an increase in perfusion pressure by arginine vasopressin (AVP).

2 In the absence of AVP, bolus doses of noradrenaline (NA) and phenylephrine produced pressor responses of similar time course, while UK-14,304 was practically inactive. Responses to noradrenaline were inhibited more by 0.05 μM prazosin than by 1 μM rauwolscine, suggesting the presence of α_1-adrenoceptors.

3 Following a sustained elevation in perfusion pressure by AVP, both UK-14,304 and NA (the latter in the presence of 0.05 μM prazosin to inhibit α_1-adrenoceptors) elicited dose-dependent pressor responses. The maximum response to UK-14,304 under these conditions was approximately 30% of the maximum response to NA in the absence of prazosin and AVP. Responses to phenylephrine were not affected by the AVP-induced increase in vascular tone.

4 In the presence of AVP, pressor responses to UK-14,304 were resistant to 0.05 μM prazosin and susceptible to antagonism by 1 μM rauwolscine ($-\log K_b$ 7.65 ± 0.15). Similarly, responses to NA in the presence of 0.05 μM prazosin and AVP were inhibited by 1 μM rauwolscine. This represents the first demonstration of prazosin-resistant, rauwolscine-sensitive α_2-adrenoceptor-mediated responses in the vasculature of the rat tail.

5 These results suggest that in isolated vascular preparations, functional populations of postjunctional α_2-adrenoceptors may be 'uncovered' by the presence of AVP.

Introduction

In the pithed rat, pressor responses to the bolus administration of α-adrenoceptor agonists have been shown to be mediated by two discrete receptor subtypes on arterial smooth muscle, α_1- and α_2-adrenoceptors (Docherty & McGrath, 1980; McGrath, 1982). Responses elicited via the α_1-subtype are sensitive to prazosin and resistant to yohimbine, rauwolscine and idazoxan, while responses mediated via the α_2-subtype are resistant to prazosin, but sensitive to either yohimbine, rauwolscine or idazoxan (McGrath, 1982; van Zwieten & Timmermans, 1987).

However, in spite of the availability of α-adrenoceptor agonists with proven selectivity for either subtype *in vivo*, there are few examples *in vitro* of blood vessels that possess a functional population of postjunctional α_2-adrenoceptors; contractile responses to all agonists are usually sensitive to low concentrations of prazosin. Furthermore, of the few available preparations that possess postjunctional α_2-adrenoceptors, the majority of these have been from the venous side of the circulation (Ruffolo, 1986).

Postjunctional α_2-adrenoceptors have been identified on the smooth muscle of the rat tail by radioligands for this subtype (Weiss *et al.*, 1983; Nasseri *et al.*, 1985) and several groups have shown that these receptors mediate contractions (Medgett & Langer, 1984; Rajanayagam & Medgett, 1987; Abe *et al.*, 1987). However, much of the evidence for a functional response mediated via α_2-adrenoceptors is based upon a modest differential sensitivity of contractile

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responses elicited by 'selective' α-adrenoceptor agonists to prazosin and either idazoxan or yohimbine, rather than the pronounced differences observed *in vivo*. For example, contractile responses to the selective α_1-adrenoceptor agonist methoxamine were less sensitive to the selective α_2-adrenoceptor antagonist idazoxan than were those elicited by either noradrenaline or the selective α_2-adrenoceptor agonist UK-14,304 (see: Abe *et al.*, 1987). Moreover, responses to both agonists were similarly sensitive to the α_1-adrenoceptor antagonist prazosin. These apparently contradictory observations have led to the suggestions that this preparation either does not possess a functional population of postjunctional α_2-adrenoceptors (Marwood *et al.*, 1986), or that the α-adrenoceptor population is unlike the α_1- and α_2-subtypes described *in vivo* (Su *et al.*, 1986).

In the present study, we provide evidence for a population of postjunctional α_2-adrenoceptors in the perfused isolated whole tail of the rat. The pharmacological characteristics of responses mediated by this subtype (prazosin-resistant, rauwolscine-sensitive) appear to be critically dependent upon the presence of pre-existing 'tone' and, as such, may give insight into the elusive nature of this subtype in isolated vascular smooth muscle.

Methods

Male Wistar rats weighing 275–325 g were killed by stunning followed by cervical dislocation. The ventral surface of the tail was shaved at the base before amputation by cutting through an intervertebral disc. The skin was reflected around the proximal first 3–5 cm of the tail, and a longitudinal incision was made through the midline of the fascia on the underside of the tail. The tail artery was exposed using blunt forceps and cleaned of fat and connective tissue. Following insertion of a polyethylene cannula into the tail artery, the whole tail was gently placed inside a glass water-heated vessel maintained at 30°C, a temperature similar to that found in anaesthetized rats (Wade & Beilen, 1970).

The arterial cannula was attached to a perfusion circuit and the tail vasculature perfused with modified physiological salt solution, maintained at 30°C and gassed with 95% O_2: 5% CO_2, at a constant rate of 0.6 ml min^{-1}. After approximately 60 s of perfusion, blood filled drops were observed from the cut end of the tail and this indicated a successful cannulation. The perfusion pressure was initially high but soon declined and stabilised between 40–80 mmHg, approximately 45 min after the start of perfusion.

At the end of the equilibration period, 1 nmol noradrenaline (NA) was injected into the perfusion solution via an injection port close to the point of cannulation of the tail. This was repeated until three similar pressor responses to noradrenaline were obtained. Two different experimental procedures were then performed.

(1) *α-Adrenoceptor-mediated responses at basal perfusion pressure*

A dose-response curve to noradrenaline (0.01–10 nmol) was constructed by bolus injection (10 μl) into the perfusate. A minimum of 2 min was allowed between individual responses. Following a 45 min equilibration period with perfusate containing either 0.05 μM prazosin or 1 μM rauwolscine, the curve was repeated. In a number of experiments, the curve to NA was again repeated after a further 45 min equilibration with perfusate containing both 0.05 μM prazosin and 1 μM rauwolscine.

(2) *α-Adrenoceptor-mediated responses after elevation of perfusion pressure*

A dose-response curve was constructed to either UK-14,304 (0.001–3 nmol) or phenylephrine (0.01–10 nmol) in normal saline, or noradrenaline in the presence of 0.05 μM prazosin in saline. [Arg]-vasopressin (AVP) 1–5 nM was then included in the solution until the perfusion pressure was elevated by 40–80 mmHg to approximately 120 mmHg. In general, it was found that a gradual increase in the concentration of AVP, 0.5 nM every 20–30 min, was the most effective method of achieving a steady elevated perfusion pressure. Dose-response curves to all three agonists (for noradrenaline, still in the presence of 0.05 μM prazosin) were then repeated in the presence of AVP. In the case of UK-14,304, responses were again repeated after a further 45 min equilibration with either 0.05 μM prazosin or 1 μM rauwolscine. For noradrenaline (in the presence of both 0.05 μM prazosin and AVP), responses were repeated in the presence of 1 μM rauwolscine.

In the majority of the preparations with AVP-induced tone, acetylcholine (0.1–10 nmol in 10 μl) was found to produce a dose-related transient drop in perfusion pressure (5–20 mmHg), presumably by the release of endothelium-derived relaxing factor (EDRF) from endothelial cells (Furchgott, 1984).

Measurement of responses and analysis of results

Changes in perfusion pressure were detected by a pressure transducer (Elcomatic EM 751) placed in the perfusion circuit and recorded on a Linseis Flatbed recorder. Unless stated otherwise, responses were measured as the peak pressor response to the bolus injection of the agonist. Dose-response

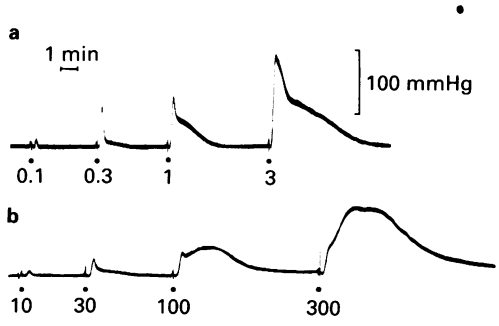


Figure 1 Representative recordings of pressor responses of the rat isolated perfused tail to noradrenaline (a) before and (b) after exposure to 0.05 μM prazosin. Inhibition by prazosin was associated with a marked change in the time course of responses.

relationships were determined for all agonists by plotting the change in perfusion pressure against the log of the mol dose of the agonist. The negative logarithm of the agonist dose required to elicit 50% of the maximum response ($-\log EC_{50}$ or pD_2) was determined in each preparation (NB: since this is a perfusion system this does not represent a true maximum indicating maximal tissue activation, but is the largest response obtained with the doses employed). When responses were elicited in the presence of an antagonist, the results are shown as a percentage of the 'maximum' response attained in the control curve. In addition, where the responses before and after the antagonist were qualitatively similar, the negative logarithm of the antagonist dissociation constant ($-\log K_b$) was determined by the agonist dose-ratio method described by Furchgott (1972).

Differences between means were compared by either a paired or unpaired Student's t test.

Drugs and solutions

The composition of the physiological salt solution was (in mM) NaCl 118.4, $NaHCO_3$ 25, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 2.5, glucose 11, Na_2EDTA 0.023. Two % Ficoll (molecular weight approximately 70,000) was also included to prevent excessive water retention by the tail. The following compounds were used: prazosin HCl (Pfizer); rauwolscine HCl (Roth); UK-14,304 (5-bromo-6-[2-imidazolin-2-ylamino]-quinoxaline bitartrate, Pfizer); phenylephrine HCl (Sigma); noradrenaline bitartrate (Sigma); acetylcholine chloride (Sigma); arginine vasopressin (AVP, Sigma). All drugs were made up in distilled water except AVP, which was diluted with the physiological salt solution, and NA which was made up in distilled water containing 23 μM EDTA.

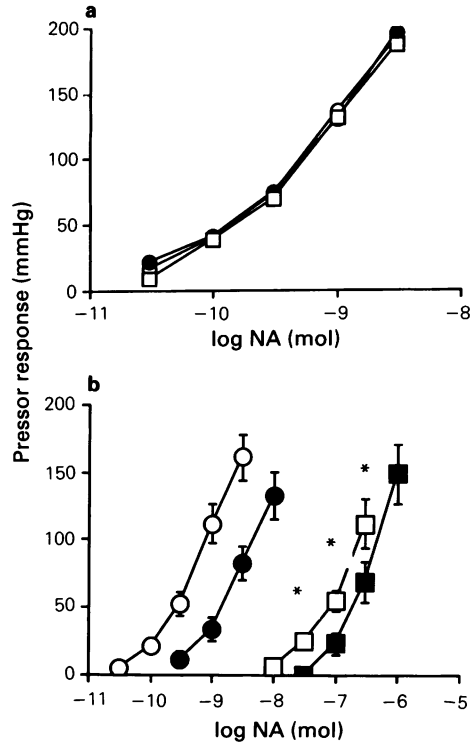


Figure 2 (a) Comparison of successive dose-response curves to noradrenaline in the rat isolated perfused tail; 1st (○) 2nd (●) and the 3rd (□) dose-response curves, respectively. In order to preserve clarity the s.e.mean of the responses have been omitted, but for each curve this never exceeded ±10% ($n = 7$). (b) The effect of 1 μM rauwolscine (●), 0.05 μM prazosin (□) and 1 μM rauwolscine and 0.05 μM prazosin combined (■) on responses to noradrenaline (control curve - ○) in the rat isolated perfused tail. All responses are the mean of the 6–8 observations ± s.e.mean (vertical bars). *Denotes a statistically significant difference ($P < 0.05$ paired Student's t test) between responses to noradrenaline in the presence of 0.05 μM prazosin before and after the addition of 1 μM rauwolscine.

Results

Responses to noradrenaline and the effect of 0.05 μM prazosin and 1 μM rauwolscine

Noradrenaline produced a dose-dependent increase in perfusion pressure in the tail artery following

bolus injection (Figure 1a). At low doses (0.03–0.3 nmol), responses were characterized by a peak increase in pressure 30 s after injection and a return to baseline perfusion pressure within 90 s. At higher doses (1 nmol and above), the peak responses were achieved within 60–120 min of injection and complete recovery effected only after 10 min. Responses to greater than 3 nmol noradrenaline were not elicited due to the length of time taken for the perfusion pressure to return to baseline.

Rauwolscine 1 μM caused a 5 fold rightward parallel displacement of the noradrenaline dose-response curve without a change in either the slope of the curve, the 'maximum' response or the time course of responses (Figure 2). Based upon the assumption of a competitive interaction with a single population of receptors, the $-\log K_b$ value is 6.6 ± 0.12 ($n = 7$).

Prazosin 0.05 μM produced greater than a 100 fold rightward displacement of the noradrenaline curve (measured at the 50% level of the control curve), significantly greater ($P < 0.05$) than that produced by 1 μM rauwolscine (Figure 2). The inhibition produced was associated with marked changes in the time course of pressor responses; the time to peak response to noradrenaline was significantly greater than for equivalent-sized responses in the control curve (Figure 1b). No attempt was made to determine a dissociation constant for prazosin. The combination of 0.05 μM prazosin and 1 μM rauwolscine produced a significantly greater inhibition of responses to NA than that observed for either antagonist alone (Figure 2), and the remaining pressor responses were still characterized by an increase in the time to peak (compared to control responses).

Responses to the agonists in the presence and absence of tone induced by AVP

Figure 3 shows the effect of AVP-induced tone on responses to UK-14,304, phenylephrine and noradrenaline (the latter in the presence of 0.05 μM prazosin to inhibit α_1-adrenoceptors). In the absence of AVP, the selective α_2-adrenoceptor agonist UK-14,304 (0.01–100 nmol) failed to elicit responses greater than 5 mmHg. Following the elevation of perfusion pressure with AVP (40–80 mmHg), UK-14,304 elicited a dose-dependent increase in perfusion pressure (Figure 3a), but the maximum response was significantly less than the maximum response to noradrenaline under control conditions (compare with Figure 2). At all doses, the time to peak response was 60 s or greater, and at high doses the return to basal perfusion pressure was not complete even 20 min after the injection of the agonist (all responses were measured from the level of the previous pressor response) (Figure 4). Similarly, responses to noradrenaline in the presence of 0.05 μM prazosin (α_1-adrenoceptors inhibited) were significantly increased following elevation of perfusion pressure by AVP (Figure 3b). The time courses of responses to low doses of noradrenaline (0.3–10 nmol) were similar to those elicited by UK-14,304.

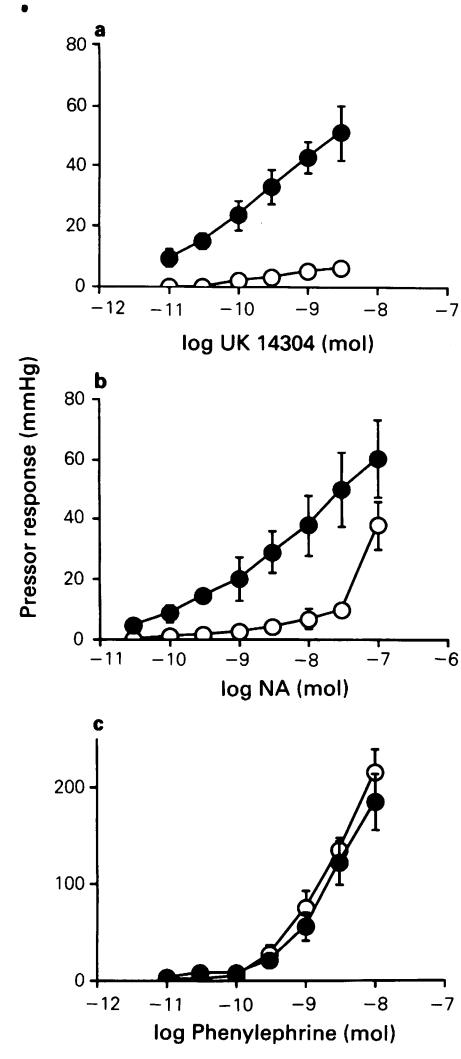


Figure 3 Pressor responses to (a) UK-14,304, (b) noradrenaline (in the presence of 0.05 μM prazosin to block α_1-adrenoceptors) and (c) phenylephrine before (○) and after (●) the induction of 'tone' with arginine-vasopressin (AVP) (range of concentrations used explained in Methods). The increase in perfusion pressure induced by AVP varied between (40–80 mmHg). All response are the mean of 5–8 observations ± s.e.mean (vertical bars).

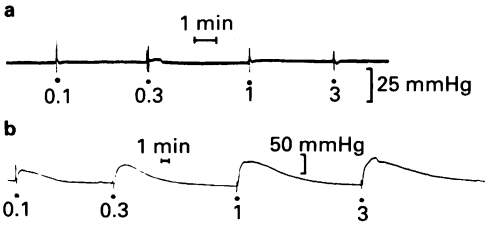


Figure 4 Representative recording of the pressor responses of the rat isolated perfused tail to UK-14,304 (a) before and (b) after induction of 'tone' with 0.5 nM arginine-vasopressin. Resting perfusion pressure before 0.5 nM arginine-vasopressin was 55 mmHg and 120 mmHg following 'tone' induction. Note the change in both the time base and the response calibration between (a) and (b).

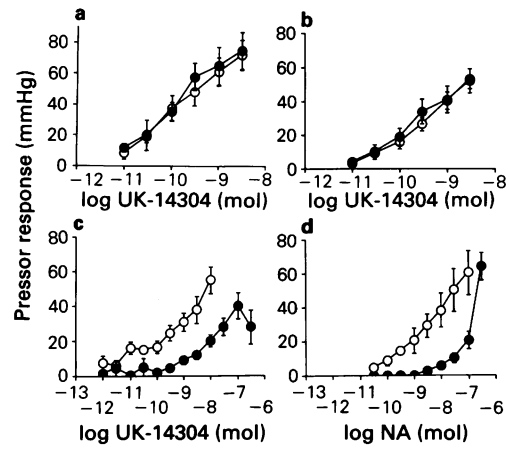


Figure 5 The effects of rauwolscine and prazosin on pressor responses to UK-14,304 and noradrenaline in the rat isolated perfused tail with 0.5–1.5 nM arginine-vasopressin. (a). Successive dose-response curves to UK-14,304 separated by 60 min; 1st dose-response curve (○), 2nd dose-response curve (●). (b) UK-14,304 dose-response curve before (○) and after (●) 0.05 μM prazosin. (c) UK-14,304 dose-response curve before (○) and after (●) 1 μM rauwolscine. (d) Noradrenaline dose-response curve (in the presence of 0.05 μM prazosin) before (○) and after (●) 1 μM rauwolscine. All responses are the mean of 5–8 observations ± s.e.mean (vertical bars).

In marked contrast, pressor responses to the selective α_1-adrenoceptor agonist phenylephrine were similar to those elicited by noradrenaline in the absence of prazosin and were unaffected by elevation of perfusion pressure with AVP (Figure 3c). In the absence of AVP, 0.01 μM prazosin produced a 100 fold rightward displacement of the phenylephrine dose-response curve, indicating that responses to this agonist are mediated via α_1-adrenoceptors.

The effects of antagonists on responses to α-adrenoceptor agonists in the presence of AVP

Following elevation of perfusion pressure with AVP, consecutive dose-response curves to UK-14,304 were reproducible (Figure 5a). Prazosin 0.05 μM failed to affect responses to UK-14,304 under these conditions (Figure 5b). In contrast, rauwolscine 1 μM caused a 45 fold parallel rightward displacement of the UK-14,304 curve (Figure 5c). The $-\log K_b$ value for rauwolscine against UK-14,304 in the presence of AVP (7.65 ± 0.15; $n = 7$) is greater than that observed against noradrenaline in control preparations (6.6 ± 0.12; $n = 7$).

In the presence of both 0.05 μM prazosin and AVP, 1 μM rauwolscine produced a 50 fold rightward displacement of the noradrenaline dose-response curve (Figure 5d). Interestingly the position of the noradrenaline curve following inhibition of responses by 1 μM rauwolscine is similar to that for noradrenaline (and 0.05 μM prazosin) in the absence of AVP-induced tone. However, since the inhibition was associated with a change in the time course of the pressor responses, no attempt was made to estimate the dissociation constant for rauwolscine under these conditions.

Discussion

Evidence for two populations of postjunctional α-adrenoceptors mediating contractions in the isolated vascular bed of the rat tail

In the present study, two observations favour the view that the major population of α-adrenoceptors in the perfused isolated vascular bed of the rat tail is an α_1-subtype. First, both noradrenaline and the selective α_1-adrenoceptor agonist phenylephrine (McGrath, 1982) elicited dose-dependent pressor responses, while the selective α_2-adrenoceptor agonist UK-14,304 was practically inactive. Secondly, the selective α_1-adrenoceptor antagonist prazosin (Cambridge *et al.*, 1977) was more potent against noradrenaline pressor responses than was the selective α_2-adrenoceptor antagonist rauwolscine (Weitzell *et al.*, 1979). The finding that prazosin produced changes in the time course of pressor responses to noradrenaline cannot, however, be taken as evidence of receptor heterogeneity. In both the isolated thoracic aorta and perfused isolated

mesenteric bed of the rat, preparations with a homogeneous population of functional postjunctional α_1-adrenoceptors, similar changes in non-equilibrium responses to noradrenaline following prazosin have been described (Downing *et al.*, 1983; 1985).

In a single receptor system, the overall effect of a combination of two antagonists, in concentrations that produce widely different displacements of the dose-response curve of the agonist is, in effect, determined solely by the antagonist which alone produces the greatest inhibition, i.e. there is no additivity or synergism predicted (see Downing *et al.*, 1988, for experimental example). Since the combination of rauwolscine 1 μM and prazosin 0.05 μM produced a significantly greater inhibition of noradrenaline pressor responses than 0.05 μM prazosin alone, the presence of more than one receptor subtype is suggested. This concurs with other observations in the perfused isolated tail artery, where prazosin and the selective α_2-adrenoceptor antagonist idazoxan (Doxey *et al.*, 1984) produced a greater inhibition of responses to noradrenaline than prazosin alone (Rajanayagam & Medgett, 1987). However, it is clear from the magnitude of the additional inhibition produced by 1 μM rauwolscine (following 0.05 μM prazosin), that the responses to noradrenaline in the presence of 0.05 μM prazosin are attributable to a combination of both α_1- and α_2-subtypes. Thus, there is no evidence for a prazosin-resistant, rauwolscine-sensitive α_2-adrenoceptor response as described in the pithed rat (see McGrath, 1982).

The possibility of the presence of two postjunctional α-adrenoceptors is further supported by observations with the agonists following induction of tone with AVP. Responses to UK-14,304 and noradrenaline (the latter in the presence of 0.05 μM prazosin to inhibit α_1-adrenoceptors) were significantly potentiated showing leftward displacement of the dose-response curve. For UK-14,304, the maximum response was approximately 30% of that produced by noradrenaline in the absence of both AVP-induced tone and prazosin. Furthermore, the time courses of submaximal responses to both UK-14,304 and noradrenaline (in the presence of AVP) were different from the equivalent-sized responses to noradrenaline in the absence of prazosin and AVP, in that there was longer time to the peak response and a longer duration (compare Figures 1a and 4). In marked contrast, pressor responses to the selective α_1-adrenoceptor agonist phenylephrine were unaffected by the increase in the perfusion pressure. This indicates that responses potentiated by AVP-induced tone arise from the stimulation of receptors other than α_1-adrenoceptors.

In the presence of AVP, rauwolscine inhibited responses to UK-14,304 but prazosin was ineffective. Thus, the uncovered α-adrenoceptor appears to be of the α_2-subtype. Under these conditions, the $-\log K_b$ value calculated from the effect of 1 μM rauwolscine (7.65), is consistent with an effect at α_2-adrenoceptors in the rat (see Daly *et al.*, 1988) and significantly greater than that observed against responses to noradrenaline mediated via α_1-adrenoceptors in the absence of prazosin and AVP. Finally, responses produced by noradrenaline (the endogenous ligand) in the presence of prazosin and AVP were also inhibited by 1 μM rauwolscine. This provides unambiguous evidence for a prazosin-resistant, rauwolscine-sensitive α_2-adrenoceptor-mediated response in the perfused isolated vascular bed of the rat tail.

Identification of functional postjunctional α_2-adrenoceptors in isolated vascular preparations

A number of other observations appear to indicate that the use of AVP to induce a 'physiological' degree of tone in isolated blood vessels, may generally be more applicable in the identification of functional postjunctional α_2-adrenoceptors.

In both the perfused isolated mesenteric bed of the rat (Fiotakis & Pipili, 1983) and the perfused isolated femoral artery of the rat (Dyke & Widdop, 1987), non-phenylethylamine agonists with selectivity for α_2-adrenoceptors were found to elicit small pressor responses. However, the magnitude of these responses precluded quantitative assessment of the effects of antagonists. Furthermore, although there is evidence from radioligand studies for a population of postjunctional α_2-adrenoceptors in the mesenteric circulation, no functional response could be attributed to these receptors since contractions of isolated strips of the mesenteric artery to all α-adrenoceptor agonists were sensitive to low concentrations of prazosin (Agrawal *et al.*, 1985). This latter study is remarkably similar to earlier work on the tail artery by Weiss *et al.* (1983) and seems to suggest that functional postjunctional α_2-adrenoceptors (prazosin-resistant, rauwolscine-sensitive) in these two vascular beds may also be uncovered by the elevation of tone with AVP. The elevation of tone *per se* by AVP may not be the critical factor since a study by Karmazyn *et al.* (1978) has shown that in the isolated perfused mesenteric aetery of the rat, pressor responses to NA, angiotensin II and KCl were potentiated by the presence of AVP at a concentration that had no effect on the baseline perfusion pressure. However, our results with phenylephrine, whose responses in the presence of AVP-induced tone are not potentiated would not agree with these results. Also it appears likely that the ability of various agents to potentiate or uncover postjunctional α_2-adrenoceptor-mediated responses is not restricted to AVP:

5-hydroxytryptamine produces similar effects to AVP in the perfused vascular bed of the rat tail (Templeton, 1988), while Furuta (1988) has shown that the selective α_2-adrenoceptor agonist B-HT 920 produced prazosin-resistant, yohimbine-sensitive contractile responses of the canine portal vein only in the presence of prostaglandin-induced 'tone'. Similarly, in the rabbit isolated saphenous vein, the rank order of agonist potency suggests the presence of postjunctional α_2-adrenoceptors (UK-14,304 > noradrenaline ≫ phenylephrine). However, responses to the selective α_2-adrenoceptor agonist B-HT 920 (Kobinger & Pichler, 1980) are sensitive to low concentrations of both prazosin and rauwolscine (Schümann & Lues, 1983). In the presence of either angiotensin II or the calcium channel facilitator Bay K 8644 (Schramm *et al.*, 1983), contractions to B-HT 920 possess the characteristics of an α_2-adrenoceptor-mediated response; prazosin-resistant, rauwolscine-sensitive (Schümann & Lues, 1983; authors' unpublished observations). Furthermore, α_2-adrenoceptor-mediated responses to B-HT 920 in the plantaris artery of the dog are only apparent in the presence of Bay K 8644 (Sulpizio & Hieble, 1987).

The feature common to all of these interventions is the ability of the 'potentiator' to elevate $[Ca^{2+}]_i$ to a level that presumably permits expression of the stimulation of the postjunctional α_2-adrenoceptor. In fact, manipulation of the isolated preparations in such a manner achieves conditions similar to that observed in the pithed rat, in which vascular tone is maintained by high levels of circulating angiotensin II (Grant & McGrath, 1988), and in human platelets, where pro-aggregatory responses to some 'selective' α_2-adrenoceptor agonists are dependent upon an existing aggregatory response to a submaximal concentration of ADP (Clare *et al.*, 1984). The obligatory role of pre-existing 'tone' for α_2-adrenoceptor-mediated responses (prazosin-resistant, rauwolscine-sensitive) is highlighted by many accounts of susceptibility of these responses to physiological and non-receptor pharmacological antagonism (see Reid & McGrath, 1985). In most instances, stimulation of postjunctional α_1-adrenoceptors elevates $[Ca^{2+}]_i$ by the entry of extracellular Ca^{2+} into the cell and also the release of Ca^{2+} from intracellular stores (Reid & McGrath, 1985).

In the light of the results in the present study, it would appear that responses mediated through activation of α_1-adrenoceptors in isolated blood vessels are more apparent in the absence of induced 'tone' because of the duality of the effector coupling mechanism (both intracellular and extracellular Ca^{2+}), while responses mediated via α_2-adrenoceptor (extracellular calcium only; see Abe *et al.*, 1987) are critically dependent upon pre-existing tone by an agent that does not stimulate α_1-adrenoceptors and, is therefore, prazosin-resistant. The essential difference between responses elicited via the two subtypes under these conditions is borne out by the different time courses of responses in the present study. In those instances where pre-existing tone arises from stimulation of α_1-adrenoceptors, e.g. noradrenaline or at high concentrations of α_2-agonists, two factors may combine to render the 'α_2-responses' sensitive to low concentrations of prazosin. First, the initial transient response to α_1-adrenoceptor stimulation in several isolated blood vessels, which for full agonists is associated with the release of intracellular Ca^{2+}, is antagonized in an unsurmountable manner by potent antagonists (Downing *et al.*, 1983; 1985; McGrath & Wilson, 1987; present study). Secondly, partial agonists at postjunctional α_1-adrenoceptors are even more affected than full agonists by the ability of prazosin to delay the onset of responses (Wilson *et al.*, 1987). Paradoxically, therefore, the difficulty of demonstrating postjunctional α_2-adrenoceptors *in vitro* may be attributable, in part, to the antagonist that has provided much of the existing framework for subtypes of α-adrenoceptors, prazosin. Thus, the perfused isolated vascular bed of the rat tail is similar to the rabbit isolated saphenous vein (Daly *et al.*, 1988), in that α-adrenoceptor subtypes that mediate pharmacological responses of the same polarity appear to interact functionally distal to the receptor.

In conclusion, our observations support the suggestions of a number of previous studies that the vascular bed of the rat tail possesses a population of functional postjunctional α_2-adrenoceptors (Weiss *et al.*, 1983; Medgett & Langer, 1984; Rajayanagam & Medgett, 1987; Abe *et al.*, 1987). We have demonstrated that by elevating 'tone' in the vascular bed with AVP, prazosin-resistant, rauwolscine-sensitive α_2-adrenoceptor responses can be obtained with both the selective α_2-adrenoceptor agonist UK-14, 304 and the endogenous ligand, noradrenaline.

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Subclassification of atrial and intestinal muscarinic receptors of the rat—direct binding studies with agonists and antagonists

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1 Although extensively investigated, the extent of differences between receptors mediating negative inotropic and chronotropic responses is still unclear. In the present study atrial and intestinal muscarinic receptors were identified by [^{3}H]-N-methyl-scopolamine ([^{3}H]-NMS) binding and the affinities of some presumably inotropy- or chronotropy-selective agonists and several antagonists determined.

2 All the agonists tested showed similar affinity for right and left atrial receptors. Accepting an affinity difference of 0.4 log units as experimental error, none of the agonists tested was selective for either atrium.

3 Affinity differences of the cardioselective antagonists himbacine, AF-DX 116 and methoctramine and the M_1-selective antagonist dicyclomine for right and left atrial muscarinic receptors were also minimal (less than 2 fold selective). When compared to intestinal receptors, AF-DX 116 was 3 to 4 fold, methoctramine 10 to 13 fold selective and himbacine and dicyclomine non-selective.

4 These data provide evidence for differences between atrial and intestinal but not between right and left atrial muscarinic receptors.

Introduction

Within the past few years it has become apparent that peripheral muscarinic receptors originally classified as M_2 (Hammer *et al.*, 1980; Barlow *et al.*, 1981; Hammer & Giachetti, 1984) do not form a homogeneous class. Antagonists such as 4-diphenylacetoxy N-methylpiperidine methiodide (4-DAMP) (Barlow *et al.*, 1976; 1980; Choo & Mitchelson, 1985), silaprocyclidine, hexahydro siladifenidol (Mutschler & Lambrecht, 1984) and cyclohexylphenyl (1-piperidinoethyl) silanol (CPPS; Eglen & Whiting, 1986a) show higher affinity for ileal smooth muscle than cardiac receptors, while gallamine (Clark & Mitchelson, 1976), pancuronium (Leung & Mitchelson, 1982), stercuronium (Li & Mitchelson, 1980), himbacine (Anwar-ul *et al.*, 1986; Gilani & Cobbin, 1987), AF-DX 116 (Giachetti *et al.*, 1986b; Micheletti *et al.*, 1987) and methoctramine (Melchiorre *et al.*, 1987; Giraldo *et al.*, 1988) show slightly or considerably higher affinity for cardiac than ileal muscarinic receptors. In most but not all cases binding studies have confirmed the selectivity patterns (Choo & Mitchelson, 1985; Doods *et al.*, 1987; Michel & Whiting, 1987; for two recent reviews see Eglen & Whiting, 1986b and Mitchelson, 1988).

While the distinct nature of cardiac versus smooth muscle muscarinic receptors becomes increasingly apparent, it is still unclear if right atrial receptors differ from left atrial or atrial from ventricular receptors. Thus, Barlow *et al.* (1980) using 3-acetoxy N-methylpiperidine methiodide (3-AMP) have suggested that atrial muscarinic receptors mediating effects on atrial contractility differ from those mediating effects on atrial rate. A similar conclusion has been reached by Chassaing *et al.* (1984) and Mutschler & Lambrecht (1984) employing various agonists in functional assays. Subsequent studies by Barlow & Shepherd (1985) and Barlow & Weston-Smith (1985), however, showed no convincing differences between effects on atrial rate and effects on atrial force and the original selectivity observed with 3-AMP was probably due to different cholinesterase activities in right and left atrium. According to Clague *et al.* (1985) neither antagonist data nor agonist potencies support the concept of 2 different receptors in right and left atrium, respectively.

Since all the affirmative evidence has been obtained with agonists for which selectivity may be due to differences in affinity and/or efficacy, it

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seemed interesting to supplement the functional results with radioligand binding data. This paper describes the binding properties of some reputedly selective agonists to right and left atrial and intestinal smooth muscle muscarinic receptors. In addition, some cardioselective antagonists and the M_1 selective dicyclomine have also been included.

Methods

Membrane preparation

Rats of either sex (300–500 g) were killed by decapitation, the hearts and small intestine removed and rinsed in homogenization buffer. The atrial appendages (= atria throughout this paper) were removed and immediately frozen in liquid nitrogen. The entire small intestine was cut into pieces (5 cm) and the mucosal layer removed with a blunt scalpel leaving essentially smooth muscle with the adherent nervous plexuses. All subsequent membrane preparation procedures were conducted at 4–7°C. Atria and intestines were homogenized in 65 mM phosphate buffer (pH 7.4) containing 250 mM sucrose and 5 mM $MgCl_2$ (Ultra-Turrax, 2 × 20 s bursts), passed through 2 sieves (pore sizes: 1 and 0.2 mm^2) and centrifuged at 500 *g* for 10 min. The pellet was washed once, and the supernatants combined and centrifuged at 30,000 *g* in an RC5B centrifuge for 10 min. The membrane pellet so obtained was washed twice and finally taken up in assay buffer at 1 mg ml^{-1} (atria) and 4 mg ml^{-1} (intestine) and stored in liquid nitrogen.

Binding assays

All experiments were performed using 65 mM phosphate buffered saline (pH 7.4 at 25°C) containing 5 mM $MgCl_2$, 0.5 mM $CaCl_2$ and 10 mM glucose. A volume of 50 μl membrane suspension (50 μg atrial and 200 μg intestinal protein), 50 μl [^{3}H]-N-methylscopolamine ([^{3}H]-NMS), 100 μl water or competitor (agonist or antagonist) and 200 μl buffer (total volume: 400 μl) were incubated at 25°C for 45 min. [^{3}H]-NMS was used at ≈0.3 nM which is close to its K_D value. When used, physostigmine was added to the membrane suspension at a concentration of 8 μM, yielding a final concentration of 1 μM in the assay. In each assay at least 11 (antagonist) or 15 (agonist) competitor concentrations were used. Bound ligand was separated from free ligand by vacuum filtration through Whatman GF/B glass fibre filters. The filters were rinsed 4 times with 3 ml assay buffer and their radioactivity content determined by liquid scintillation counting. Non-specific binding was defined as residual binding in the presence of 10 μM atropine sulphate and amounted to 5% (atria) and 10% (intestine), respectively at the radioligand concentration used in competition experiments (0.2–0.4 nM).

Data analysis

All binding data were analysed by a nonlinear least-squares curve fitting procedure on a Hewlett-Packard 9825 A desk top computer using a generalized model for ligand-receptor interactions (Wiemer *et al.*, 1982). Competition binding data were also analysed by use of a linearization procedure to provide estimates of IC_{50} values and Hill coefficients (nH) for competing compounds. For calculation of nH values the binding data between 10 and 90% of the control value were used. IC_{50} values so obtained were corrected for the presence of radioligand (Cheng & Prusoff, 1973) and expressed as IC_{50} corr. All values are the arithmetic mean ± s.e.mean, derived from 4–5 experiments.

Materials

[^{3}H]-NMS (specific activity: 72 Ci mmol^{-1}) was purchased from Amersham, Bucks, U.K., atropine sulphate from Sigma, Munich, FRG, acetylcholine chloride from Fluka, Vienna. The following drugs were kindly donated by the indicated companies and people: 3-AMP (3-acetoxy N-methylpiperidine methiodide), its isomer 4-AMP methiodide, EOE (ethoxyethyl trimethylammonium iodide): Dr R.B. Barlow, Univ. Bristol, UK; himbacine hydrochloride: Dr W.C. Taylor, Univ. Sydney, Australia; AF-DX 116 (11-[2-(diethylamino-methyl)-1-piperidinyl-acetyl]-5,11-dihydro-6H-pyrido(2,3-b)1,4-benzodiazepine-6-one): Thomae, Biberach, FRG; methoctramine tetrahydrochloride: Dr C. Mechiorre, Univ. Camerino, Camerino, Italy; APE (arecaidine propargylester): Dr E. Mutschler, Univ. Frankfurt, FRG; dicyclomine: Merrell Dow Research Institute, Cincinnati, U.S.A.

Results

Saturation binding of [^{3}H]-NMS

Binding of [^{3}H]-NMS to both atrial and intestinal homogenates was conducted at ligand concentrations between 0.1 and 8.5 nM and comprised a saturable and a non-saturable component. Affinity constants (K_D) and total number of binding sites (R_T) were: $K_D = 0.45 \pm 0.039$ nM, $R_T = 1330 \pm 79$ fmol mg^{-1} protein for atrial and $K_D = 0.28 \pm 0.058$ nM, $R_T = 644 \pm 56$ fmol mg^{-1} protein for intestinal receptors ($n = 3$ each) (data not shown). These disso-

Table 1 Affinity of agonists for [^{3}H]-N-methyl scopolamine ([^{3}H]-NMS) binding sites in rat intestinal and atrial membrane homogenates

	Small intestine				*Right atrium*				*Left atrium*			
Agonist	pIC_{50} *corr*	pK_{IH}	pK_{IL}	*nH*	pIC_{50} *corr*	pK_{IH}	pK_{IL}	*nH*	pIC_{50} *corr*	pK_{IH}	pK_{IL}	*nH*
Acetylcholine*	6.50	7.94	5.81	0.54	6.88	7.75	5.74	0.47	6.85	7.63	5.61	0.47
	(0.10)	(0.19)	(0.08)	(0.02)	(0.09)	(0.12)	(0.07)	(0.01)	(0.09)	(0.11)	(0.05)	(0.01)
3-AMP*	4.57	6.09	3.61	0.56	4.18	5.75	4.00	0.64	4.21	5.32	3.81	0.62
	(0.14)	(0.34)	(0.06)	(0.09)	(0.04)	(0.21)	(0.07)	(0.03)	(0.10)	(0.43)	(0.13)	(0.07)
4-AMP	5.61	6.67	4.62	0.43	5.60	6.18	4.49	0.54	5.54	6.39	4.47	0.51
	(0.04)	(0.22)	(0.16)	(0.05)	(0.06)	(0.08)	(0.04)	(0.04)	(0.03)	(0.17)	(0.07)	(0.02)
APE	7.01	8.19	6.10	0.49	7.60	8.34	6.26	0.44	7.52	8.76	6.47	0.42
	(0.11)	(0.08)	(0.03)	(0.03)	(0.11)	(0.15)	(0.05)	(0.02)	(0.05)	(0.08)	(0.04)	(0.01)
EOE	5.52	6.85	4.73	0.48	5.84	6.95	4.59	0.46	5.52	6.37	4.37	0.52
	(0.10)	(0.39)	(0.19)	(0.03)	(0.08)	(0.12)	(0.12)	(0.04)	(0.12)	(0.29)	(0.15)	(0.04)

Competition curves were linearized (logit/log) and the IC_{50} values calculated and corrected for the presence of [^{3}H]-NMS. Each curve was fitted to a two binding site model yielding estimates for the high (K_{IH}) and low affinity (K_{IL}) site. The following range of percentages of high (H) and low (L) affinity sites were found for the 3 tissues: small intestine: acetylcholine: 19–44H, 56–81L; 3-AMP: 53–72H, 28–47L; 4-AMP: 36–45H, 55–64L; APE: 44–57H, 43–56L; EOE: 30–64H, 56–70L. Right atrium: 46–62H, 38–54L; 3-AMP: 21–41H, 59–79L; 4-AMP: 53–65H, 35–47L; APE: 50–61H, 39–50L; EOE: 41–58H, 42–59L. Left atrium: acetylcholine: 48–62H, 38–52L; 3-AMP: 22–55H, 45–78L; 4-AMP: 47–66H, 34–53L; APE: 34–53H, 47–66L; EOE: 45–48H, 52–55L. All affinity values are expressed as negative log and are in $mol l^{-1}$. Each value is the mean of 4–5 experiments. Values in parentheses represent s.e.mean. All nH values are significantly lower than unity.

* Experiments were done in the presence of 1 μM physostigmine.

ciation constants were used in all subsequent competition experiments to calculate apparent affinities of agonists and antagonists (K_I, IC_{50}).

Affinity of agonists for atrial and intestinal binding sites

The affinity estimates for 5 agonists which might possibly be selective for one of the three tissues studied are listed in Table 1. Figure 1 shows a representative example of competition curves from which the affinity estimates were obtained. In all cases competition curves were flat resulting in Hill coefficients between 0.4 and 0.6 (Table 1), in accordance with results obtained in other tissues. Fitting of a two binding site model to the data yielded dissociation constants for the high affinity site (K_{IH}) which were approximately two orders of magnitude lower than those for the low affinity site (K_{IL}) in all three tissues.

To facilitate multiple comparisons of agonist affinities, IC_{50} values corrected for ligand occupancy were calculated and these are also listed in Table 1. The affinity of acetylcholine (+1 μM physostigmine) to right and left atrial tissue was identical (pIC_{50} corr = 6.9) but somewhat (factor 2.2) higher than that to intestinal binding sites. The affinity patterns of 3-AMP (+1 μM physostigmine), albeit in the high micromolar range, were similar to those of acetylcholine. The 4-isomer of AMP showed no affinity differences at all. The quaternary ether compound ethoxyethyl trimethylammonium iodide (EOE) showed equally low affinity for intestinal and atrial

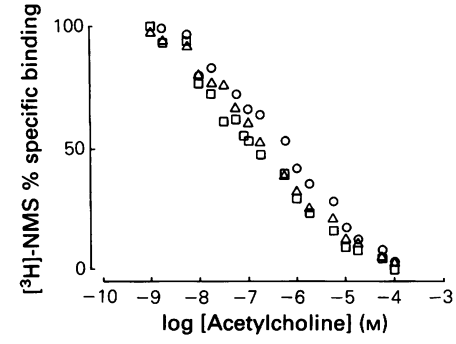


Figure 1 Displacement of specific [^{3}H]-N-methyl-scopolamine ([^{3}H]-NMS) binding by acetylcholine in the presence of 1 μM physostigmine from intestinal (○), right (□) and left (△) atrial membranes. Individual fitting of displacement curves yielded the parameters given in Table 1. Fitting all the data simultaneously yielded the following parameters: $K_{IH} = 8.3 \times 10^{-9}$ M, $K_{IL} = 1.2 \times 10^{-6}$ M; H = 50%, L = 50% ($r = 0.985$). The data shown in this figure are from a single representative experiment done in duplicate (variation coefficient less than 8%). Total binding was 2000 to 3000 d.p.m., non-specific binding 150–280 d.p.m. (intestine) and 1000–1500 d.p.m. and 40–70 d.p.m. (atria), respectively.

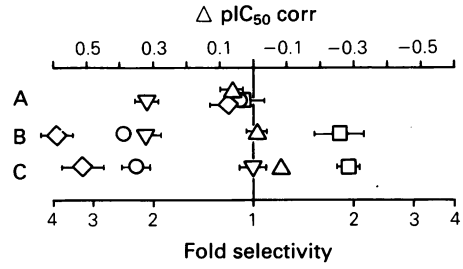


Figure 2 Comparison of the affinity of agonists for right versus left atrial (A), right atrial versus intestinal (B) and left atrial versus intestinal (C) binding sites. The upper axis shows the difference between the pIC_{50} corr values for the 3 comparisons, the lower axis the selectivity factors (antilog of ΔpIC_{50} corr values). Selectivities of less than 2.5 (0.4 log units) were considered as experimental error and thus without pharmacological significance. Each point represents the mean of 4–5 experiments each; horizontal lines show s.e.mean.

binding sites (pIC_{50} corr = 5.5–5.8), although a higher affinity for intestinal than atrial receptors would be expected from functional studies. The affinity of arecaidine propargylester (APE) was identical in both atria (pIC_{50} corr = 7.5 and 7.6) and 3 to 4 fold lower in intestine.

A graphical representation of the affinity comparisons right versus left atrium (A), right atrium versus small intestine (B) and left atrium versus small intestine (C) is shown in Figure 2. For A, no agonist showed more than 2 fold and for B and C more than 4 fold selectivity.

Affinity of antagonists for atrial and intestinal binding sites

The affinities of the 3 cardioselective antagonists AF-DX 116, himbacine and methoctramine and the M_1-selective antagonist dicyclomine to atrial and intestinal muscarinic binding sites are listed in Table 2. None of the compounds differentiated between right and left atrial receptors, AF-DX 116 and methoctramine were clearly cardioselective while himbacine and dicyclomine showed less than 2 fold affinity differences. Affinity comparisons of these 4 compounds are shown in Figure 3. In intestinal homogenates, the interaction of [^{3}H]-NMS with himbacine, AF-DX 116 and dicyclomine and in atria with himbacine seemed to involve heterogeneous binding sites, resulting in Hill coefficients less than unity.

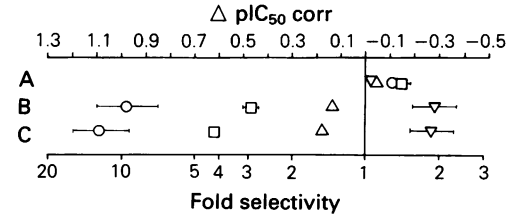


Figure 3 Comparison of the affinity of methoctramine (○), AF-DX 116 (□), himbacine (△) and dicyclomine (▽) for right versus left atrial (A), right atrial versus intestinal (B) and left atrial versus intestinal (C) binding sites. For description of axes see Figure 2. Each point represents the mean of 5 experiments; horizontal lines show s.e.mean.

Discussion

The aim of the present study was to characterize the apparent binding affinity of some muscarinic agonists and antagonists for muscarinic receptors present in right and left atrium and the small intestine of the rat. Since attenuation of cardiac frequency and contractility may theoretically be mediated by different receptor subtypes or identical receptors but different receptor-effector coupling mechanisms, binding

Table 2 Affinity of antagonists for [^{3}H]-N-methyl scopolamine ([^{3}H]-NMS) binding sites in rat intestinal and atrial membrane homogenates

Antagonist	*Small intestine* pK_I, *nH*	*Right atrium* pK_I, *nH*	*Left atrium* pK_I, *nH*
AF-DX 116	6.51 (0.07)	7.11 (0.02)	7.27 (0.05)
	0.69 (0.03)*	0.89 (0.06)	0.88 (0.03)
Dicyclomine	7.35 (0.14)	7.08 (0.08)	7.15 (0.07)
	0.74 (0.06)*	0.94 (0.06)	0.94 (0.06)
Himbacine	8.07 (0.03)	8.23 (0.01)	8.28 (0.04)
	0.72 (0.04)*	0.85 (0.01)*	0.86 (0.04)*
Methoctramine	6.48 (0.02)	7.48 (0.17)	7.60 (0.16)
	0.96 (0.04)	1.38 (0.06)*	1.11 (0.05)

All affinity values are expressed as the negative log of the inhibitory affinity constant (K_I) and are in mol l^{-1}.
Values in parentheses are the s.e.mean. The number of experiments was 5.
* Represents a nH significantly different ($P < 0.05$) from unity.

studies might be expected to help locate the origin of selectivity.

The saturation data should be commented upon briefly. While the affinity constants of [^{3}H]-NMS for both atria and small intestine agreed well with published data ($\approx$0.2–0.4 nM), the number of [^{3}H]-NMS binding sites in the atria was considerably (ca 4 to 5 fold) higher than normally found. The reason for this discrepancy may lie in the particular portion of the atrial tissue used. In the present case only the atrial appendages were used while many investigators use the atrium proper in order to obtain more tissue. Thus, the receptor density seems to be higher in the appendages (auricles) than the atrial wall. A preliminary comparison (unpublished) seems to bear out this conclusion.

In terms of the agonists studied, the major finding was the absence of affinity differences between right and left atrial tissues. In no case was selectivity more than 2 fold, a factor within the 0.4 log units suggested by Barlow & Shepherd (1985) as experimental error. Acetylcholine and 3-AMP methiodide which are substrates of cholinesterase showed similar affinity in all 3 tissues in the presence of physostigmine. The isolated finding of acetylcholine selectivity (5 fold) for inotropic responses (Chassaing *et al.*, 1984) which, however, could not be confirmed by Clague *et al.* (1985) and Barlow & Weston-Smith (1985) thus finds no support in binding studies. In physiological *in vitro* experiments, ethoxyethyl trimethylammonium iodide (EOE) has been found to be 2 to 4 times more active on ileum than on atria (Barlow & Dawson, 1986; Barlow & Weston-Smith, 1985). In the present binding studies, EOE showed similar affinities for both atria and small intestine. The small selectivity observed by Barlow and collaborators may therefore reflect different receptor-effector coupling. The affinity of arecaidine propargylester (APE) for atria was 3 to 4 times higher than to intestine, in accord with results from physiological studies. The latter also indicate no differences between right and left atrium (Barlow & Weston-Smith, 1985; Clague *et al.*, 1985) which was also found in this study. The methylene-bridged form of APE for which a more than 50 fold affinity difference between atria has been described (Mutschler & Lambrecht, 1984) was unfortunately not available.

In a thorough assessment of the binding mechanism of agonists to cardiac muscarinic receptors, Wong *et al.* (1986) have recently obtained affinity constants of a number of agonists for their interaction with ventricular and atrial (right plus left) receptors prepared from the hearts of Syrian golden hamsters. In agreement with published data, all agonists recognized 2 (and in some cases 3) classes of receptor in both atrial and ventricular tissue. A careful analysis of binding data revealed differences between atrial and ventricular receptors for some, but not all agonists. Guanylyl imidodiphosphate was shown to alter the binding of carbachol by reducing the fraction of the high (or highest) affinity component. Thus, with respect to differences between atrial and ventricular receptors, these authors have reached similar conclusions to those of another recent study by Brunner & Kukovetz (1988) using a kinetic approach.

The use of 3 cardioselective antagonists AF-DX 116, himbacine and methoctramine was also unable to reveal any differences between right and left atrium. As to the affinity differences between atrial and smooth muscle receptors, they were generally smaller than expected from physiological experiments. Himbacine, however, for which a 10 fold higher affinity for atrial over ileal, tracheal or uterine smooth muscle has been shown (Gilani & Cobbin, 1986; Anwar-ul *et al.*, 1986), was non-selective in binding studies. Lack of himbacine selectivity was also found in competition studies against [^{3}H]-quinuclidinyl benzilate (Choo & Mitchelson, 1988). AF-DX 116 has been shown to antagonize cardiac muscarinic responses with 7.5 to 10 times higher potency than smooth muscle or secretory responses (Giachetti *et al.*, 1986b). In binding studies the cardioselectivity found was 8 fold compared to neuronal M_1 receptors (Hammer *et al.*, 1986) and 3 to 4 fold compared to ileal receptors (Michel & Whiting, 1987). The 3 to 4 fold cardiac selectivity found in this study is in accord with published results and confirms the tendency towards lower selectivity in binding than functional studies, as was also found for some other antagonists (Michel & Whiting, 1987). Methoctramine was found to have a pA_2 in atrial preparations of 7.7 to 7.9 and the highest cardioselectivity yet obtained (up to 130 fold) (Melchiorre *et al.*, 1987). In the binding experiments described here, the compound was 10 to 13 fold cardioselective compared to intestinal receptors. In another study a 7 fold selectivity for atria compared to circular ileum has been obtained (Michel & Whiting, 1987). Hill coefficients calculated for methoctramine in this study were close to unity except for the right atrium (nH = 1.38) which may reflect an interaction with muscarinic receptors which is not strictly competitive; this has also been found in functional studies (Melchiorre *et al.*, 1987; Giraldo *et al.*, 1988). The low Hill slope of AF-DX 116 in intestinal tissue probably reflects the heterogeneity of muscarinic receptors in both the longitudinal and circular smooth muscle of this organ (Giraldo *et al.*, 1987; Michel & Whiting, 1987). The low Hill coefficients for himbacine in all 3 tissues studied are presented here for the first time; the reasons are unknown at present.

Dicyclomine, which is capable of distinguishing

between the muscarinic receptor in the cerebral cortex and the heart or urinary bladder (Nilvebrant & Sparf, 1986) and in one study also between the cardiac and glandular subtype (Giachetti *et al.*, 1986a), was unable to differentiate between right and left atrium or atrial and intestinal receptors. This is probably due to the mixed population of receptors present in small intestine and which comprises in part the cardiac type of receptor. The low nH value found for dicyclomine in this study (0.74) is in support of this view.

In conclusion, the present study demonstrates that the muscarinic receptors present in the right and left atrium of the rat are indistinguishable in radioligand binding experiments employing both agonists and antagonists. A similar conclusion has recently been reached with a different, i.e. kinetic approach (Brunner & Kukovetz, 1988). The affinity estimates for the agonist APE and the antagonists AF-DX 116 and methoctramine, however, show clear differences between atrial and intestinal receptors lending further support for the heterogeneity of peripheral M_2 muscarinic receptors.

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Local opioid-sensitive afferent sensory neurones in the modulation of gastric damage induced by Paf

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1 The role of local sensory neurones in modulating the extent of gastric mucosal damage induced by close-arterial infusion of platelet-activating factor (Paf 50 ng kg^{-1} min^{-1} for 10 min) has been investigated in the anaesthetized rat.

2 Local intra-arterial infusion of the neurotoxin, tetrodotoxin (TTX), substantially augmented the mucosal damage induced by Paf, as assessed by both macroscopic and histological techniques.

3 In rats pretreated with capsaicin 2 weeks prior to study, to induce a functional ablation of primary afferent neurones, gastric damage induced by Paf was significantly augmented.

4 Administration of morphine (0.75–3 mg kg^{-1} i.v.) or its peripherally acting quaternary analogue, N-methyl morphine (15 mg kg^{-1} i.v.), also significantly enhanced the gastric damage induced by Paf.

5 The potentiation by morphine of Paf-induced gastric damage was inhibited by administration of the opioid antagonists, naloxone (1 mg kg^{-1} i.v.) or the peripherally acting N-methyl nalorphine (3 mg kg^{-1} i.v.).

6 Administration of TTX or morphine alone, or pretreatment with capsaicin did not induce any detectable mucosal damage, suggesting that interference with local sensory neuronal activity itself does not directly induce mucosal disruption.

7 These results indicate that peripheral opiate-sensitive afferent sensory neurones play a physiological defensive role in the mucosa, attenuating the extent of gastric damage induced by Paf.

Introduction

Platelet-activating factor (Paf) is a potent ulcerogen in the rat gastric mucosa (Rosam *et al.*, 1986). Local intra-arterial infusion of Paf has been shown to induce mucosal damage and, in addition, to stimulate motility in the rat stomach (Esplugues & Whittle, 1988a, b). After termination of the local infusion of Paf, a prolonged phase of gastric motility has been observed, which was not prevented by administration of α- or β-adrenoceptor antagonists, by atropine or vagotomy or by ganglion blockade with hexamethonium. However, this prolonged motility phase following Paf infusion was abolished by administration of the neurotoxin, tetrodotoxin (TTX), suggesting that it was brought about by activation of local non-cholinergic, non-adrenergic neurones (Esplugues & Whittle, 1988b; 1989).

It has been proposed that local neuronal mechanisms can modulate the integrity of the gastric mucosa. Thus, gastric damage induced by pylorus ligation, acid distension, ethanol and indomethacin is increased in rats pretreated with capsaicin, an extract of red peppers that causes the functional ablation or degeneration of primary afferent neurones (Szolcsanyi & Bartho, 1981; Holzer & Sametz, 1986; Holzer, 1988). In the current study, in which both TTX and capsaicin pretreatment were used, we have investigated whether a local protective neuronal mechanism can attenuate the extent of gastric mucosal injury induced by the intravascular administration of the ulcerogen, Paf. In addition, since opioids can affect sensory neurones by actions at peripheral sites (Ferreira & Nakamura, 1979; Lembeck & Donnerer, 1985), we have compared the effects of opioids and their antagonists, including peripherally-acting quaternary derivatives, on Paf-induced damage, in order to evaluate their potential actions on any afferent sensory neurones in the gastric mucosa.

Methods

Gastric damage induced by the local intra-arterial infusion of Paf

Male Wistar rats (230–260 g), deprived of food but not water for 18–20 h before the experiments, were

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anaesthetized with sodium pentobarbitone (60 mg kg^{-1}, i.p.) and the stomach exposed by a midline incision. The left gastric artery was cannulated with a short 23 g teflon cannula, and Paf was infused for 10 min (12.5 μl min^{-1}); in most experiments the dose was 50 ng kg^{-1} min^{-1}, which has previously been found to cause consistent damage to the gastric mucosa (Esplugues & Whittle, 1988a), and in some experiments a lower dose, 10 ng kg^{-1} min^{-1}, was also used.

In one series of studies, after ligating the oesophagus and the pylorus, a solution in saline of the neurotoxin, tetrodotoxin (TTX 0.0625, 0.125 and 0.25 μg kg^{-1} min^{-1}) was infused locally (12.5 μl min^{-1}) and 3 ml of a 5, 10 or 20 μM solution of TTX respectively was instilled into the gastric lumen. After 10 min, a solution containing both TTX and Paf (50 ng kg^{-1} min^{-1}) was infused locally for 10 min. After termination of the Paf infusion, the local intra-arterial infusion with TTX was maintained and the stomach removed 20 min later.

In a further series of experiments, Paf (50 ng kg^{-1} min^{-1}) was infused locally for 10 min in rats that had been pretreated 10 min previously with morphine (0.75, 1.5 and 3 mg kg^{-1}, i.v.) or its analogue N-methyl morphine (15 mg kg^{-1}, i.v.), and the stomachs were removed 20 min later. In other experiments, naloxone (1 mg kg^{-1}), N-methyl nalorphine (3 mg kg^{-1}), mepyramine (1 mg kg^{-1}) or cimetidine (50 mg kg^{-1}) were administered i.v., 10 min before the administration of morphine.

Mean systemic arterial blood pressure (BP) was measured from a cannula in a carotid artery connected to a pressure transducer (Elcomatic) and a chart recorder (Rikadenki, model R-50).

Gastric damage in capsaicin-treated rats

Additional experiments were performed in animals pretreated with capsaicin to investigate the possibility of sensory neuronal involvement in the aetiology of gastric mucosal damage induced by intra-arterial Paf. Adult rats (190–210 g) were treated with increasing doses of capsaicin for three consecutive days (20, 30 and 50 mg kg^{-1}, s.c.). This regimen has been shown to deplete neuropeptides in primary afferent neurones in adult rats (Martling, 1987). All capsaicin injections were made under halothane anaesthesia and, to counteract the respiratory impairment associated with the administration of capsaicin, the rats were pretreated with terbutaline (0.1 mg kg^{-1}, i.m.) and aminophylline (10 mg kg^{-1}, i.m.) before the capsaicin injection. The animals were used 2 weeks after completion of the capsaicin treatment. Control animals received a similar regimen of treatment with the vehicle.

Assessment of mucosal damage

Twenty minutes after the cessation of the local intra-arterial infusion of Paf, the stomachs were removed and opened along the greater curvature. The stomachs were pinned out, mucosal side out, to a wax block and immersed in neutral buffered formalin and then photographed on colour transparency film. The extent of macroscopically-visible damage was determined from these projected transparencies via computerised planimetry. The area of mucosal damage was calculated as the % of the total gastric mucosa showing macroscopically visible damage.

Two samples of the fundus were excised from standardised regions and were processed by routine techniques before being embedded in paraffin. Sections (4 μm) were stained with haematoxylin and eosin and examined under a light microscope. The 1 cm length of each histological section was divided into 4 subsections. Each subsection was histologically assessed for epithelial cell damage (a score of 1 being assigned); glandular disruption, vasocongestion or oedema in the upper mucosa (a score of 2 being assigned); haemorrhagic damage in the mid to lower mucosa (a score of 3) and deep necrosis and ulceration (a score of 4). Each subsection was evaluated on a cumulative basis (the maximum score being 10) and the overall mean score value was taken as the histological index for that section. All determinations were performed in a randomised manner with both transparencies and histological sections coded to eliminate observer bias.

Drugs

Paf [1-O-alkyl-2-O-acetyl-*sn*-glyceryl-3-phosporylcholine, Sigma Chemical Co. St. Louis, MO] was stored at −20°C as a stock solution in chloroform (2 mg ml^{-1}). On the day of an experiment, an aliquot was dried under nitrogen and redissolved in 0.25% bovine serum albumen in isotonic saline and kept on ice.

Tetrodotoxin (Sigma Chemical Co.), morphine hydrochloride (MacFarlane Smith), naloxone hydrochloride (SALARS), mepyramine (May & Baker Ltd.), cimetidine (intravenous injection, Smith, Kline & French Ltd.), N-methyl-nalorphine and N-methyl morphine (synthesized in the Dept. of Medicinal Chemistry, WRL) were dissolved in isotonic saline immediately before use. Capsaicin (Fluka Chemic AG) was prepared in a 50 mg ml^{-1} solution containing absolute ethanol, tween 80 and isotonic saline (10:10:80 v/v/v).

Statistical analysis

All data are expressed as mean ± s.e.mean. Comparisons between groups of parametric data were made

by Student's t test for unpaired data. Comparisons between groups of non-parametric data (histological index) were made by the Mann-Whitney U-Test. P values of less than 0.05 were taken as significant.

Results

Potentiation by tetrodotoxin of gastric damage by local Paf

Close-arterial infusion of Paf ($50\,ng\,kg^{-1}\,min^{-1}$ for 10 min), with the stomach being removed 20 min later, induced macroscopically apparent damage affecting $33 \pm 4\%$ ($n = 17$) of the total area of the mucosa, as determined by computerised planimetry. The nature of this damage was similar to that described before (Esplugues & Whittle, 1988a) and was characterised by extensive hyperaemia and haemorrhage, located mainly in the acid secreting corpus region of the stomach. In control experiments, local infusion of saline ($12.5\,\mu l\,min^{-1}$) induced minimal mucosal damage ($0.5 \pm 0.5\%$ of total mucosal area, $n = 10$).

Local intra-arterial infusion of TTX (0.05, 0.125 and $0.25\,\mu g\,kg^{-1}\,min^{-1}$ with intragastric instillation of TTX), significantly potentiated the degree of Paf-induced gastric damage, to a level where virtually all of the glandular mucosa was affected (Figure 1). The damage that resulted from this interaction between Paf and TTX was of haemorrhagic nature, and of diffuse location with both the corpus and the antrum being affected. Administration of the highest dose of TTX alone, however, did not induce any macroscopically apparent mucosal damage (Figure 1).

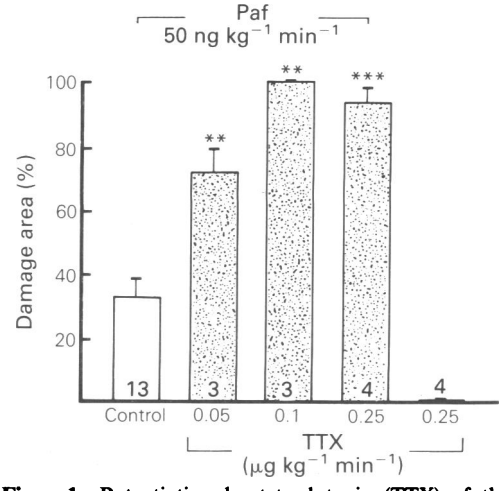


Figure 1 Potentiation by tetrodotoxin (TTX) of the gastric mucosal damage induced by the local intra-arterial infusion of Paf ($50\,ng\,kg^{-1}\,min^{-1}$ for 10 min). TTX was infused locally (0.05, 0.1 and $0.25\,ng\,kg^{-1}\,min^{-1}$ with 5, 10 or 20 μM instilled into the gastric lumen). Results, shown as the % of the total mucosal area that exhibited macroscopically assessed damage are expressed as mean of n (number in columns) experiments: vertical lines show s.e.mean. TTX alone did not induce any significant damage. Significant increase from the control intra-arterial infusion of Paf is given as $^{**}P < 0.01$, $^{***}P < 0.001$.

Potentiation by morphine of gastric damage by local Paf

Administration of morphine ($0.75–3\,mg\,kg^{-1}$, i.v.) caused a significant and dose-dependent increase in the extent of mucosal damage induced by local infusion of Paf ($50\,ng\,kg^{-1}\,min^{-1}$), as shown in Figure 2. The damage was also of haemorrhagic nature and again of diffuse location, with both the antrum and the corpus being affected. Pretreatment with morphine ($3\,mg\,kg^{-1}$ i.v.) alone did not cause any macroscopically detectable damage to the rat gastric mucosa (Figure 2).

Administration of the peripherally-acting morphine analogue, N-methyl morphine ($15\,mg\,kg^{-1}$, i.v.) which itself did not induce any macroscopic damage to the mucosa ($n = 3$) likewise significantly enhanced the gastric damage induced by local Paf infusion (from $33 \pm 4\%$, $n = 17$ to $56 \pm 3\%$, $n = 6$ of total mucosal area; $P < 0.001$).

Pretreatment with either the opioid antagonist, naloxone ($1\,mg\,kg^{-1}$, i.v.), which acts on both central and peripheral opioid receptors, or N-methyl nalor-

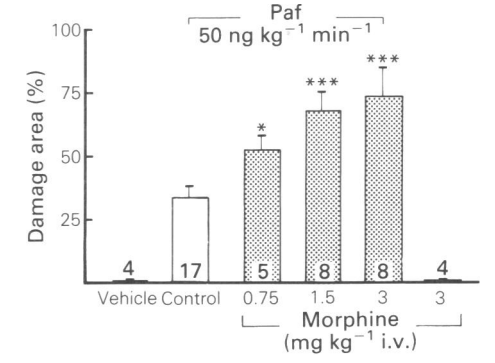


Figure 2 Potentiation by morphine ($0.75–3\,mg\,kg^{-1}$, i.v.) of the gastric mucosal damage induced by the local intra-arterial infusion of Paf ($50\,ng\,kg^{-1}\,min^{-1}$ for 10 min). Results, shown as the % of the total mucosal area that exhibited macroscopically assessed damage, are expressed as mean of n (number in columns) experiments; vertical lines show s.e.mean. Morphine ($3\,mg\,kg^{-1}$ i.v.) alone did not significantly damage the mucosa. Significant increase from the control intra-arterial infusion of Paf is given as $^{*}P < 0.05$ and $^{***}P < 0.001$.

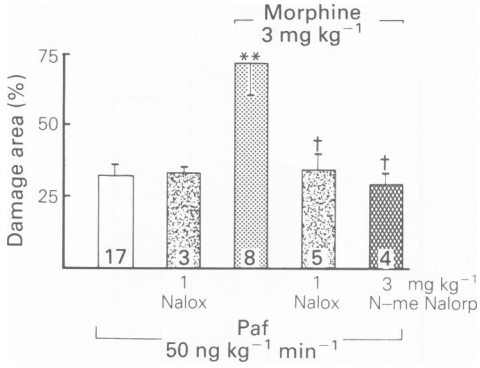


Figure 3 Effects of the opiate antagonist naloxone (Nalox; 1 mg kg^{-1}, i.v.) and N-methyl nalorphine (N-me Nalorp; 3 mg kg^{-1} i.v.), on the potentiation by morphine (3 mg kg^{-1} i.v.) of gastric damage induced by local intra-arterial infusion of Paf (50 ng kg^{-1} min^{-1} for 10 min). Results, shown as the % of the total mucosa area that exhibited macroscopically assessed damage, are expressed as mean of n (number in columns) experiments; vertical lines show s.e.mean. Naloxone (1 mg kg^{-1} i.v.) did not significantly affect the damage induced by Paf alone. Significant difference from the control intra-arterial infusion of Paf is given as $^{**}P < 0.01$, and from the combination of morphine and Paf by $^{\dagger}P < 0.05$.

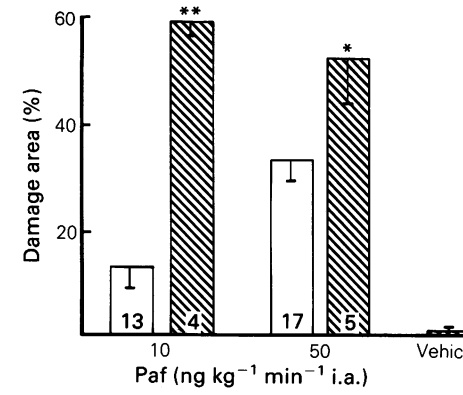


Figure 4 Potentiation by pretreatment (2 weeks earlier) with capsaicin, of the gastric mucosal damage induced by local intra-arterial infusion of Paf (10 and 50 ng kg^{-1} min^{-1}) for 10 min. Open columns: effect of Paf alone; hatched columns: effect of Paf after capsaicin pretreatment. Results, shown as the % of the total mucosal area that exhibited macroscopically assessed damage, are expressed as mean of n (number in columns) experiments; vertical lines show s.e.mean. Significant difference from the control intra-arterial infusion of Paf is given as $^{*}P < 0.05$ and $^{**}P < 0.01$.

phine (3 mg kg^{-1}, i.v.), a peripherally acting quaternary opioid receptor antagonist, significantly ($P < 0.05$) prevented the potentiation of Paf-induced damage by morphine (3 mg kg^{-1}, i.v.), as shown in Figure 3. Pretreatment with naloxone (1 mg kg^{-1}, i.v.) did not affect the degree of mucosal damage induced by Paf alone (Figure 3).

Prior i.p. administration of the H_1 and H_2 receptor antagonists, mepyramine (1.5 mg kg^{-1}) and cimetidine (50 mg kg^{-1}) failed to modify the potentiation by morphine (3 mg kg^{-1}) of Paf-induced gastric damage, with the area of damage being 69 ± 2% of the total mucosal area ($n = 4$), comparable to that obtained with the combination of Paf and morphine alone (72 ± 12%, $n = 8$).

Local intra-arterial infusion of saline had no significant action on resting BP, which remained between 100–120 mmHg. The local intra-arterial administration of Paf (50 ng kg^{-1} min^{-1}) induced a rapid hypotension, reaching plateau levels within 2 min (−50 ± 5 mmHg, $n = 4$) which was maintained throughout the 10 min period of infusion, as found previously (Esplugues & Whittle, 1988a). After the termination of the Paf infusion, the BP gradually increased, and by the end of the following 20 min period, the BP was similar to the basal levels. Pretreatment with morphine (1.5 and 3 mg kg^{-1} i.v.), which itself did not significantly alter BP ($n = 4$), did not significantly influence the hypotension induced by Paf, the fall in BP being −45 ± 3 mmHg and −57 ± 10 mmHg ($n = 4$) for each dose of morphine respectively.

Potentiation by capsaicin of gastric damage by local Paf

Pretreatment with capsaicin, 2 weeks prior to study, significantly ($P < 0.05$) augmented the extent of gastric mucosal damage induced by local infusion of Paf (10 and 50 ng kg^{-1} min^{-1}), as shown in Figure 4. The mucosal damage was haemorrhagic and diffuse, affecting both corpus and antral regions.

The gastric mucosa from capsaicin-pretreated rats did not exhibit any macroscopic damage in the absence of the Paf infusion ($n = 3$). Furthermore, in capsaicin pretreated rats, local intra-arterial infusion of saline (12.5 μl min^{-1}) did not cause any macroscopic damage to the gastric mucosa ($n = 4$).

Histological assessment of gastric damage

The nature of the histological damage induced by Paf (50 ng kg^{-1} min^{-1} for 10 min) was similar to that described before (Esplugues & Whittle, 1988a). It was characterised by vascular congestion throughout the corpus mucosal layer with extensive damage to surface epithelium, dilatation of the gastric glands, sub-epithelial oedema and, in some cases, areas of necrosis and/or partial disappearance of the mucosa.

Table 1 Effect of morphine ($3\,mg\,kg^{-1}$ i.v.), tetrodotoxin (TTX; $0.25\,\mu g\,kg^{-1}\,min^{-1}$ locally with intragastric $20\,\mu M$) or prior capsaicin treatment on the damage to the rat gastric mucosa following local intra-arterial infusion of Paf ($50\,ng\,kg^{-1}\,min^{-1}$ for 10 min), as determined histologically

Treatment	*Histological index*	(n)
Paf alone	2.1 ± 0.5	(9)
+ Morphine	3.8 ± 0.8*	(5)
+ TTX	4.3 ± 0.5*	(4)
+ Capsaicin	3.5 ± 0.2*	(6)

Results, shown as the histological index (0–10 scale), are the mean ± s.e.mean of *n* animals. * Statistical difference from the Paf alone group, $P < 0.05$.

Pretreatment with morphine ($3\,mg\,kg^{-1}$ i.v.) or local administration of TTX ($0.25\,\mu g\,kg^{-1}\,min^{-1}$ with intragastric instillation of a $20\,\mu M$ solution), significantly ($P < 0.05$) augmented the degree of Paf-induced histological damage (Table 1). The nature of this latter damage was characterised by the same histological features as observed with Paf alone, but with a much more profound degree of vasocongestion throughout the whole mucosa.

Animals pretreated with capsaicin also had a significantly ($P < 0.05$) higher degree of histological damage than those receiving Paf alone (Table 1). This increase in the level of damage was related to an enhancement of vascular congestion throughout the mucosa, with extensive subepithelial oedema being observed. In control studies, administration of TTX, morphine or capsaicin-pretreatment alone did not induce significant histological damage ($n = 3$ for each).

Discussion

Our results indicate that local neuronal mechanisms can modulate the degree of Paf-induced gastric mucosal injury. Thus, the damaging effects of local Paf were increased in a dose-dependent manner by the local administration of tetrodotoxin (TTX). This neurotoxin specifically binds to and blocks axonal fast sodium channels and thus prevents nervous conduction and mediator release from nerve terminals (Kao, 1966). In previous studies, we have shown that gastric motility changes that followed local infusion of Paf were not affected by vagotomy, or by pretreatment with atropine, ganglion blockade with hexamethonium, or by α- and β-adrenoceptor blockade, but were prevented by TTX administration in doses comparable to those of the present study (Esplugues & Whittle, 1988b; 1989). These observations thus suggested that a neuronal mechanism was involved in this contractile action of Paf which was local and non-adrenergic, non-cholinergic in nature, and such a mechanism may have some similarities with the mucosal neuronal processes observed in the current study.

In the present study, administration of TTX alone did not lead to any macroscopically detectable mucosal damage, indicating that inactivation of the mucosal neuronal mechanism did not directly disrupt the mucosa, at least under these acute conditions, although the mucosa was considerably more susceptible to injury following challenge.

Capsaicin-sensitive sensory fibres are present in the gastric mucosa (Sharkey *et al.*, 1984) and a role for such neurones in the regulation of gastric mucosal defense has been proposed. Thus, desensitization or destruction of these nerve fibres by prior treatment with capsaicin increased gastric damage induced by intragastric acid or ethanol and by administration of cysteamine or indomethacin (Szolcsanyi & Bartho, 1981; Holzer & Sametz, 1986). In the present investigation, adult rats were pretreated with capsaicin over a three day period, to induce 'desensitization' or a functional ablation of primary afferent neurones as a result of mediator depletion of the sensory nerve terminals (Russell & Burchiel, 1984; Buck & Burks, 1986; Martling, 1987; Holzer, 1988). Our findings that such capsaicin pretreatment increased the mucosal damage induced by Paf further supports a role of sensory afferent nerves in the regulation of mucosal integrity following challenge. As with TTX, capsaicin pretreatment itself did not damage the mucosa on macroscopic inspection.

Studies on vasodilatation and plasma leakage have suggested that opioids may modulate the activity of primary afferent neurones in the skin (Bartho & Szolcsanyi, 1981; Lembeck & Domnerer, 1985; Russell *et al.*, 1985; Smith & Buchan, 1984). Furthermore, studies on pain and analgesia have demonstrated that opioids can affect afferent sensory neurones by an action at peripheral sites (Ferreira & Nakamura, 1979; Smith *et al.*, 1982; Ferreira *et al.*, 1984; Russell *et al.*, 1987). The suggestion that local afferent neurones can modulate gastric mucosal integrity thus raises the possibility that opioids could affect the response to mucosal injury, by a peripheral action on such neuronal mechanisms. However, previous studies with opioid agonists in various rat models of gastric damage have produced contradictory findings. Thus, subcutaneous or intraperitoneal administration of morphine enhanced mucosal damage induced by pylorus ligation (Ho *et al.*, 1984), restraint stress (Till *et al.*, 1988) or by indomethacin (Gyres *et al.*, 1985). Likewise, subcutaneous or intravenous administration of enkephalinamide analogues augmented stress-induced gastric ulceration

(Morley et al., 1982; Till et al., 1988). In contrast, others have shown that morphine (in doses of 8–32 mg kg^{-1}) can reduce the ulceration associated with restraint-stress (Arrigo-Reina & Ferri, 1980; Glavin et al., 1986). However, these latter findings may reflect the high doses of morphine used as well as the use of models of ulceration with complex pathophysiology. In the current study, we have therefore investigated the effects of morphine administered intravenously in low doses on the gastric damage induced by direct local infusion of Paf.

Pretreatment with morphine dose-dependently increased the extent of mucosal damage induced by local Paf administration. This effect appears to be exerted at peripherally-located opioid receptors since the quaternary analogue, N-methyl morphine, which does not penetrate the blood-brain barrier and thus acts at peripheral sites (Smith et al., 1982), likewise substantially augmented Paf-induced damage. The effect of morphine appeared to be mediated through interaction with classical opiate receptors since intravenous administration of low doses of the opiate antagonist, naloxone, abolished this response. In addition, the peripherally-acting quaternary antagonist, N-methyl nalorphine (Smith et al., 1982) also substantially inhibited the potentiating action of morphine, giving further support to a peripheral site of action of the opioids in this model. Although the opioid receptors have not been fully characterised, the specificity of morphine, naloxone and N-methyl nalorphine (Magan et al., 1982) suggest the involvement of μ receptors in this mechanism.

As with TTX and capsaicin pretreatment, administration of morphine alone in the present study did not damage the mucosa. The potentiating effect of morphine on Paf-induced gastric damage was not due to the release of endogenous mast-cell histamine since H_1- and H_2-receptor blockade did not affect the degree of damage. Furthermore, morphine did not enhance the fall in systemic arterial blood pressure induced by Paf, obviating any non-specific augmentation of damage by this mechanism. Any involvement of a change in luminal acidity underlying the potentiation of Paf-induced damage by morphine also appears unlikely, since antisecretory doses of cimetidine did not affect the gastric damage induced by this combination, while previous studies have shown that intragastric acid did not augment Paf- induced gastric damage (Rosam et al., 1986).

Although TTX was the more potent, the similarity between the effects of TTX, capsaicin pretreatment and the opioid agonists in potentiating Paf-induced gastric mucosal damage suggest the involvement of opiate-sensitive sensory afferent neurones in this interaction. The macroscopic observations were confirmed by histological evaluation, with the major characteristic underlying the enhanced damage of extensive vasocongestion implicating vascular or endothelial cell damage as a primary or contributing factor to this mucosal injury. It is therefore pertinent that capsaicin-sensitive nerve fibres, identified using immunohistochemical techniques for neuropeptides in the rat stomach, have been located in the submucosa in association with the microvasculature (Sharkey et al., 1984; Ekblad et al., 1985), these being the blood vessels that regulate blood flow to the mucosal capillaries.

The present findings indicate that the degree of mucosal damage induced by intravascular administration of the ulcerogen, Paf is modulated by a local neuronal mechanism. Indeed, challenge of the gastric mucosa by Paf or other ulcerogens may lead to the activation of local opioid-sensitive sensory afferent neurones which attenuates the subsequent degree of damage, although the mechanisms underlying such activation are not clear. The proposition that the release of vasoactive mediators from local sensory nerves may contribute to the protection of the gastric mucosa from injury (Szolcsanyi & Bartho, 1981; Holzer & Sametz, 1986; Holzer, 1988) thus warrants further consideration. The current observations could also imply that gastric damage resulting from noxious stimuli and conditions such as stress may be augmented by morphine or endogenous enkephalins, which would act by inhibiting the activation of these peripheral afferent neurones and the release of neuropeptides from the sensory nerve terminals.

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Presynaptic muscarinic receptors inhibiting endogenous noradrenaline release in the portal vein of the freely moving rat

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1 In the portal vein of the freely moving unanaesthetized rat, the existence of presynaptically located inhibitory muscarinic receptors was investigated by use of the muscarinic agonist methacholine (MCh)

2 Infusion of MCh ($0.3\,\mu g\,min^{-1}$) did not significantly inhibit the endogenous noradrenaline (NA) overflow in portal plasma. However, after inducing high intra-synaptic concentrations of NA by blocking the presynaptic α_2-adrenoceptors with yohimbine ($1\,mg\,kg^{-1}$), MCh ($0.3\,\mu g\,min^{-1}$) was able to reduce the yohimbine-induced enhanced NA overflow by 38%.

3 The MCh-induced inhibition was almost completely abolished after blockade of the presynaptic muscarinic receptors with atropine ($0.6\,mg\,kg^{-1}$).

4 During electrical stimulation of the portal vein nervous plexus the evoked NA overflow was strongly inhibited (95%) during MCh-infusion ($0.3\,\mu g\,min^{-1}$). Again atropine ($0.6\,mg\,kg^{-1}$) was able to reverse this inhibition.

5 These results show the existence of presynaptic muscarinic receptors inhibiting endogenous NA overflow from the portal vein nervous plexus under conditions of enhanced sympathetic activity in the freely moving rat.

Introduction

Modulation of the noradrenaline (NA) overflow by prejunctional cholinoceptors has been reported in a number of tissues (see Vanhoutte & Levy, 1980; Shepherd & Vanhoutte, 1985, for reviews). It has become clear that these prejunctional cholinoceptors are of the muscarinic subtype, because atropine and not hexamethonium fully antagonized the acetylcholine-induced reduction of stimulus-evoked NA overflow (Löffelholz & Muscholl, 1969; Rorie *et al.*, 1981).

To the best of our knowledge, no *in vivo* data are available on the presynaptic modulation of sympathetic transmitter release mediated by postganglionic prejunctional muscarinic receptors in the freely moving unanaesthetized rat.

Recently, we introduced a new technique which allows the study of presynaptic modulation of the endogenous NA release from the portal vein nervous plexus of the freely moving rat (Remie & Zaagsma, 1986). Using this method we investigated several types of presynaptic receptors and striking differences were found when compared to the results obtained both *in vitro* and *in vivo* in the presence of anaesthetics (Remie *et al.*, 1988).

In the present study we investigated the influence of the prejunctional cholinoceptor on basal and electrically-evoked NA overflow in the vasculature of freely moving rats, both in the absence and presence of presynaptic α_2-adrenoceptor blockade.

Methods

Male normotensive Wistar rats weighing 270–350 g were used. All animals were housed in individual cages and permitted tap water and standard laboratory chow *ad libitum*. Lights were on from 07h 00min–19h 00min. Rats were prepared surgically according to the method described recently (Remie *et al.*, 1988). Briefly, under ether anaesthesia a five fine-stitch purse-string suture was made in the wall

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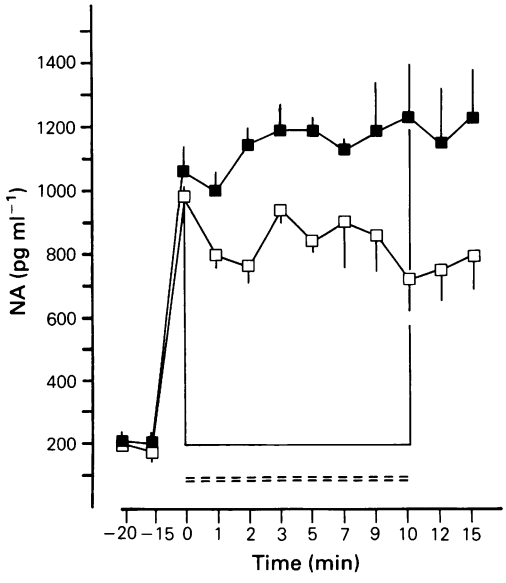


Figure 1 Effect of yohimbine (1 mg kg^{-1}), given via the infusion cannula in the portal vein at $t = -15$ min, on the basal noradrenaline (NA) overflow measured in the portal vein of the freely moving unanaesthetized rat (control, closed symbols). The open symbols represent the effect of yohimbine (1 mg kg^{-1}) followed by a 10 min infusion of methacholine (0.3 μg min^{-1}) starting at $t = 0$ min (indicated by = = = = =) ($n = 3$).

of the portal vein at the side opposite the gastroduodenal vein; a Zeiss Opmi 7 operation microscope was used. A silicon cannula was inserted downstream into the vessel and anchored with the drawstrings of the suture. A second cannula, used for the infusion of drugs was inserted in the vessel in a similar way, at the side opposite the superior pancreatico-duodenal vein. A bipolar stimulation electrode was placed around the portal vein, between the two cannulae. Both the cannulae and the electrode wires were driven subcutaneously to emerge at the crown of the head, where they were fixed to the skull.

Using this technique, we were able to stimulate the local nervous plexus of the portal vein, to apply drugs locally and to take blood samples from the portal vein without disturbing the animal. The blood samples (200 μl) were collected as described previously (Remie & Zaagsma, 1986) into small ice-cooled cups containing some EDTA crystals and red blood cells were spun down immediately (Beckmann Microfuge).

For the determination of catecholamines we used 100 μl plasma which was extracted according to the method described recently (Remie *et al.*, 1988). The analytical column was an Alltech Rosil C18 5 μm cartridge (150 × 4.6 mm) and the mobile phase was composed of ultra pure water/methanol (94.0:6.0, v/v), citric acid 1.35%, NaH_2PO_4 1.3%, heptanesulphonic acid 0.06%, and 0.01% of both EDTA and NaCl. The pH was adjusted to 4.1 with citric acid. Before use the solution was filtered (Schleicher and Schull, 0.2 μm) and degassed by sonification at low pressure.

An ESA Coulochem 5100 A detector with a high sensitivity analytical cell (5011) was used for the detection of the catecholamines. The guard potential was set to +450 mV, the first detector potential to −350 mV and the second high sensitivity detector to +350 mV. The chromatograms were recorded on a Kipp BD 41 dual channel flatbed recorder. Corrections for incomplete recovery were performed.

Experiments were started approximately ten days after surgery. Blood samples of 200 μl were taken at the following times: $t = -10, 0, 1, 2, 3, 5, 7, 9, 10, 12, 15$ min. The intravenous infusion of methacholine (MCh) lasted from $t = 0$ until $t = 10$ min and in electrical stimulation experiments until $t = 12$ min. Yohimbine was given intravenously as a bolus injection at $t = -15$ min, as was the combination of yohimbine and atropine. These administrations were preceded by two blood samples taken at $t = -20$ and $t = -15$ min.

The nervous plexus of the portal vein was stimulated via bipolar circular electrodes around the vessel (Remie & Zaagsma, 1986), with square wave constant current (5 mA) biphasic pulses having a frequency of 2 Hz and a duration of 3 ms. When electrical stimulation was applied, it started at $t = 10$ min and lasted 2 min. During stimulation a blood sample was taken every 15 s. The increase or decrease in the amount of basal NA in portal plasma after administration of drugs, was expressed as a percentage of the yohimbine-induced enhanced level (100 ± 8%). The amount of NA was calculated from the area under the curve, from $t = 0$ until $t = 10$ min, as illustrated in Figure 1. The ratio between the two areas reflects the change in NA overflow. The increase or decrease in the amount of NA overflow during electrical stimulation is expressed as a percentage of the control stimulation area (100 ± 6%). In experiments involving electrical stimulation no yohimbine was administered.

The results are given as means ± s.e.mean: number of experiments is indicated by n. Statistical evaluation was performed with Student's t test for unpaired data. P values less than 0.05 were regarded as significant.

Drugs

Yohimbine hydrochloride (Brocacef, Maarssen, The Netherlands), acetyl-β-methylcholine chloride (MCh,

Aldrich Chemical Company Inc., Milwaukee, WIS, U.S.A.) and atropine sulphate (Brocacef, Maarssen, The Netherlands) were used. All drugs were dissolved in sterile saline.

Results

Basal overflow

Infusion of MCh ($0.3\,\mu g\,min^{-1}$; $3\,\mu g\,ml^{-1}$ at a rate of $6\,ml\,h^{-1}$) had no significant effect on the basal NA overflow from sympathetic varicosities of the freely moving rat. At a ten-times higher concentration of MCh, plasma NA concentrations were rather unstable and tended to be elevated. At this concentration, plasma adrenaline (Ad) levels were enhanced (data not shown). Figure 1 depicts the rise of the portal NA level after the administration of yohimbine ($1\,mg\,kg^{-1}$). The mean basal NA level of $205 \pm 15\,pg\,ml^{-1}$ increased by a factor 5.18 to $1061 \pm 85\,pg\,ml^{-1}$. Infusion of $0.3\,\mu g\,min^{-1}$ MCh significantly decreased the NA overflow.

The total amount of NA overflow during 10 min of MCh infusion in the presence of yohimbine, calculated from the area under the curve, was reduced to $62 \pm 9\%$ of the control (yohimbine only) overflow. When atropine ($0.6\,mg\,kg^{-1}$) was given together with yohimbine, the MCh-induced decrease of NA overflow reverted to $92 \pm 8\%$ of the control value (Figure 2). Atropine itself had no effect on basal NA overflow (data not shown).

Electrically-evoked overflow

During electrical stimulation of the portal vein nervous plexus in the presence of a MCh infusion ($0.3\,\mu g\,min^{-1}$), the electrically-evoked NA overflow was reduced to $5 \pm 15\%$ of the control stimulation area (Figure 3). In the presence of atropine ($0.6\,mg\,kg^{-1}$), given 15 min before stimulation, the evoked release during the infusion of MCh ($0.3\,\mu g\,min^{-1}$) reverted to $97 \pm 12\%$ of the control stimulation value. Atropine itself tended to enhance the electrically-evoked NA overflow ($122 \pm 8\%$), but failed to reach a significant level.

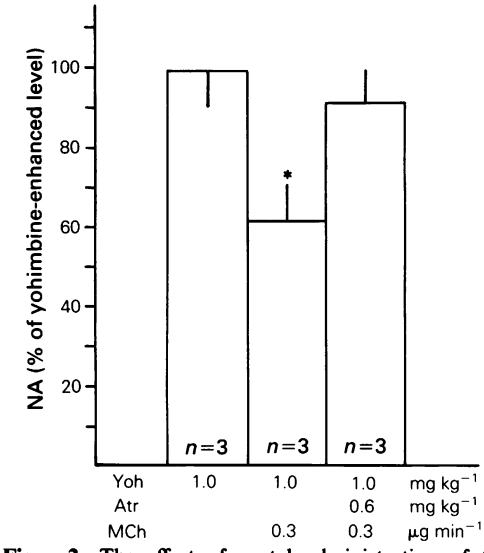


Figure 2 The effect of portal administration of the muscarinic agonist methacholine (MCh, dosage indicated) on endogenous noradrenaline (NA) overflow in the portal vein of freely moving unanaesthetized rats after α_2-adrenoceptor blockade in the absence or presence of atropine (Atr, $0.6\,mg\,kg^{-1}$). Values represent the cumulative NA overflow determined by the area under the curve. The effect of yohimbine (Yoh) alone on the basal NA overflow was set as $100 \pm 8\%$. (* $P < 0.05$, versus both control and atropine-treated rats).

Discussion

The release of NA from sympathetic varicosities is modulated by a number of inhibitory and facilitatory receptors located on postganglionic presynaptic nerves (Langer, 1981). In the cardiovascular system, inhibition of NA release by muscarinic agonists is well established (Löffelholz & Muscholl, 1969; Vanhoutte & Levy, 1980; Rorie *et al.*, 1981; Shepherd & Vanhoutte, 1985).

In our model we are able to study the presynaptic modulation of endogenous NA overflow in the portal vein of freely moving unanaesthetized rats during pharmacological and/or electrical stimulation of the portal vein nervous plexus. Basal endogenous NA measured in the portal vein originates not only from the portal vein nervous plexus but contains NA from other sympathetically innervated tissues or vascular beds as well. During electrical stimulation, however, the increase in NA concentration is unequivocally caused by NA overflow from the local portal vein nervous plexus only (Remie & Zaagsma, 1986; Remie *et al.*, 1988).

Drugs given in the portal vein will reach their highest tissue concentration at the place of administration. After elimination by the liver the amount of unmetabolized drug will be lower in the rest of the body. Moreover, at an infusion rate of $0.3\,\mu g\,min^{-1}$ the concentration of MCh in the portal vein will be very low due to dilution in the blood stream. Based on a portal blood flow of about $15\,ml\,min^{-1}$, measured by using an electromagnetical flow probe

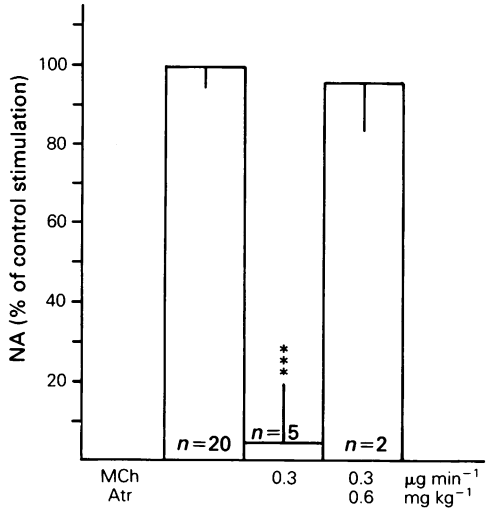


Figure 3 The effect of methacholine (MCh, 0.3 μg min^{-1}) on the noradrenaline (NA) overflow in the portal vein, evoked during 120 s of electrical stimulation (2 Hz, 3 ms, 5 mA) in the absence or presence of atropine (Atr, 0.6 mg kg^{-1}) and the effect of atropine (0.6 mg kg^{-1}) alone. Data given are the percentage of the control stimulation area which was set as 100 ± 6% (*** $P < 0.001$, versus both control and atropine-treated rats).

(Daemen *et al.*, 1989) and on data of Tsuchiya *et al.* (1978), a concentration of approximately 0.1 μM can be calculated. Higher MCh concentrations (5–320 μM) have been found to decrease dose-dependently the NA overflow of the perfused rabbit heart (Muscholl *et al.*, 1979). Fuder *et al.* (1985) also demonstrated an inhibition of the NA overflow using this selective muscarinic agonist at concentrations of 1 and 10 μM in guinea-pig isolated atria.

In our model, infusion of MCh (0.3 μg min^{-1}) has no effect on the basal NA level, indicating that possible interfering effects of MCh like vasodilatation or bradycardia have no influence on the plasma NA level measured in the portal vein. Higher dosages (3 and 30 μg min^{-1}) caused unstable NA levels, which tended to be elevated, and a small rise in plasma Ad levels. This rise could be caused by stimulation of the adrenal medulla. Critchley *et al.* (1986) showed that in perfused adrenal glands of dogs and cats, 1 μM MCh preferentially released Ad from the medulla. Ad could act as a neuromodulator on the presynaptically located β_2-adrenoceptors thus enhancing the NA overflow (Remie *et al.*, 1988). Thus, under conditions of low sympathetic activity, cholinergic presynaptic inhibition of NA release plays a minor or negligible role.

When yohimbine (1 mg kg^{-1}) is used, the presynaptically located inhibitory α_2-adrenoceptors are blocked (central as well as peripheral) and the NA overflow is strongly (5 fold) enhanced. Under these circumstances, with high concentrations of NA present in the synaptic clefts, infusion of a low dosage of MCh (0.3 μg min^{-1}) clearly decreases the NA overflow.

During stimulation of the portal vein nervous plexus, the synaptic concentration of NA is also elevated. Again, MCh (0.3 μg min^{-1}) decreases the amount of NA overflow from the synaptic clefts.

Atropine (0.6 mg kg^{-1}) suppressed the MCh-induced inhibition of both the stimulus-evoked and the yohimbine-induced rise in NA overflow almost completely, confirming the muscarinic nature of these presynaptic cholinoceptors. Atropine alone showed a tendency to enhance the electrically-evoked NA overflow, although the difference just failed to reach the significance level. This could, however, indicate functional inhibition of NA overflow at the sympathetic varicosity by endogenous acetylcholine released during co-stimulation of parasympathetic neurones present in the portal vein nervous plexus (Boekelaar, 1985). A similar moderate atropine (0.1 μM)-induced enhancement of [^{3}H]-NA overflow was found by Bognar *et al.* (1988) during electrical field stimulation (10 Hz, 1 ms and 75 mA) of the rabbit iris *in vitro*.

Our results clearly show the existence of physiologically active inhibitory prejunctional muscarinic receptors in the portal vein varicosities of the freely moving unanaesthetized rat. This cholinergic inhibition of the NA overflow can be measured only under conditions of enhanced sympathetic activity. This modulation probably plays an important physiological role in the maintenance of the balance between the sympathetic and the parasympathetic nervous system.

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Ketamine-induced relaxation in intact and skinned smooth muscles of the rabbit ear artery

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1 The effects of ketamine, an intravenous anaesthetic, on the rabbit ear artery were investigated by measuring the tension in intact and saponin-treated skinned smooth-muscle fibres.

2 Ketamine dose-dependently inhibited contractions of intact smooth-muscle fibres induced by high K^+ solution and by noradrenaline (NA) or histamine in Krebs solution. This drug similarly attenuated both phasic and tonic contractions induced by high K^+ solution.

3 Ketamine also inhibited NA- or histamine-induced contractions in Ca^{2+}-free solution containing 2 mM EGTA, but it did not affect the caffeine-induced contraction in this solution.

4 Because the pCa-tension relationship of saponin-treated skinned smooth-muscle fibres was not affected, it can be proposed that ketamine does not have an effect on the contractile proteins.

5 In the presence of 5 mM NaN_3, 20 μM inositol 1,4,5-trisphosphate ($InsP_3$) or 25 mM caffeine produced a contraction in skinned smooth-muscle fibres after accumulation of Ca^{2+} by intracellular stores. Analysis of the $InsP_3$- or caffeine-induced contractions indicates that ketamine does not have an effect on the Ca^{2+} accumulation into and Ca^{2+} release from the intracellular stores.

6 These results indicate that the relaxant effects produced by ketamine in the rabbit ear artery are not likely to be due to an intracellular action. The inhibitory effects of ketamine could be caused by a decrease of the Ca^{2+} influx through the plasma membrane or interference with the process of signal transduction between receptors on the plasma membrane and intracellular stores.

Introduction

Ketamine, an intravenous anaesthetic, has various effects on the cardiovascular system. Amongst them, ketamine is known for its hypertensive properties (Hug, 1979). However, this drug has also been demonstrated to produce biphasic blood-pressure responses (initially hypotensive, later hypertensive) in man, rats and dogs (Domino *et al.*, 1965; Virtue *et al.*, 1967; Dowdy & Kaya, 1968) or profound hypotension in certain species including rabbits (Clanachan *et al.*, 1976). The mechanisms of the hypertensive actions are thought to be via the central nervous system, baroreceptors and/or vascular sympathetic neurotransmission (Fukuda *et al.*, 1986). However, the hypotension is thought to be caused by a direct effect on vascular smooth muscles.

The contraction and relaxation of vascular smooth muscles depends on changes of the intracellular free Ca^{2+} concentration. The cytoplasmic Ca^{2+} is increased by an influx of external Ca^{2+} through voltage-dependent or receptor-operated channels and by the release of this ion from intracellular stores, mainly from the sarcoplasmic reticulum (SR) (Kuriyama *et al.*, 1982). The inhibitory effects of ketamine on vascular contraction have been shown to be due to an interference with transmembrane Ca^{2+} influx (Altura *et al.*, 1980; Fukuda *et al.*, 1983), but other mechanisms, such as effects on contractile proteins and on intracellular stores, were not excluded.

The present studies were therefore undertaken to investigate further the effects of ketamine on vascular smooth muscles using intact and skinned smooth-muscle fibres of the rabbit ear artery.

Methods

Preparation

Rabbits of either sex, weighing 2 to 3 kg were stunned and exsanguinated. The central ear artery was dissected out of the ear and cleaned of its periarterial connective tissues under a binocular microscope while being kept in warmed and oxgenated Krebs solution. Thin circular strips (0.3–0.5 mm in

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length, 0.05–0.08 mm in width and 0.02–0.03 mm in thickness) were prepared under a binocular microscope by use of fine forceps and small knives made from pieces of razor blades. In all experiments, the endothelium was carefully removed with the small knives as indicated previously (Itoh *et al.*, 1985).

Recording of mechanical activity

Mechanical activity of intact and skinned smooth-muscle fibres was measured by attaching a circular strip to a strain gauge (U-gauge, Shinko, Tokyo, Japan). Each end of the strip was knotted by a fine silk fibre which was then fixed to a piece (about 1 mm × 1 mm) of Scotch double-sided adhesive tape (3M Co., St. Paul, MN, U.S.A.). One tape was fixed to a side of the chamber and the other tape to the strain gauge. The chamber had a volume of 0.9 ml. The solutions were rapidly injected by a syringe from one end of the chamber and simultaneously siphoned off from the other end. The tissue was not superfused constantly but remained exposed to the solution in the chamber until the next solution was injected (Kanmura *et al.*, 1988).

Chemically skinned muscle fibres were obtained by exposing the fibres to saponin (35 $\mu g\,ml^{-1}$) in relaxing solution for 20 min (Itoh *et al.*, 1981). The Ca^{2+}-induced contractions were recorded at various concentrations of free Ca^{2+} buffered by ethyleneglycol *bis*-(β-aminoethylether)-N,N,N',N'-tetra-acetic acid (EGTA). The intracellular Ca^{2+} stores were loaded by exposing the skinned fibres to a solution containing 0.6 μM free Ca^{2+} (buffered with 4 mM EGTA). The release of Ca^{2+} from these stores was then examined by recording the tension induced by inositol 1,4,5-trisphosphate ($InsP_3$) or by caffeine in Ca^{2+}-free solution supplemented with 0.1 mM EGTA. NaN_3 5 mM was present throughout the experiments in order to prevent Ca^{2+} accumulation by the mitochondria. To prevent the deterioration of the function of the contractile proteins, 0.1 μM calmodulin was present throughout the experiments. The relaxation of Ca^{2+}-induced contraction was accelerated by supplementing the relaxing solution needed for washing away the Ca^{2+}-containing solution with 6 mM K phosphate (Itoh *et al.*, 1986). The temperature was kept at 25°C (Iino, 1981; Itoh *et al.*, 1981) because skinned fibres deteriorate more rapidly at higher temperatures.

Solutions

The composition of Krebs solution was as follows (mM): NaCl 135.5, KCl 5.9, $MgCl_2$ 1.2, $CaCl_2$ 1.5, glucose 11.5 and 2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethanesulphonic acid (HEPES) 11.6. It was bubbled with O_2 and the pH was adjusted to 7.3 with NaOH. High K^+ solution was prepared by replacing NaCl with KCl. In Ca^{2+}-free solutions, $CaCl_2$ was replaced with $MgCl_2$ and 2 mM EGTA was added. The relaxing solution used for skinned fibres contained (mM): K methanesulphonate (KMs) 114, Tris maleate 20, $Mg(Ms)_2$ 5.1, adenosine 5'-triphosphate (ATP) 5.2 (10.4 mM Na^+ as Na_2-ATP) and EGTA 4. The free Ca^{2+} concentration was changed by adding appropriate amounts of $Ca(Ms)_2$ to EGTA. A value of $10^6\,M^{-1}$ was used for the apparent binding constant of Ca^{2+} to EGTA at pH 6.8 and 25°C (Itoh *et al.*, 1981). To observe the effects of ketamine on Ca^{2+} release from intracellular stores, the concentration of EGTA was reduced to 0.1 mM.

Drugs

Chemicals used were: ketamine hydrochloride from Parke Davis (Bornem, Belgium), noradrenaline (NA) and EGTA from Fluka (Switzerland), ATP from Boehringer (Manheim, F.R.G.). $InsP_3$ from Sigma Chemical Co. (St. Louis, MO, U.S.A.), saponin from ICN Pharmac. Inc. (Cleveland, OH, U.S.A.), caffeine from BDH Chemicals (Poole, England), HEPES and histamine from Merck (Darmstadt, F.R.G.). Calmodulin was prepared from bovine brain according to Gopalakrishna & Anderson (1982).

Results

Effects of ketamine on mechanical responses induced by high K^+ or agonists in intact smooth-muscle fibres

In intact muscle strips of the rabbit ear artery, high K^+ solution evoked a rapid phasic and sustained tonic contraction. Ketamine (100 μM and 300 μM) inhibited these phasic and tonic responses to the same extent (Figure 1Aa). Figure 1B shows the effects of various concentrations of ketamine on the phasic and tonic responses of the high K^+-induced contraction. The IC_{50} values measured for the action of ketamine on the phasic and tonic responses evoked by 138 mM K^+ were 176 μM and 145 μM, respectively. In this tissue, 10 μM NA produced the maximal contraction and the amplitude of contraction evoked by 138 mM K^+ solution was 0.45 $\pm$ 0.05 times that of the 10 μM NA-induced contraction ($n = 5$). Fifty nM NA, 500 nM histamine and 138 mM K^+ produced approximately the same amplitude of contraction, and we investigated the effects of ketamine on these similar contractions. It was observed that NA- or histamine-induced contractions in Krebs solution were also inhibited by ketamine (Figure 1Ab,c). Figure 1C shows the dose-dependent

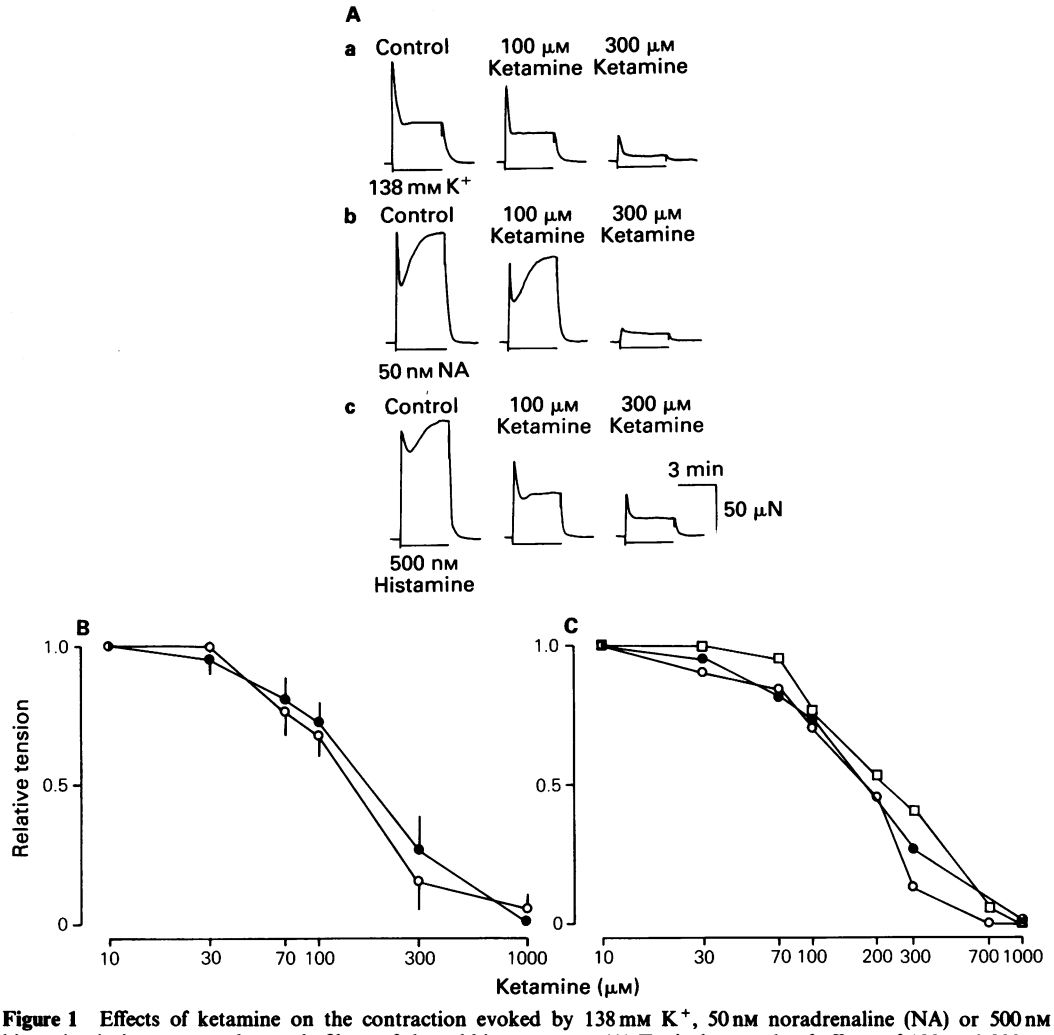


Figure 1 Effects of ketamine on the contraction evoked by 138 mM K^+, 50 nM noradrenaline (NA) or 500 nM histamine in intact smooth-muscle fibres of the rabbit ear artery. (A) Typical example of effects of 100 and 300 μM ketamine on the contraction evoked by K^+ (Aa), NA (Ab) or histamine (Ac). (B) Concentration-response curves of ketamine on the phasic (●) and tonic (○) responses of the 138 mM K^+-induced contraction. The amplitude of phasic or tonic responses evoked in the absence of ketamine was normalized as 1.0. Vertical bars indicate s.d., $n = 4$. Curves were fitted by eye. (C) Concentration-response curves of ketamine on contraction evoked by 138 mM K^+ (●), 50 nM NA (○) or 500 nM histamine (□). Amplitude of the phasic contraction evoked by each stimulant in the absence of ketamine was normalized as 1.0. Vertical bars which represent s.d. were omitted to make curves clear, $n = 4$. Curves were fitted by eye.

action of ketamine on the phasic response of contractions evoked by 138 mM K^+, 50 nM NA or 500 nM histamine. The maximum amplitude of contraction evoked by each agent in the absence of ketamine was normalized to 1.0. The IC_{50} values for 138 mM K^+-, NA- and histamine-induced contractions were 176 μM, 170 μM and 200 μM, respectively.

Effects of ketamine on contractions of intact muscle fibres induced by NA and caffeine in Ca^{2+}-free solution

In the rabbit ear artery, in Ca^{2+}-free solution containing 2 mM EGTA, NA produces a contraction due to the release of Ca^{2+} from intracellular stores

(Casteels & Droogmans, 1981). Caffeine also produces a contraction due to the release of Ca^{2+} from intracellular stores that are closely related to or are the same as those on which NA acts. We further investigated the effects of ketamine on these agonist-induced contractions in Ca^{2+}-free solution.

After releasing the stored Ca^{2+} completely by repeated application of 10 mM caffeine in Ca^{2+}-free solution containing 2 mM EGTA, the tissues were exposed to 1.5 mM Ca^{2+} for 10 min. It is known that the intracellular Ca^{2+} stores are completely filled by this procedure (Casteels & Droogmans, 1981). Thereupon the tissues were superfused for 3 min with Ca^{2+}-free solution and 50 nM NA was then applied for 3 min. Ketamine was applied before and during the application of NA. Ketamine 300 μM significantly inhibited this NA-induced contraction (0.35 ± 0.05 times the control, $n = 4$), and at 1 mM ketamine this contraction was completely blocked (Figure 2A). Ketamine also inhibited the histamine-induced contraction in Ca^{2+}-free solution (data not shown). In contrast, ketamine had little effect on the contraction evoked by 10 mM caffeine (Figure 2B). Ketamine (up to 300 μM) applied only during the application of Ca^{2+} did not inhibit the subsequent contraction induced by NA, but 1 mM ketamine in Ca^{2+}-containing solution slightly inhibited the contraction induced by NA in Ca^{2+}-free solution (data not shown).

To see whether this inhibitory effect of ketamine is due to the inhibition of Ca^{2+} release from intracellular stores or to an enhancement of the Ca^{2+} extrusion through the plasma membrane, the effects of ketamine were investigated on contractions induced in Ca^{2+}-free solution by successive applications of NA and caffeine. After eliciting a contraction to 50 nM NA, a small contraction could still be induced by subsequent application of 10 mM caffeine. However, if a contraction was first induced by 10 mM caffeine, neither 50 nM NA nor 10 mM caffeine could induce a further contraction. Treatment with 300 μM ketamine significantly inhibited the NA-induced contraction (0.43 ± 0.08 times the control, $n = 4$), but enhanced the caffeine-induced contraction (1.66 ± 0.32 times the control, $n = 4$) (Figure 3). This result indicates that ketamine probably inhibits the NA-induced Ca^{2+}-release from stores rather than enhancing the Ca^{2+} extrusion through the plasma membrane.

Effects of ketamine on the contractile proteins of skinned smooth-muscle fibres

The minimum concentration of Ca^{2+} required to produce a contraction in saponin-treated skinned muscle fibres of the rabbit ear artery was 0.3 μM and

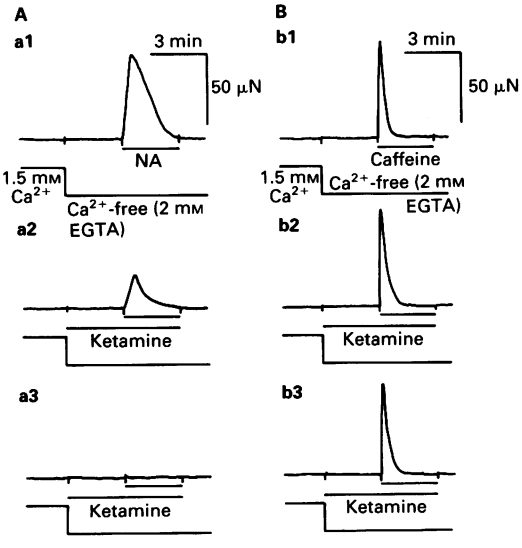


Figure 2 Effects of ketamine on noradrenaline (NA)- and caffeine-induced contraction in Ca^{2+}-free solution containing 2 mM EGTA. (A) After 1.5 mM Ca^{2+} was applied for 10 min, the smooth muscle fibres were washed with Ca^{2+}-free solution containing 2 mM EGTA and subsequently 50 nM NA was applied. (B) 10 mM caffeine was applied in Ca^{2+}-free solution instead of NA in (A). (a1 and b1) Control; (a2 and b2) 300 μM ketamine was applied before and during application of stimulants in Ca^{2+}-free solution; (a3 and b3) 1 mM ketamine was applied. These results are typical of 4 experiments.

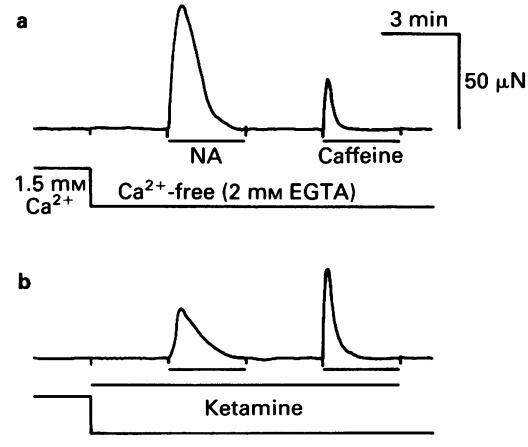


Figure 3 Effects of ketamine on successive noradrenaline (NA)- and caffeine-induced contractions in Ca^{2+}-free solution containing 2 mM EGTA. After 1.5 mM Ca^{2+} was applied for 10 min, 50 nM NA and 10 mM caffeine were applied successively for 3 min with 3 min interval. (a) Control; (b) 300 μM ketamine was applied in Ca^{2+}-free solution. These results were typical of 4 experiments.

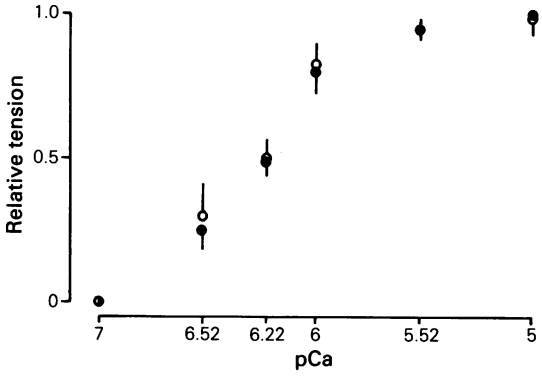


Figure 4 Effects of 1 mM ketamine (○) on the pCa-tension relation in saponin-treated skinned smooth-muscle fibres (●, control). The amplitude of contraction evoked by 10 μM Ca^{2+} in the absence of ketamine was normalized to 1.0. Vertical bars indicate s.d. (n = 3 to 5).

the maximum amplitude of contraction was reached in 10 μM Ca^{2+}. After the Ca^{2+}-induced contraction had reached a steady state, 1 mM ketamine was applied. Ketamine did not exert any effect on these Ca^{2+}-induced contractions (Figure 4).

Effects of ketamine on caffeine- and $InsP_3$-induced contractions in skinned smooth-muscle fibres

To study the effects of ketamine on the release of Ca^{2+} from intracellular stores directly, its effects on caffeine- and $InsP_3$-induced contractions were observed in saponin-treated skinned smooth-muscle fibres. After skinning, 0.6 μM Ca^{2+} buffered with 4 mM EGTA was applied for 2 min, the tissue was rinsed with relaxing solution containing 0.1 mM EGTA and 25 mM caffeine was added (Figure 5a). On the basis of the findings of Endo (1977) and of Itoh *et al.* (1981), it is accepted that high concentrations of caffeine release most of the Ca^{2+} present in intracellular stores, and that the amplitude of caffeine-induced contraction can be used as an indication of the amount of Ca^{2+} present in the stores.

When 1 mM ketamine was added to the relaxing solution containing 0.1 mM EGTA before and during application of 25 mM caffeine, the amplitude of this caffeine-induced contraction remained the same as that of the control (Figure 5b). When 1 mM ketamine was added to the solution containing 0.6 μM Ca^{2+} which was used to load the stores, the subsequent caffeine-induced contraction was not affected (Figure 5c).

$InsP_3$, the hydrolytic product of phosphatidylinositol 4,5-bisphosphate (PIP_2), is thought to act as a second messenger in vascular smooth muscles, by releasing Ca^{2+} from intracellular stores

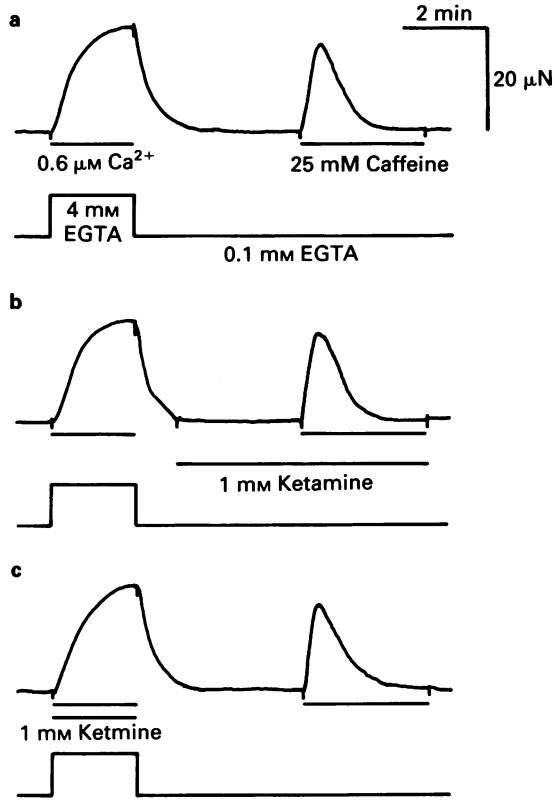


Figure 5 Effects of ketamine on caffeine-induced contraction in saponin-treated skinned smooth-muscle fibres. Caffeine 25 mM was applied after a 2 min exposure to a solution with 0.6 μM Ca^{2+} and a 4 min wash in the relaxing solution with 0.1 mM EGTA. (a) Control; (b) 1 mM ketamine was added 3 min before the application of caffeine; (c) 1 mM ketamine was applied during application of 0.6 μM Ca^{2+}. These results were typical of 4 experiments.

(Somlyo *et al.*, 1985; Hashimoto *et al.*, 1986). We investigated the effects of ketamine on this $InsP_3$-induced contraction in skinned smooth-muscle fibres. After accumulation of Ca^{2+} in the stores, application of 20 μM $InsP_3$ for 3 min elicited a transient contraction (Figure 6a). Treatment with 1 mM ketamine after or during Ca^{2+} loading did not inhibit the $InsP_3$-induced contractions (Figure 6b,c). These results suggest that ketamine does not act on the final step of the mechanism responsible for the release of Ca^{2+} by agonists.

Discussion

Ketamine is known for its hypertensive properties (Hug, 1979), and this effect has been ascribed to its

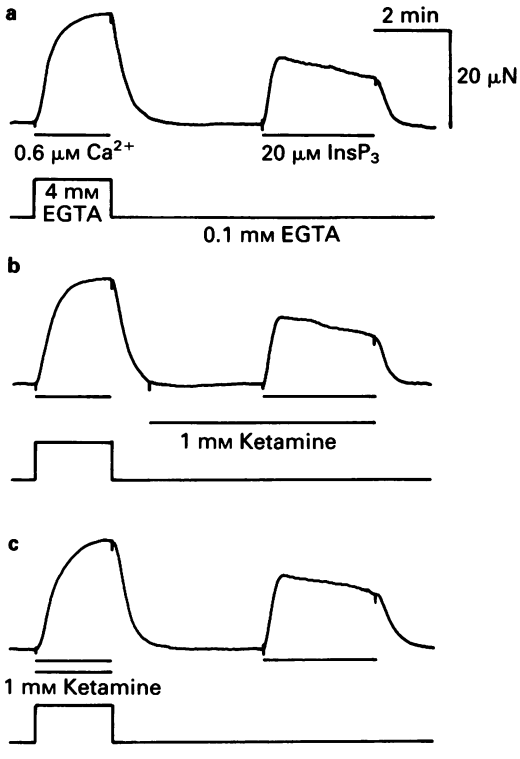


Figure 6 Effects of ketamine on inositol 1,4,5-trisphosphate ($InsP_3$)-induced contraction of saponin-treated skinned smooth-muscle fibres. $InsP_3$ 20 μM was used instead of 25 mM caffeine used in Figure 5. Other procedures were the same as in Figure 5.

action on the central nervous system (Wong & Jenkins, 1974), on the baroreceptors (Dowdy & Kaya, 1968) or on vascular sympathetic neuromuscular junctions (Fukuda *et al.*, 1986). However, *in vitro*, this drug has been shown to inhibit contractions of vascular smooth muscles (Altura *et al.*, 1980; Fukuda *et al.*, 1983) and the mechanism of this inhibitory action has been thought to be due to its interference with transmembrane influx of Ca^{2+} in a way similar to that of Ca antagonists. However, the present results indicate that ketamine possesses different characteristics from Ca antagonists.

In vascular tissues Ca antagonists inhibit preferentially tonic responses rather than phasic responses (Itoh *et al.*, 1984). In contrast, the inhibitory effects of ketamine on contraction induced by high K^+ were similar for both the phasic and the tonic responses. Moreover, ketamine inhibited agonist-induced contractions in the same manner as contractions induced by high K^+ (Figure 1C). In the rabbit ear artery, NA and histamine can cause contractions without modifying the membrane potential (Droogmans *et al.*, 1977). It is therefore unlikely that the voltage-dependent Ca^{2+} influx plays an important role in these contractions. From these results, we suspect that the inhibitory effects of ketamine are not limited to the voltage-dependent Ca^{2+} influx.

Agonists induce contractions of the rabbit ear artery in Ca^{2+}-free solution by releasing Ca^{2+} from intracellular stores, mainly from sarcoplasmic reticulum. Ketamine inhibits agonist-induced contractions in Ca^{2+}-free solution, but not the contractions induced by caffeine. This difference could be due to the fact that caffeine acts directly on intracellular stores. It can therefore be proposed that ketamine blocks some step(s) between receptor occupancy on the plasma membrane and the Ca^{2+} release from intracellular stores.

The mechanism responsible for Ca^{2+} release from intracellular stores by agonists is thought to be due to $InsP_3$, one of the hydrolytic products of PIP_2. Receptor occupancy by agonists induces the activation of phosphodiesterase (phospholipase C), which causes hydrolysis of PIP_2. Guanine nucleotide binding proteins (G-proteins) are assumed to be involved in this process.

It is possible that ketamine could block the receptor occupation by agonists, but because it exerts its inhibitory action on many different kinds of agonist-induced contractions (Altura *et al.*, 1980), this hypothesis is unlikely. Because ketamine penetrates rapidly into cells (Cohen *et al.*, 1973), an intracellular action of ketamine has been suggested (Altura *et al.*, 1980). However, intracellular application of ketamine in skinned smooth-muscle fibres did not reveal any effect on contractile proteins or on $InsP_3$- or caffeine-induced Ca^{2+} release from intracellular Ca^{2+} stores. It is therefore possible that ketamine could interfere with the functions of G-proteins and/or phosphodiesterase.

Recently, hydrolysis of PIP_2 has been shown to be induced not only by agonists but also by high K^+ solutions in guinea-pig ileum smooth muscles (Best & Bolton, 1986; Sasaguri & Watson, 1988). Part of the inhibition of ketamine of high K^+-induced contractions could therefore be due to an attenuated activity of G-proteins and/or phosphodiesterase. Further investigations about the effects of ketamine on the hydrolysis of PIP_2 by agonists or high K^+ solution are needed.

In conclusion, ketamine inhibits high K^+- and agonist-induced contractions in the presence of Ca^{2+} and agonist-induced contractions in Ca^{2+}-free solutions. Ketamine does not exert an effect on contractile proteins or on intracellular Ca^{2+} stores in skinned smooth-muscle fibres. The main site of the inhibitory effects of ketamine may be the process of hydrolysis of PIP_2.

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Inhibition of bradykinin-induced bronchoconstriction in the guinea-pig by a synthetic B_2 receptor antagonist

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1 Intravenous bradykinin (Bk) elicited bronchoconstriction in the anaesthetized ventilated guinea-pig which was not mimicked by the B_1 receptor agonist, des-Arg^9-Bk.

2 Bradykinin-induced bronchoconstriction was inhibited by the B_2 receptor antagonist B4881, but not by the B_1 receptor antagonist des-Arg^9-Leu^8-Bk. The effect of B4881 was short-lived.

3 The B_2 receptor antagonist as B4881 was selective for bradykinin as B4881 did not significantly inhibit bronchoconstriction induced by i.v. bombesin, platelet activating factor, acetylcholine, histamine or vagal stimulation.

4 These results suggest that bradykinin-induced bronchoconstriction in the guinea-pig is via activation of a B_2 receptor population and that B4881 is a selective B_2 antagonist that may be useful for investigating the involvement of bradykinin in the lung.

Introduction

Bradykinin has long been known to induce bronchoconstriction in a number of mammalian species including man (Collier *et al.*, 1960; Bhoola *et al.*, 1962; Herxheimer & Stresemann, 1963). However, the receptor type which mediates this effect has not been characterized. Recent research has shown that bradykinin can act via two distinct receptor populations, designated as B_1 and B_2 (Regoli & Barabe, 1980) which has led to the development of a number of antagonists for the B_2 receptor population (Vavrek & Stewart, 1985) based on synthetic peptide analogues. These B_2 receptor antagonists have been used to antagonize the activity of bradykinin in a number of tissues including blood vessels (Schachter *et al.*, 1987; Whalley, 1987), and smooth muscles of intestine, bladder and uterus (Vavrek & Stewart, 1985; Regoli *et al.*, 1986; Griesbacher & Lembeck, 1987). Additionally, these B_2 antagonists have been used to antagonize the pain producing activity of bradykinin on the human blister base (Whalley *et al.*, 1987; Steranka *et al.*, 1988), the ability of bradykinin to induce mucosal electrolyte transport (Kachur *et al.*, 1987) and to demonstrate the involvement of bradykinin in endotoxin-induced hypotension (Weipert *et al.*, 1988). In the present study, we have evaluated one of the more potent B_2 antagonists (B4881) D-Arg-[Hyp^3-Thi^{5,8}-D-Phe^7]-Bk, on bradykinin-induced bronchoconstriction in the anaesthetized, ventilated guinea-pig in comparison with the B_1 antagonist des-Arg^9-Leu^8-Bk. Furthermore, we have examined the ability of the B_1 receptor agonist, des-Arg^9-Bk, to mimick the bronchoconstrictor effect of bradykinin and have investigated the selectivity of the B_2 antagonist B4881 by evaluating the effect of this drug on bronchoconstriction induced by acetylcholine, histamine, bombesin, platelet activating factor (Paf) and vagal stimulation.

Methods

Male Dunkin-Hartley guinea-pigs (body weight 250–350 g) were used throughout this study. Animals were anaesthetized with urethane (25% w/v i.p., 7 ml kg^{-1}). The trachea was cannulated for measurement of airways obstruction and the polyethylene cannula was connected to a Harvard small animal ventilator. Animals were ventilated with room air (1 ml 100 g^{-1} body weight) at 70 strokes min^{-1}. Air overflow was measured at the side arm of the tracheal cannula by means of a pressure transducer (Ormed Engineering) and expressed as pulmonary inflation pressure (PIP) in mmHg as an index of bronchoconstriction. Arterial blood pressure was recorded via a cannula in the carotid artery and

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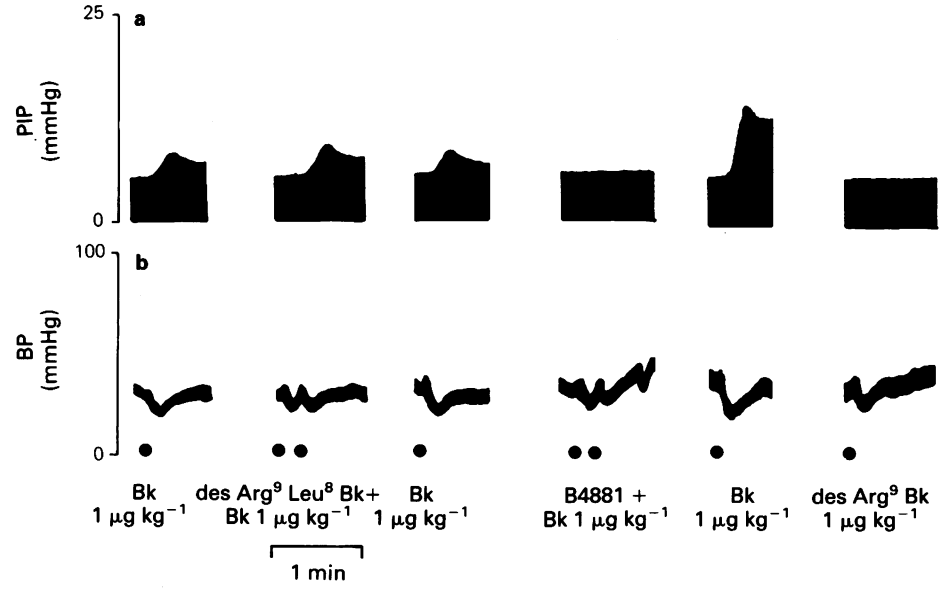


Figure 1 Representative trace showing the increase in pulmonary inflation pressure (PIP) as an index of bronchoconstriction (a) or blood pressure (b) in response to i.v. bradykinin (Bk 1 $\mu g kg^{-1}$) alone or in the presence of the B_1 antagonist des-Arg^9-Leu^8-Bk or the B_2 antagonist B4881. Responses to i.v. des-Arg^9-Bk are shown for comparison. PIP and BP are expressed as mmHg. Bradykinin was administered at 10 min intervals.

intravenous injections were made via a cannulated jugular vein.

In some experiments the cervical vagus nerve was carefully dissected free and placed on platinum electrodes. The vagus nerve was stimulated with 10 V pulses of 0.5 ms at 30 Hz for 10 s using a variable pulse stimulator (Scientific and Research Instruments Ltd.).

Bronchoconstriction was induced by i.v. administration of a spasmogen or by electrical stimulation of the vagus; 10 min later, the antagonists were injected 30 s before a second injection of spasmogen or electrical stimulation of the vagus in order to assess the efficacy of these drugs. In order to determine the time course of action of B4881, in some experiments bradykinin was administered 5 min, 10 min and 25 min as well as 30 s after administration of B4881.

Drugs and solutions

Urethane, bovine serum albumin (BSA), histamine, acetylcholine, bombesin, bradykinin, des-Arg^9-bradykinin and des-Arg^9-(Leu^8)-bradykinin were purchased from Sigma and prepared for i.v. administration in sterile 0.9% sodium chloride solution. Paf was obtained from Novabiochem (Nottingham,

Table 1 The effect of B4881 and des-Arg^9-(Leu^8)-Bk on bradykinin (Bk)-induced bronchoconstriction

Bk	*B4881*	*des-Arg^9-(Leu^8)-Bk*
Before	116.7 ± 17.8 (n = 12)	103.0 ± 28.8 (n = 5)
30 s after	14.1 ± 8.3 (n = 12)**	80.2 ± 14.8 (n = 5)
5 min after	60.8 ± 15.7 (n = 5)*	ND
10 min after	96.6 ± 16.2 (n = 6)	ND
25 min after	87.9 ± 28.9 (n = 12)	ND

Bronchoconstriction induced by bradykinin (1 $\mu g kg^{-1}$) before, and at various times after pretreatment with B4881 (400 $\mu g kg^{-1}$, i.v.) or des-Arg^9-(Leu^8)-Bk. Results are expressed as mean ± s.e.mean of % increase in pulmonary inflation pressure at various times after treatments in comparison with responses to Bk before. Significance is indicated by *$P < 0.05$; **$P < 0.01$. ND = not done.

England) and prepared in 0.25% BSA. The B_2 receptor antagonist, B4881, was a kind gift from Drs J.M. Stewart and R. Vavrek, Department of Biochemistry, University of Colorado School of Medicine, Denver, U.S.A.

Statistical analysis

The significance of the responses before and after treatment with either B_1 or B_2 antagonists was assessed by a paired t test. All results have been expressed as mean ± s.e.mean.

Results

Bradykinin (1 $\mu g kg^{-1}$, i.v.) induced a marked increase in PIP which was completely abolished by prior treatment with the B_2 antagonist B4881 (400 $\mu g kg^{-1}$, i.v.) administered immediately before the injection of bradykinin (Figure 1 and Table 1). The antagonist activity of B4881 was of short duration as the response to bradykinin had recovered 25 min after administration of the B_2 antagonist (Figure 2 and Table 1).

In contrast, no significant inhibition of bradykinin-induced bronchoconstriction was observed following pretreatment with the B_1 receptor antagonist, des-Arg^9-(Leu^8)-bradykinin (Figure 1 and Table 1). Occasionally, the response to bradykinin recovered to a level greater than before administration of B4881 (see Figure 1), although this was not significant in a group of animals.

Furthermore, no significant bronchoconstriction was observed following i.v. administration of des-Arg^9-Bk at both similar and higher concentrations to that used with bradykinin (Figure 3). However, this concentration of des-Arg^9-Bk induced marked changes in blood pressure indicating that the material was active (Figure 3).

Intravenous administration of histamine (10 $\mu g kg^{-1}$), acetylcholine (10 $\mu g kg^{-1}$), bombesin (0.5 $\mu g kg^{-1}$) or Paf (20 $ng kg^{-1}$) all induced bronchoconstriction assessed as a significant increase in PIP. However, bronchoconstriction induced by these spasmogens was not significantly attenuated by prior treatment with B4881 at a concentration of

Table 2 The effect of B4881 on the bronchoconstrictor effect of spasmogens and vagal stimulation

	n	*B4881* (400 $\mu g kg^{-1}$, i.v.) *Before*	*After*
Histamine (10 $\mu g kg^{-1}$)	4	270 ± 37.1	258 ± 36.1
Acetylcholine (10 $\mu g kg^{-1}$)	4	124 ± 14.4	136 ± 11.3
Bombesin (0.5 $\mu g kg^{-1}$)	4	65.4 ± 10.3	98.9 ± 11.5
Paf (20 $ng kg^{-1}$)	5	202 ± 18.2	233 ± 13.5
Vagal Stimulation	3	116 ± 16.3	97.3 ± 21.0

Bronchoconstriction induced by histamine, acetylcholine, bombesin, Paf and vagal stimulation before and after treatment with B4881. Results are expressed as mean ± s.e.mean of % increase in pulmonary inflation pressure.

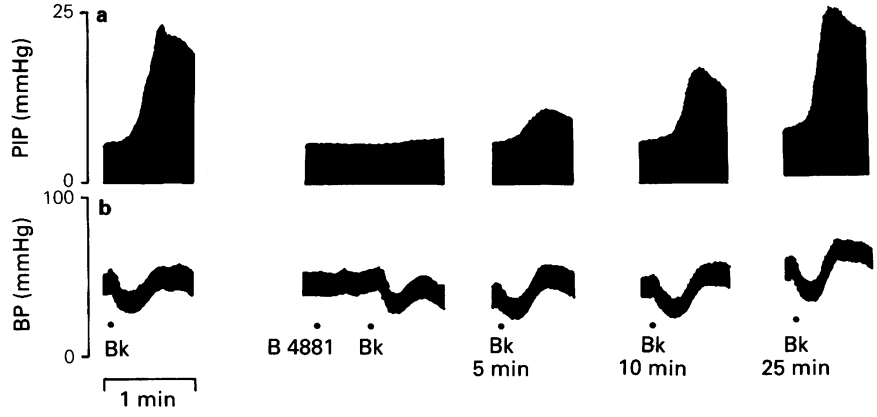


Figure 2 Representative trace showing the increase in pulmonary inflation pressure (PIP) as an index of bronchoconstriction (a) or blood pressure (BP) (b) in response to i.v. bradykinin (Bk, 1 $\mu g kg^{-1}$) before and at various times after treatment with B4881 (400 $\mu g kg^{-1}$). PIP and BP are expressed in mmHg.

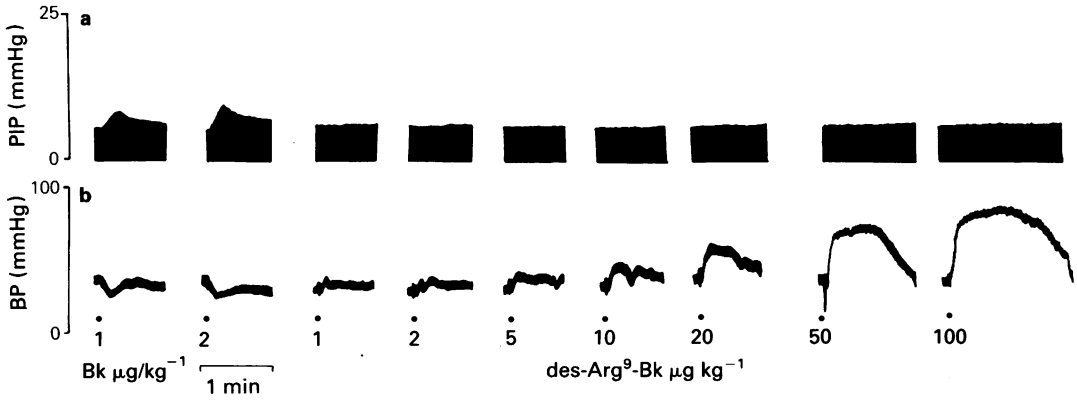


Figure 3 Representative trace showing the increase in pulmonary inflation pressure (PIP) as an index of bronchoconstriction (a) or blood pressure (BP) (b) in response to i.v. bradykinin (Bk 1 $\mu g kg^{-1}$, i.v.). PIP and BP are expressed in mmHg. Responses to i.v. des-Arg^9-Bk are shown for comparison.

400 $\mu g kg^{-1}$ i.v., a dose sufficient to abolish completely bradykinin-induced bronchoconstriction (Table 2). Moreover, bronchoconstriction induced by electrical stimulation of the vagus was not attenuated by prior treatment with B4881 (400 $\mu g kg^{-1}$, i.v.) (Table 2).

Discussion

There is now substantial evidence indicating that bradykinin will induce a wide array of biological activities including bronchoconstriction and that there are two distinct bradykinin receptor populations, namely B_1 and B_2 (Regoli & Barabe, 1980). Our results demonstrate that bradykinin-induced bronchoconstriction in the anaesthetized guinea-pig can be abolished by pretreatment with the selective B_2 antagonist B4881. In contrast, the suggested B_1 agonist des-Arg^9-Bk (Regoli & Barabe, 1980) had no significant effect on bradykinin-induced bronchoconstriction. These results taken together suggest that the effect of bradykinin on airway smooth muscle is predominantly via the activation of the B_2 rather than via the B_1 receptor population. Whilst we were not formally analysing blood pressure it was noted that the B_1 agonist had a marked effect on systemic blood pressure indicating that this molecule had biological activity in our preparations. Furthermore, the B_2 antagonist did not appear to attenuate the blood pressure fall induced by i.v. bradykinin which may suggest that the activity of bradykinin on the vasculature is via the B_1 receptor.

B4881 has been described elsewhere as a selective B_2 antagonist but to date such observations have not been made in the lung. In the present study, however, B4881 had no significant effect on bronchoconstriction induced by other spasmogens, either acting on peptide receptors (bombesin), or not (histamine, acetylcholine and Paf). These results suggest that B4881 does not possess significant antagonistic activity for histamine (H_1), muscarinic or Paf receptors. Although the mechanisms underlying histamine and acetylcholine-induced bronchoconstriction are well documented, the precise mechanism of Paf and bombesin-induced bronchoconstriction are less well described. However, the present results suggest that neither Paf nor bombesin induced bronchoconstriction is via the activation of B_2 receptors. Furthermore, B4881 had no significant effect on bronchoconstriction induced by electrical stimulation of the vagus suggesting that bradykinin is not involved in this phenomenon. The present results suggest that B4881 is a selective B_2 antagonist in the guinea-pig lung and it is hoped that this compound will provide a useful tool in assessing the suggested contribution of bradykinin to respiratory diseases (Proud *et al.*, 1988).

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Inhibition of histamine-stimulated inositol phospholipid hydrolysis by agents which increase cyclic AMP levels in bovine tracheal smooth muscle

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1 The effect on histamine-stimulated [^{3}H]-inositol phosphate accumulation of a range of agents which increase the accumulation, or mimic the actions, of cyclic AMP has been investigated in bovine tracheal smooth muscle.

2 Salbutamol (1 μM), forskolin (1 μM) and vasoactive intestinal peptide (VIP, 1 μM) inhibited the inositol phosphate response to 0.1 mM histamine and increased the accumulation of [^{3}H]-cyclic AMP in [^{3}H]-adenine-labelled slices of bovine tracheal smooth muscle. The effect on inositol phospholipid hydrolysis was mimicked by the membrane permeant analogues of cyclic AMP, dibutryl-cyclic AMP (1 mM) and 8-bromo-cyclic AMP (1 mM).

3 In contrast to salbutamol, which was equally effective at producing the two effects, forskolin produced large increases in [^{3}H]-cyclic AMP accumulation (EC_{50} = 1.2 μM) at much higher concentrations than those required for inhibition of histamine-stimulated [^{3}H]-inositol phosphate accumulation (EC_{50} = 0.09 μM). However, significant increases in [^{3}H]-cyclic AMP accumulation, of similar magnitude to those obtained with salbutamol and VIP, were observed over the concentration range appropriate for inhibition of the inositol phosphate response to histamine.

4 In the presence of histamine (0.1 mM), isobutylmethylxanthine (IBMX, 1 mM) and rolipram (0.1 mM) both significantly ($P < 0.05$) elevated tissue [^{3}H]-cyclic AMP levels. IBMX, rolipram and (to a lesser extent) SKF 94120 significantly ($P < 0.05$) reduced histamine-stimulated [^{3}H]-inositol phosphate accumulation by 81%, 68% and 20%, respectively. M&B 22948 was without a significant effect on either [^{3}H]-cyclic AMP or histamine-induced [^{3}H]-inositol phosphate accumulation.

5 Both rolipram and forskolin reduced the increase in incorporation of [^{3}H]-inositol into membrane phospholipids which followed stimulation with histamine. However, a significant inhibition of [^{3}H]-inositol phosphate accumulation could be demonstrated under conditions in which there was no change in the level of [^{3}H]-inositol incorporation.

Introduction

Histamine H_1-receptor stimulation in tracheal smooth muscle produces a contractile response which appears to be initiated via the mobilization of calcium from intracellular stores (Takuwa *et al.*, 1987; Kotlikoff *et al.*, 1987) and maintained by the activation of protein kinase C (Park & Rasmussen, 1985; Dale & Obianime, 1985; Takuwa *et al.*, 1987). Studies in canine tracheal smooth muscle (Hashimoto *et al.*, 1985) and other tissues (Berridge, 1987) have shown that inositol-1,4,5-trisphosphate, which is produced following agonist-stimulated inositol phospholipid hydrolysis, can mobilise calcium ions from intracellular stores whilst 1,2-diacylglycerol, another product of inositol phospholipid hydrolysis, has been shown to activate protein kinase C (Nishizuka, 1986; Berridge, 1987).

Histamine H_1-receptor-activation in bovine tracheal smooth muscle stimulates the accumulation of [^{3}H]-inositol phosphates following prelabelling of tracheal slices with [^{3}H]-myo-inositol (Barnes *et al.*, 1986; Hall & Hill, 1988). β_2-Adrenoceptor agonists are potent relaxants of airway smooth muscle (Barnes, 1986a) and we have shown previously that β_2-adrenoceptor stimulation can markedly inhibit the hydrolysis of inositol phospholipids elicited by histamine in bovine tracheal smooth muscle (Hall & Hill, 1988). It is thought that the relaxant effects of β_2-agonists on airway smooth muscle tone are

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related to their stimulating effects on adenylate cyclase activity leading to an increase in the intracellular levels of cyclic AMP (Rinard *et al.*, 1983; Barnes, 1986a; Fujiwara *et al.*, 1988) and subsequent activation of the adenosine 3':5'-cyclic monophosphate (cyclic AMP)-dependent protein kinase A (Torphy *et al.*, 1982). The present study was therefore undertaken to determine whether the inhibitory effects of β-agonists on histamine-stimulated inositol phospholipid hydrolysis are mediated via the production and action of cyclic AMP. We now demonstrate that a number of agents, which either increase cyclic AMP accumulation or mimic the actions of cyclic AMP in bovine tracheal smooth muscle, also inhibit the histamine-induced accumulation of [^{3}H]-inositol phosphates in this tissue. A preliminary account of this work has been presented to the British Pharmacological Society (Hall *et al.*, 1988).

Methods

Accumulation of [^{3}H]-inositol phosphates

The accumulation of [^{3}H]-inositol phosphates in slices (300 × 300 μm) of bovine tracheal smooth muscle was measured as described by Hall & Hill (1988). Briefly, washed slices were labelled in a minimal volume of Krebs-Henseleit buffer containing 30 μCi [^{3}H]-myo-inositol (final concentration 0.4 μM, total volume 8 ml) for 75 min at 37°C under an atmosphere of 95% O_2/5% CO_2. The slices were then washed in Krebs-Henseleit buffer containing 5 mM lithium chloride and resuspended in 8 ml of medium. One hundred μl aliquots of the slice suspension were then transferred to flat-bottomed insert vials containing Krebs-Henseleit buffer and 5 mM LiCl (final volume 300 μl). The vials were gassed with 95% O_2/5% CO_2, capped and incubated for 20 min at 37°C in the presence or absence of phosphodiesterase inhibitors. Agonists or cyclic AMP analogues were finally added in 10 μl of medium and the incubation terminated after 45 min by the addition of 100 μl of ice-cold perchloric acid (10% w/v). Samples were neutralised with 0.75 ml KOH (0.15 M), centrifuged (3000 *g*, 10 min, 4°C) and 0.75 ml aliquots of the supernatant were diluted to 3 ml with 50 mM Tris buffer (pH 7.0). Total [^{3}H]-inositol phosphates were finally separated from free [^{3}H]-myo-inositol by anion-exchange chromatography (Hill & Kendall, 1987).

For measurement of the incorporation of [^{3}H]-inositol into inositol-containing phospholipids, 1.2 ml chloroform/methanol/10 M HCl (100 : 200 : 1 v/v/v) was added to each sample after removal of the supernatant as described above. The tubes were vortexed and left to stand for 30 min. Chloroform (0.4 ml) and water (0.4 ml) were then added and the tubes centrifuged at *circa* 1500 *g* for 5 min to separate the phases. A portion (200 μl) of the chloroform phase was then removed into a scintillation vial and dried overnight. Tritium was determined by liquid scintillation counting. In some experiments histamine (0.1 mM) was added during the last 45 min of the 75 min labelling period with [^{3}H]-inositol in order to stimulate incorporation. The slices were then washed with histamine-free Krebs medium and the experiment continued as described above for measurement of inositol phosphate accumulation.

Accumulation of [^{3}H]-cyclic AMP

Cyclic AMP production was measured essentially as described previously for brain slices (Donaldson *et al.*, 1988a). Washed slices (300 × 300 μm) of bovine tracheal smooth muscle were incubated for 40 min at 37°C in 20 ml Krebs-Henseleit medium containing 0.08 μM (40 μCi) [^{3}H]-adenine under an atmosphere of 95% O_2/5% CO_2. The prelabelled slices were then washed with Krebs solution and resuspended in 8 ml of medium. Aliquots (100 μl) of the slice suspension were then added to insert vials containing Krebs medium and where appropriate phosphodiesterase inhibitors (final volume 300 μl) and incubated for 20 min as described above. Agonists were finally added in 10 μl of medium and the incubations terminated after 10 min by the addition of 200 μl of 1 M HCl. Samples were left on ice for at least 5 min before being diluted with 0.75 ml distilled water. Slices were precipitated by centrifugation at 1500 *g* for 10 min and 1 ml samples of the aqueous supernatant were taken for analysis of [^{3}H]-cyclic AMP by column chromatography as described by Donaldson *et al.* (1988b). An additional 100 μl sample of the aqueous supernatant was taken to determine the total radioactivity present in each sample. These 'totals' were used to correct for variations in the numbers of slices in each sample.

In studies of the time course of [^{3}H]-cyclic AMP accumulation, slices (3 ml of slice suspension) which had been prelabelled with [^{3}H]-adenine were transferred to a 20 ml glass vial containing 6 ml Krebs-Henseleit solution and where appropriate 1 mM 3-isobutyl-1-methylxanthine (IBMX). The slices and incubation medium were stirred magnetically at a rate sufficient to buoy up the slices and were gassed with 95% O_2/5% CO_2 via a glass Pasteur pipette as described previously (Donaldson *et al.*, 1988b). Following agonist addition, 300 μl aliquots of the slice-containing incubation medium were taken at appropriate times and added to tubes containing 200 μl of ice-cold M HCl. Samples were then processed as described above.

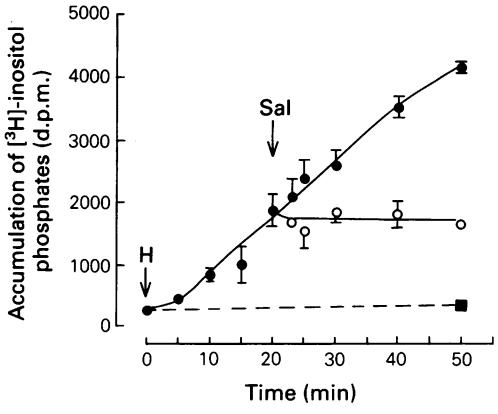


Figure 1 The effect of salbutamol on the inositol phosphate response to 0.1 mM histamine in slices of bovine tracheal smooth muscle pre-labelled with [^{3}H]-myo-inositol. Histamine (H) was added at time zero and 1 μM salbutamol (Sal) (○) or 10 μl Krebs medium (●) was added 20 min later. The basal accumulation of [^{3}H]-inositol phosphates after 50 min in the absence of histamine is also shown (■). Each data point represents the mean of triplicate determinations and vertical lines indicate s.e.mean. If not shown the s.e.mean lies within the symbol. The experiment was repeated twice more with similar results.

Data analysis

Statistical analysis of the effect of various agents on inositol phospholipid hydrolysis was normally performed by both the Wilcoxon signed rank test and the paired *t* test. Dose-response curves were drawn by inspection and, where appropriate, EC_{50} values were compared by unpaired *t* test. All values in the text of the results section represent mean ± s.e.mean of *n* separate experiments.

Chemicals

Dowex 50 W, H^+-form (200–400 mesh), Dowex-1 (X8, 100–200 mesh, chloride form) neutral alumina (type WN-3), imidazole, IBMX, salbutamol, N^6,2′-O-dibutyryl cyclic AMP, 8-bromo-cyclic AMP and forskolin were obtained from Sigma. Histamine dihydrochloride was purchased from BDH and vasoactive intestinal peptide (VIP) from Peninsular Laboratories. 8-[^{3}H]-adenine (specific activity 26 Ci mmol^{-1}) was obtained from Amersham and [^{3}H]-myo-inositol (16.5 Ci mmol^{-1}) and 8-[^{14}C]-cyclic AMP (specific activity 42.4 mCi mmol^{-1}) from N.E.N. Gifts of rolipram (Schering AG), zaprinast (M&B 22948, May and Baker) and SKF 94120 ({5-(4-acetamido-phenyl)pyrazin-2[1H]-one}, Smith, Kline and French) are gratefully acknowledged.

Results

Effect of salbutamol on inositol phosphate and cyclic AMP accumulation

We have shown previously that addition of salbutamol markedly reduces the inositol phosphate response to histamine, when the two agonists are added simultaneously (Hall & Hill, 1988). In the present study, addition of 1 μM salbutamol 20 min after 0.1 mM histamine produced a significant and rapid reduction in the accumulation of [^{3}H]-inositol phosphates elicited by histamine alone (Figure 1). This effect of salbutamol in bovine tracheal smooth muscle slices was accompanied by a significant ($P < 0.05$) 3.2 ± 0.3 fold ($n = 3$) stimulation of the accumulation of [^{3}H]-cyclic AMP (Figure 2) which reached a steady level within 5 min and was maintained for at least a further 40 min (Figure 3). Histamine (0.1 mM) produced only a small effect (1.52 ± 0.11 fold, basal = 1.0, $n = 19$) on cyclic AMP levels in this tissue. There was no significant difference between the EC_{50} values for salbutamol-stimulated cyclic AMP accumulation when measured in the presence ($EC_{50} = 3.5 \pm 1.3 \times 10^{-7}$ M, $n = 3$) or absence ($EC_{50} = 2.0 \pm 0.6 \times 10^{-7}$ M, $n = 3$) of 0.1 mM histamine. These values were in excellent agreement with the EC_{50} value (2.9×10^{-7} M) obtained previously for salbutamol from inhibition of histamine-stimulated [^{3}H]-inositol phosphate accumulation (Hall & Hill, 1988). [^{3}H]-cyclic AMP levels rose rapidly following stimulation of β_2-adrenoceptors with salbutamol (1 μM) in the presence or absence of the phosphodiesterase inhibitor IBMX (1 mM, Figure 3). The large effect of IBMX on the size of the cyclic AMP response to salbutamol suggests that phosphodiesterase enzymes are very active in this tissue. The half-times ($t_{1/2}$) for the rise in cyclic AMP levels elicited by salbutamol (1 μM) were similar in the presence (0.72 ± 0.11 min) and absence (0.63 ± 0.09 min, $n = 3$) of IBMX.

Effect of other cyclic AMP stimulants and analogues.

The rapid nature of the stimulation of cyclic AMP accumulation produced by salbutamol and the similarity in the dose-response characteristics for the effect of this β-agonist on cyclic AMP and inositol phosphate accumulation prompted us to examine the effect of other agents, known to stimulate the formation or mimic the actions of cyclic AMP in this tissue, on histamine-stimulated [^{3}H]-inositol phosphate accumulation. Vasoactive intestinal polypeptide (1 μM, VIP), forskolin (1 μM) and two analogues of cyclic AMP (8-bromo-cyclic AMP and dibutyryl-cyclic AMP, both at 1 mM) all significantly attenuated the inositol phosphate response to 0.1 mM

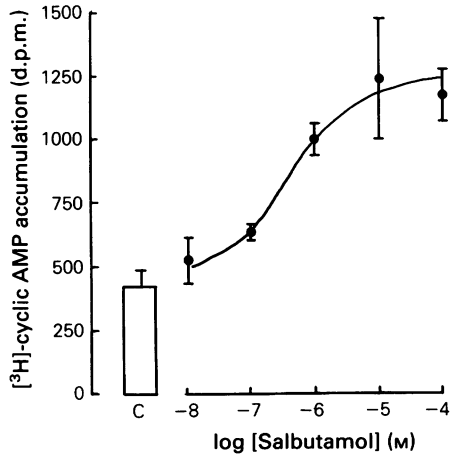


Figure 2 The effect of increasing concentrations of salbutamol on the accumulation of [^{3}H]-cyclic AMP in [^{3}H]-adenine-labelled slices of bovine tracheal smooth muscle. The basal accumulation of [^{3}H]-cyclic AMP in the absence of salbutamol is given by the column marked C. Values represent the mean of triplicate determinations and vertical lines show s.e.mean. Similar data were obtained in two other experiments.

histamine (Figure 4 and Table 1). Parallel studies in [^{3}H]-adenine-labelled slices confirmed that both 1 μM VIP and 1 μM forskolin elevated tissue levels of [^{3}H]-cyclic AMP in the presence or absence of 0.1 mM histamine (Figure 5). Studies of the time course of the cyclic AMP response to 1 μM forskolin in the presence of 0.1 mM histamine in two experiments showed that it was essentially similar to that found for salbutamol (Figure 3). A maximum

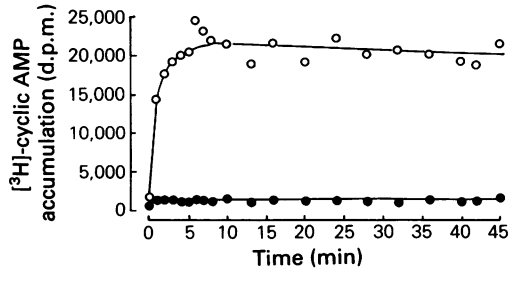


Figure 3 Time course of the accumulation of [^{3}H]-cyclic AMP in bovine tracheal smooth muscle after the addition of 1 μM salbutamol in the presence (○) or absence (●) of 1 mM isobutyl methylxanthine (IBMX). Where appropriate (○) slices were incubated for 20 min in the presence of IBMX before agonist administration. Data were obtained in a single experiment. Similar results were obtained in two other experiments.

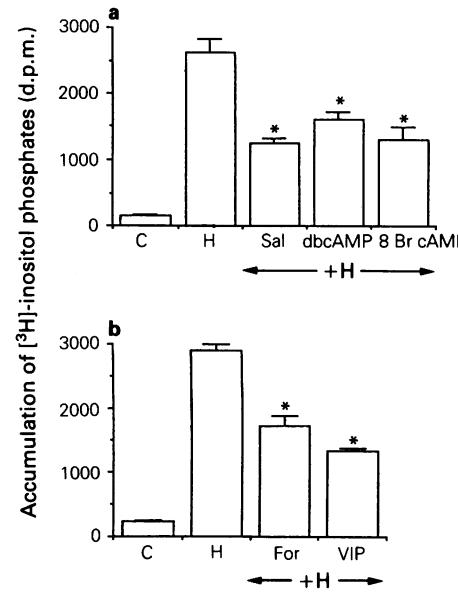


Figure 4 Inhibition of histamine-induced [^{3}H]-inositol phosphate accumulation by agents which raise, or mimic the actions of, cyclic AMP. Histograms show the basal (C) and histamine-stimulated (H, 0.1 mM) levels of [^{3}H]-inositol phosphates. (a) Shows the inhibition of the response to 0.1 mM histamine produced by 1 μM salbutamol (Sal), 1 mM dibutyryl-cyclic AMP (dbcAMP) and 1 mM 8-bromo-cyclic AMP (8BrcAMP). (b) Shows the inositol phosphate responses obtained with 1 μM forskolin (For) and 1 μM vasoactive intestinal peptide (VIP) in the presence of 0.1 mM histamine. Values represent mean (±s.e. mean, shown by bars) of triplicate determinations. *Values significantly different from the response to histamine alone ($P < 0.05$, ANOVAR). Similar data were obtained for each agent in 3–14 other experiments (see Table 1).

response was obtained within 6 min and cyclic AMP levels remained elevated for 45 min. However, it was notable that, although 1 μM forskolin produced an inhibition of histamine-stimulated [^{3}H]-inositol

Table 1 Inhibition of histamine-induced [^{3}H]-inositol phosphate accumulation in bovine tracheal smooth muscle

Agent	*% inhibition*	(n)
Salbutamol (1 μM)	66 ± 3	(15)
Dibutyryl-cyclic AMP (1 mM)	33 ± 7	(6)
8-Bromo-cyclic AMP (1 mM)	49 ± 6	(6)
Forskolin (1 μM)	48 ± 6	(8)
VIP (1 μM)	60 ± 6	(4)

Values represent mean ± s.e.mean. The number of separate experiments with each agent is given in parentheses. VIP = vasoactive intestinal peptide.

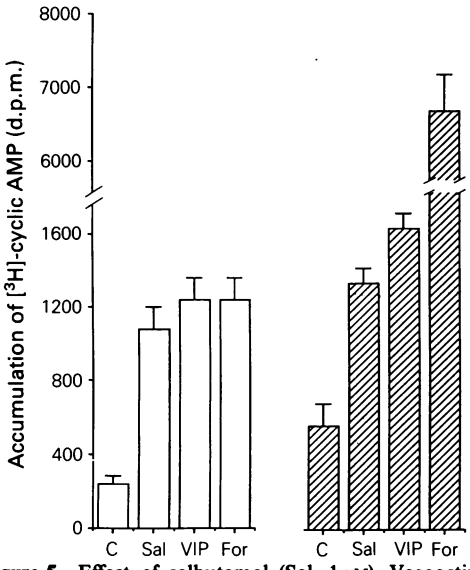


Figure 5 Effect of salbutamol (Sal, 1 μM), Vasoactive intestinal peptide (VIP, 1 μM) and forskolin (For, 1 μM) on the accumulation of [^{3}H]-cyclic AMP in the presence (hatched columns) or absence (open columns) of 0.1 mM histamine. The basal accumulations of [^{3}H]-cyclic AMP in the presence (hatched column) or absence (open column) of 0.1 mM histamine is shown by the columns marked C. Values represent the means of triplicate determinations. Vertical lines indicate s.e. mean. Similar results were obtained in two further experiments.

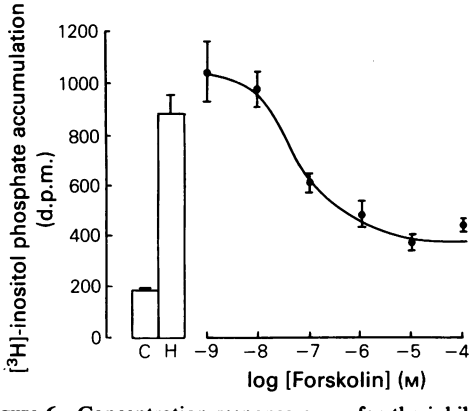


Figure 6 Concentration-response curve for the inhibition of histamine-stimulated [^{3}H]-inositol phosphate accumulation by the diterpine forskolin. The basal and control histamine (0.1 mM) responses are shown by columns C and H, respectively. Where appropriate forskolin and histamine (0.1 mM) were added simultaneously. Data represent the mean of triplicate determinations in a single experiment and vertical lines indicate s.e.mean. Each experiment was repeated twice more with similar results.

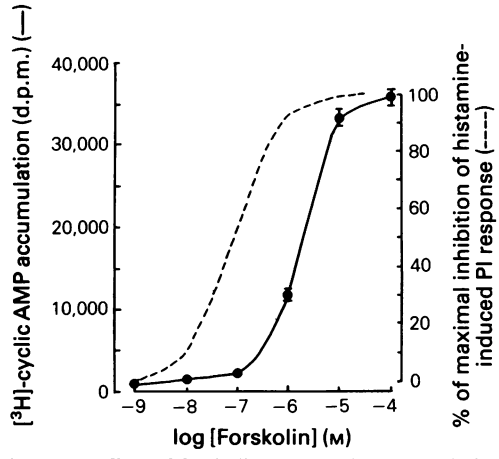


Figure 7 Effect of forskolin (●) on the accumulation of [^{3}H]-cyclic AMP in [^{3}H]-adenine-labelled slices of bovine tracheal smooth muscle. All incubations contained 0.1 mM histamine. Data represent the mean of triplicate determinations in a single experiment. Vertical lines show s.e. mean. If not shown the s.e. mean lies within the symbol. The basal accumulation of [^{3}H]-cyclic AMP in this experiment was 433 ± 50 d.p.m. and the levels obtained with 1, 10 and 100 nM forskolin (in the presence of 0.1 mM histamine) were 752 ± 46, 1047 ± 117 and 2130 ± 154 d.p.m., respectively. Similar data were obtained in two further experiments. The dashed line shows the calculated mass-action curve (0 to 100% maximal response, right ordinate scale) based on the mean EC_{50} (0.09 ± 0.01 μM) obtained for forskolin-mediated inhibition of histamine-stimulated [^{3}H]-inositol phosphate accumulation

phosphate accumulation of similar magnitude to that obtained with 1 μM salbutamol (Table 1), there was a large difference in the relative sizes of the cyclic AMP responses obtained with these agents in the presence of histamine (Figure 5).

Analysis of the concentration-response characteristics of the inhibitory effect of forskolin on histamine-stimulated [^{3}H]-inositol phosphate accumulation indicated that the EC_{50} for forskolin was 0.09 ± 0.01 μM (n = 3, Figure 6). This value was an order of magnitude lower than the EC_{50} value (1.2 ± 0.3 μM, n = 3) obtained for forskolin-mediated stimulation of [^{3}H]-cyclic AMP accumulation in the presence of histamine (0.1 mM) (Figure 7). It should be noted, however, that although there was a very large maximal stimulation of cyclic AMP accumulation produced by forskolin in the presence of histamine (Figure 7), significant accumulations of [^{3}H]-cyclic AMP over basal levels (see legend to Figure 7) were still achieved over the forskolin concentration range appropriate for inhibition of inositol phospholipid hydrolysis (1, 10 and 100 nM, respectively).

Effect of phosphodiesterase inhibitors

An alternative way of elevating tissue cyclic AMP levels in bovine tracheal smooth muscle is to inhibit the breakdown of this cyclic nucleotide with phosphodiesterase (PDE) inhibitors. In this study we have investigated the effect on cyclic AMP and inositol phosphate accumulation of the isozyme-selective PDE inhibitors rolipram (an inhibitor of the cyclic AMP-specific PDE IV, Reeves *et al.*, 1987), SKF 94120 (an inhibitor of the other cyclic AMP selective isozyme PDE III, Reeves *et al.*, 1987) and M & B 22948 (which inhibits calcium-calmodulin stimulated PDE I activity and the cyclic GMP selective isoform PDE II, Frossard *et al.*, 1981; Weishaar *et al.*, 1986), and the non-selective PDE inhibitor IBMX. The concentrations chosen were those that would produce the largest effect on the target PDE-isoform without influencing the activity of the other PDE isoenzymes (Stanley *et al.*, 1989). In the absence of 0.1 mM histamine, only IBMX produced a significant elevation of [^{3}H]-cyclic AMP over basal levels (Table 2), while in the presence of histamine both IBMX and rolipram significantly increased cyclic AMP levels over control and histamine-stimulated basal levels (Table 2). IBMX, rolipram and to a lesser extent SKF 94120 significantly reduced the accumulation of [^{3}H]-inositol phosphates elicited by histamine in bovine tracheal slices (Figure 8). The mean percentage inhibitions obtained with these PDE inhibitors were for IBMX (1 mM) 81 ± 6% ($P < 0.001$, paired t and Wilcoxon signed rank tests, 8 experiments); rolipram (0.1 mM) 68 ± 9% ($P < 0.001$, 7 experiments) and SKF 94120 (0.1 mM) 20 ± 5% ($P < 0.05$, 4 experiments). M & B 22948 (0.1 mM) was without significant effect on the histamine-induced inositol phosphate response ($n = 4$).

Table 2 The effect of phosphodiesterase (PDE) inhibitors on the accumulation of [^{3}H]-cyclic AMP in [^{3}H]-adenine-labelled slices of bovine tracheal smooth muscle

Agent	[^{3}H]-*cyclic AMP accumulation*	
	Basal	*+Histamine*
Control	1.0	1.5 ± 0.2
IBMX (1 mM)	4.0 ± 0.6*	8.6 ± 1.2*
Rolipram (0.1 mM)	1.6 ± 0.1	3.1 ± 0.5*
SKF 94120 (0.1 mM)	1.4 ± 0.1	1.9 ± 0.2
M & B 22948 (0.1 mM)	1.0 ± 0.2	1.8 ± 0.3

Values represent mean ± approximate s.e.mean of the accumulation of [^{3}H]-cyclic AMP, expressed relative to the basal accumulation (=1), in four identical experiments in which individual measurements were made in quadruplicate.
*$P < 0.05$ with respect to the basal accumulation (2-way analysis of variance with Newman Keuls *post hoc* test). IBMX = isobutyl methylxanthine

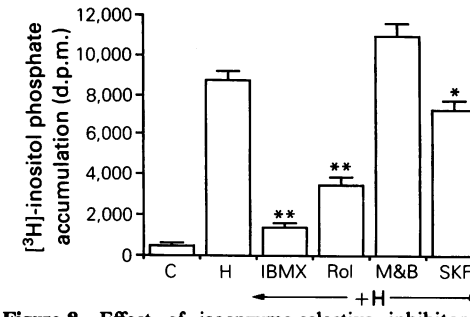


Figure 8 Effect of isoenzyme-selective inhibitors of phosphodiesterase (PDE) on histamine-stimulated [^{3}H]-inositol phosphate accumulation in bovine tracheal smooth muscle. Columns show the basal (C) and histamine-stimulated (H, 0.1 mM) levels, together with the effects of 1 mM isobutyl methylxanthine (IBMX), 0.1 mM rolipram (Rol), M & B 22948 (M & B) and SKF 94120 (SKF) measured in the presence of 0.1 mM histamine. PDE inhibitors were added 20 min before the administration of histamine. Data represent the means of triplicate determinations in a single experiment and vertical lines show s.e.mean. Similar results were obtained with each PDE inhibitor in 3–7 other experiments. *$P < 0.05$, **$P < 0.01$, with respect to the response to 0.1 mM histamine alone (ANOVAR)

Effects on the incorporation of [^{3}H]-inositol

A striking characteristic of the effect of agonists on inositol phospholipid hydrolysis in bovine tracheal smooth muscle is that the increase in accumulation of [^{3}H]-inositol phosphates is accompanied by a stimulation of the incorporation of [^{3}H]-inositol into membrane inositol phospholipids (Figure 9; Chilvers *et al.*, 1988; Hall & Hill, 1988). We have shown previously that although under normal conditions the stimulation of [^{3}H]-inositol incorporation by histamine can be inhibited by salbutamol, a marked inhibition of [^{3}H]-inositol phosphate accumulation by salbutamol can still be demonstrated under conditions in which there is no change in the level of [^{3}H]-inositol incorporation (i.e. when histamine is used to stimulate maximally incorporation during the prelabelling period, Hall & Hill, 1988). In the present study, under normal conditions, both rolipram (0.1 mM) and forskolin (Figure 9) produced a significant inhibition of the histamine-stimulated [^{3}H]-inositol incorporation. The mean EC_{50} value obtained for forskolin for this effect over three experiments (0.06 ± 0.05 μM) was similar to that

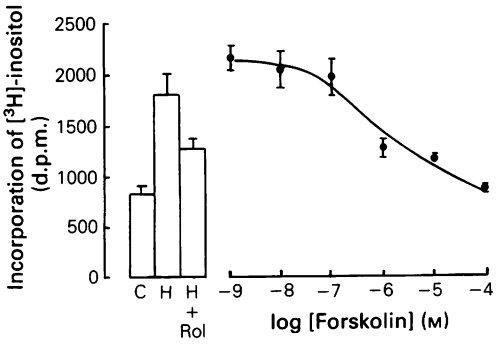


Figure 9 The effect of forskolin on the histamine-stimulated incorporation of [^{3}H]-inositol into inositol-containing phospholipids in slices of bovine tracheal smooth muscle. Columns show the incorporation under basal (C) conditions and following stimulation with 0.1 mM histamine in the absence (H) or presence of 0.1 mM rolipram (H + Rol). The concentration-response curve for forskolin (●) was obtained in the presence of histamine (0.1 mM). Values represent the means of triplicate determinations in a single experiment and vertical lines show s.e.mean. Similar data were obtained in two other experiments.

obtained when [^{3}H]-inositol phosphate accumulation was measured (Figure 6). However, the incorporation of [^{3}H]-inositol obtained in the presence of 0.1 mM histamine in three experiments (172 ± 15% of basal) was not significantly altered by 0.1 mM SKF 94120 (167 ± 30% of basal).

When histamine (0.1 mM) was present during the final 45 min of the prelabelling period with [^{3}H]-inositol, there was no further stimulation by histamine of [^{3}H]-inositol incorporation during the experimental period (Figure 10). Under these conditions, the initial lag period normally observed in the time course for histamine-stimulated [^{3}H]-inositol phosphate (Figure 1) was abolished (Figure 10). Furthermore, as observed previously with β-agonists, a significant attenuation of the histamine-stimulated [^{3}H]-inositol phosphate response by rolipram could be demonstrated under conditions in which rolipram did not interfere with the incorporation of [^{3}H]-inositol into membrane phospholipids (Figure 10).

Discussion

The present study confirms our previous observation (Hall & Hill, 1988), that β_2-agonist stimulation can markedly attenuate the accumulation of [^{3}H]-inositol phosphates elicited by histamine in slices of bovine tracheal smooth muscle, and indicates that salbutamol can produce a rapid effect even when added 20 min after the addition of histamine. The time course of this effect on inositol phosphate accumulation is similar to the rapid relaxation produced by β-agonists in bovine and canine tracheal smooth muscle strips which have been previously contracted by smooth muscle spasmogens (Fujiwara *et al.*, 1988; Takuwa *et al.* 1988). This raises the possibility that inhibition of histamine-stimulated inositol phospholipid hydrolysis by β_2-agonists may contribute to the relaxation of pre-contracted (with histamine) airway smooth muscle by preventing the formation of either the calcium-mobilising inositol-1,4,5-trisphosphate or 1,2-diacylgycerol, which appears to be responsible for the maintenance of the contractile state via the activation of protein kinase C (Park & Rasmussen, 1985; Dale & Obianime, 1985; Takuwa *et al.*, 1987).

The effect of salbutamol on inositol phospholipid hydrolysis was paralleled by an increase in the accumulation of [^{3}H]-cyclic AMP in [^{3}H]-adenine-labelled slices of bovine tracheal smooth muscle. The rapid nature of the cyclic AMP response to salbutamol ($t_{1/2}$ = 0.5 min), and the close similarity in the EC_{50} values for the two separate second messenger responses, suggested that the inhibition of histamine-stimulated inositol phosphate accumulation produced by salbutamol might be secondary to the change in the intracellular levels of the cyclic nucleotide. This hypothesis was supported by the fact that both the diterpine forskolin (a direct activator of adenylate cyclase, Seamon *et al.*, 1981) and VIP (which raises cyclic AMP levels in airway smooth muscle via an interaction with specific VIP receptors; Barnes, 1986b) increased [^{3}H]-cyclic AMP levels and inhibited histamine-stimulated [^{3}H]-inositol phosphate accumulation in this tissue. In addition two membrane permeant analogues of cyclic AMP, dibutyryl-cyclic AMP and 8-bromo-cyclic AMP, were also able to inhibit significantly the histamine-stimulated inositol phosphate response.

A notable feature of the data obtained with forskolin was that the cyclic AMP response obtained in the presence of 0.1 mM histamine was substantially greater than that obtained with the diterpine alone. No similar effect was seen with either salbutamol or VIP suggesting that forskolin, as in other tissues (Daly *et al.*, 1982; Barovsky *et al.*, 1984), raises intracellular cyclic AMP levels in airway smooth muscle by two mechanisms: (1) by a direct activation of adenylate cyclase and (2) by a potentiation of receptor-stimulated cyclic AMP accumulation. However, the large difference in maximal cyclic AMP responses observed in the presence of histamine between forskolin and other agonists which elevate cyclic AMP levels in tracheal slices, under conditions in which their relative effects on the

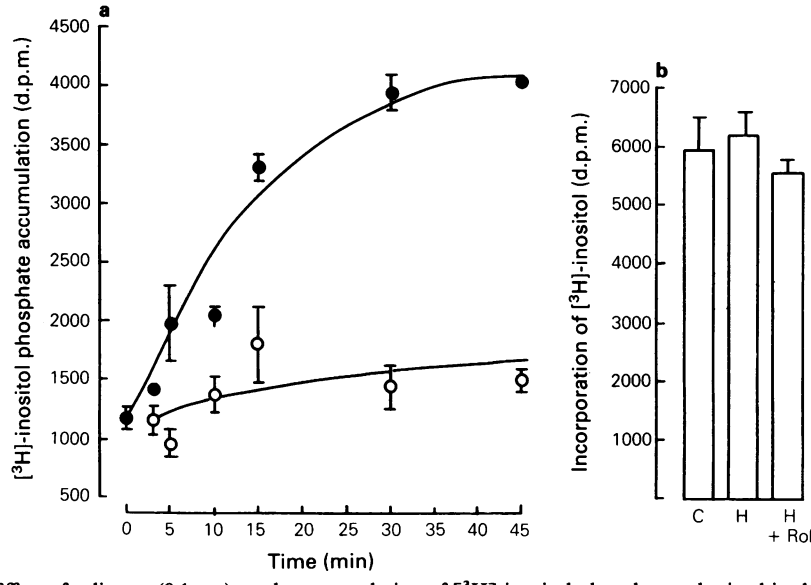


Figure 10 Effect of rolipram (0.1 mM) on the accumulation of [^{3}H]-inositol phosphates obtained in the presence of 0.1 mM histamine under conditions in which the incorporation of [^{3}H]-inositol is not affected. (a) Histamine-stimulated accumulation [^{3}H]-inositol phosphates in the presence (○) or absence (●) of 0.1 mM rolipram. (b) Incorporation of [^{3}H]-inositol into inositol phospholipids under basal conditions (45 min incubation, C) and following 45 min stimulation with 0.1 mM histamine in the absence (H) and presence (H + Rol) of 0.1 mM rolipram. Values represent mean of triplicate determinations in a single experiment and vertical lines show s.e.mean. If not shown s.e.mean lies within the symbol. Similar data were obtained in two further experiments.

histamine-induced inositol phosphate response are similar, does require explanation if cyclic AMP is proposed to mediate this latter response.

The simplest explanation is that the high levels of cyclic AMP achieved in the presence of a combination of histamine and forskolin (at concentrations of the diterpine above 1 μM) are supramaximal, and that the maximal inhibitory effect on inositol phospholipid hydrolysis is achieved at much lower cyclic AMP levels. If this is the case then one would predict that the concentration-response curve, obtained from measurement of cyclic AMP accumulation, for forskolin should lie to the right of the curve obtained for inositol phosphate accumulation. Consistent with this hypothesis, the EC_{50} values obtained for forskolin, but not salbutamol, on cyclic AMP accumulation (in the presence of 0.1 mM histamine) were one order of magnitude higher than the corresponding values obtained from measurements of inositol phosphate accumulation (Figure 7). Furthermore, the size of the increases in cyclic AMP obtained with forskolin (in the presence of histamine), over the concentration range appropriate for effects on inositol phosphate accumulation, were similar to those obtained with both VIP and salbutamol under similar conditions.

Cyclic AMP levels can also be raised in airway smooth muscle by inhibiting the PDE enzymes which break down cyclic AMP. The large extent to which the PDE enzymes are active in this tissue is illustrated by the data in Figure 3 for the time course of the effect of salbutamol on cyclic AMP levels in the presence and absence of IBMX. A new steady level of cyclic AMP is obtained with salbutamol in the presence of IBMX suggesting that, as for rolipram in guinea-pig brain slices (Donaldson *et al.*, 1988a), the inhibition of PDE can be eventually reversed by the higher intracellular levels of cyclic AMP produced in the presence of IBMX.

A number of different PDE isozymes have been described in various tissues which have different substrate specificities and sensitivities to various PDE inhibitors (Davis, 1984; Weishaar *et al.*, 1986; Reeves *et al.*, 1987; Silver *et al.*, 1988). Three PDE activities have been identified in broken cell preparations of tracheal smooth muscle (Torphy *et al.*, 1987; Silver *et al.*, 1988): (1) a calcium-calmodulin stimulated activity (PDE I), which hydrolyses both cyclic AMP and cyclic GMP with similar potencies, and is inhibitable by M & B 22948 (Weishaar *et al.*, 1986; Torphy *et al.*, 1987; Silver *et al.*, 1988); (2) a cyclic AMP-specific activity (PDE III) which is sensitive to

SKF 94120 (Gristwood *et al.*, 1986; Reeves *et al.*, 1987) and (3) a rolipram-sensitive activity, specific for cyclic AMP, with a higher Michaelis constant (K_M) for the substrate cyclic nucleotide (Torphy *et al.*, 1987; Reeves *et al.*, 1987; Silver *et al.*, 1988). Studies with partially purified isozymes suggest that the calcium-sensitive PDE I accounts for all of the cyclic GMP and 85% of the cyclic AMP metabolising activity in homogenates of tracheal smooth muscle (Torphy *et al.*, 1987). These studies also indicate that PDE III and PDE IV account for the remaining 5% and 10% respectively of the cyclic AMP metabolising activity in this tissue.

Studies with isozyme selective inhibitors in the present study, however, indicate that only rolipram and IBMX significantly raise [^{3}H]-cyclic AMP levels in intact tracheal slices incubated in the presence of 0.1 mM histamine. This is consistent with previous results obtained in brain slices (Stanley *et al.*, 1989; Challis & Nicholson, 1988) and suggests that PDE IV, although a minor component of many cells, may still have a major role in metabolising cyclic AMP in intact cells. A lack of effect of the PDE I inhibitor M & B 22948 on cyclic AMP accumulation has also been observed in canine tracheal smooth muscle, where the isoprenaline-induced smooth muscle relaxation is similarly insensitive to M & B 22948 (Torphy *et al.*, 1987). Thus, although PDE I accounts for 85% of cyclic AMP hydrolysis in the broken cell preparation of tracheal smooth muscle, it does not have a major role in intact cells. This suggests that the different PDE isozymes may be distributed within different cellular compartments in tracheal smooth muscle. The difference in the extent of the increases in cyclic AMP accumulation obtained with rolipram and IBMX (which inhibits all PDE isozymes) are difficult to explain given the lack of effect of SKF 94120 and M & B 22948, but they may be related to differences in tissue penetration or due to other properties of IBMX such as adenosine antagonism (Green & Stanberry, 1976) or antagonism of the inhibitory regulatory protein (G_i) of adenylate cyclase (Parsons *et al.*, 1988).

It is clear, however, that those PDE inhibitors which produce significant increases in cyclic AMP accumulation, i.e., rolipram and IBMX but not M & B 22948, produce a marked inhibition of histamine-stimulated [^{3}H]-cyclic AMP accumulation. The similar extent of the effects of rolipram and IBMX on inositol phospholipid hydrolysis, under conditions in which the size of the cyclic AMP responses differs, is also consistent with the hypothesis, put forward to explain the forskolin data above, that the effects of cyclic AMP on [^{3}H]-inositol phosphate accumulation are saturable.

It was notable that a small but significant inhibition of histamine-stimulated inositol phosphate accumulation was observed with SKF 94120 (Figure 8), which had no significant effect on [^{3}H]-cyclic AMP accumulation (Table 2). Small or negligible increases in cyclic AMP accumulation have been obtained with SKF 94120 in other tissues (Gristwood & Owen, 1986), under conditions where it is still possible to demonstrate activation of cyclic AMP-dependent protein kinase A (Murray *et al.*, 1987) and a pharmacological response (i.e. inotropic effect; Gristwood *et al.*, 1986) mediated by intracellular cyclic AMP. Thus, it remains a possibility that the effects of SKF 94120 on inositol phosphate accumulation are mediated via a small rise in cyclic AMP in a minor compartment of tracheal smooth muscle cells. Interestingly, a small effect of SKF 94120 on cyclic AMP accumulation and a potentiation of the isoprenaline-induced relaxation has been observed in canine tracheal smooth muscle strips (Torphy *et al.*, 1987).

A relation between intracellular cyclic AMP and inhibition of agonist-induced inositol phospholipid breakdown has been observed in a number of other tissues, including platelets (Takai *et al.*, 1982; Watson *et al.*, 1984), neutrophils (Della Bianca *et al.*, 1986), gastric mucosal cells (Puurunen *et al.*, 1987) and slices of rat kidney (Neylon & Summers, 1988). Preliminary studies by other groups in canine tracheal smooth muscle have also confirmed our observations that forskolin can inhibit histamine-induced inositol phospholipid hydrolysis (Madison & Brown, 1988; Murray *et al.*, 1988). The site of action of the cyclic AMP is at present unknown but phospholipase C (which hydrolyses phosphatidylinositol-4-5-bisphosphate, PIP_2) and the inositol phospholipid kinases (which convert phosphatidylinositol to phosphatidylinositol-4-phosphate and then to PIP_2) are the most likely sites of action.

In tracheal smooth muscle, histamine stimulates the incorporation of [^{3}H]-inositol into inositol phospholipids in addition to activating phospholipase C. This effect can be reduced by salbutamol (Hall & Hill, 1988), forskolin or rolipram, which suggests that the inositol phospholipid kinases may be the primary target for cyclic AMP. However, it seems very likely that the stimulation by histamine of [^{3}H]-inositol incorporation in airway smooth muscle is due to changes in the specific radioactivity of the inositol phospholipids that follow histamine-stimulated inositol phospholipid breakdown, as a consequence of the low turnover of inositol phospholipids under basal conditions. This means that the cyclic AMP elevating drugs have an effect on [^{3}H]-inositol incorporation which is secondary to their effect on inositol phospholipid breakdown. This suggestion is supported by the fact that inclusion of histamine in the medium during the preincubation period with [^{3}H]-inositol still revealed changes in

[^{3}H]-inositol phosphate accumulation, under conditions in which the incorporation of [^{3}H]-inositol into the phospholipid layer did not change (see Hall & Hill, 1988 and Results above). The exact site of action of cyclic AMP on the inositol phospholipid cycle in tracheal smooth muscle therefore remains to be established.

In summary, the present results show that a variety of agents which can raise (via PDE inhibition, receptor activation or direct activation of adenylate cyclase), or mimic the action of, intracellular cyclic AMP can inhibit histamine-stimulated inositol phospholipid hydrolysis. These findings may be relevant to the mechanisms by which raised intracellular cyclic AMP levels lead to relaxation of airway smooth muscle which has been pre-contracted with histamine.

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Impairment of endothelium-dependent relaxation and changes in levels of cyclic GMP in aorta from streptozotocin-induced diabetic rats

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1 Acetylcholine (ACh)-induced relaxation of aortic strips with endothelium and production of cyclic GMP between streptozotocin-induced diabetic and age-matched control rats were compared.

2 The concentration-response curve for ACh-induced relaxation was shifted to the right in diabetic rats. IC_{50} values for ACh were $4.57 \pm 0.67 \times 10^{-8}$ M and $1.00 \pm 0.87 \times 10^{-7}$ M in aortic strips from age-matched control and diabetic rats, respectively ($n = 6$, $P < 0.05$).

3 Relaxations produced by atrial natriuretic peptide (ANP) in diabetic aortae were similar to those in age-matched vessels.

4 Relaxations produced by sodium nitroprusside (SNP) in diabetic aortae were similar to those in age-matched vessels.

5 Basal levels of cyclic GMP and ACh-induced production of cyclic GMP were significantly decreased in diabetic rats.

6 These results suggest that functional changes in endothelium but not in guanylate cyclase activity in the aorta may occur in diabetes, and, thus, spontaneous and ACh-induced formation of cyclic GMP may be decreased. This decrease in production of cyclic GMP may be responsible for the decreased response of the aorta to the relaxant effect of ACh.

Introduction

It is now well-established that vascular disease is one of the complicating features of diabetes mellitus in man (Christrieb, 1973). It has been suggested that this is in part a consequence of altered sensitivity or reactivity of vascular smooth muscles to neurotransmitters and hormones (Weidmann *et al.*, 1979).

In this respect, the reactivity of vascular smooth muscles to contractile agents in diabetic rats has been extensively studied (Brody & Dixon, 1964; Pfaffman *et al.*, 1982; Scarborough & Carrier, 1984; Agrawal & McNeill, 1987; Head *et al.*, 1987; Kamata *et al.*, 1988a). However, only a few studies have been undertaken to investigate the responses to relaxant agents in diabetic animals.

The discovery that the vascular endothelium plays a vital role in the relaxation of rabbit aorta to acetylcholine (ACh) *in vitro* (Furchgott & Zawadzki, 1980) has led to the realization that many vasodilator agents act through the release of endothelium-derived relaxing factor (EDRF) (Demey & Vanhoutte, 1981). There is general agreement that endothelium-dependent relaxation is associated with increased levels of guanosine 3′ : 5′-cyclic monophosphate (cyclic GMP) in smooth muscle cells (Rapoport & Murad, 1983; Furchgott *et al.*, 1984; Ignarro *et al.*, 1984).

It has been shown, in diabetic rats that the relaxation response of blood vessels to acetylcholine (ACh) is inconsistent: both decreases (Oyama *et al.*, 1986; Durante *et al.*, 1988) and increases (White & Carrier, 1986) in endothelium-dependent relaxation have been obtained. Even though ACh-induced relaxant responses are mediated through production of cyclic GMP, there have been, until now, no data on changes in levels of cyclic GMP in blood vessels in response to ACh.

Therefore, to determine whether or not there is a causal relationship between changes in relaxation and in production of cyclic GMP, basal levels of cyclic GMP and the effect of ACh on the levels of cyclic nucleotides were examined in age-matched control and streptozotocin-induced diabetic rats.

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Methods

Male Wistar rats, 8 weeks old and 200–220 g in weight, received a single injection in the tail vein of streptozotocin (Stz) 60 mg kg^{-1}, dissolved in a citrate buffer. Age-matched control rats were injected with the buffer alone. Food and water were given *ad libitum*. The concentration of the glucose in plasma was determined by the *o*-toluidine method (Dubowski, 1962).

Eight weeks after treatment with Stz or buffer, rats were killed by decapitation. A section of the thoracic aorta between the aortic arch and the diaphragm was then removed and placed in oxygenated, modified Krebs-Henseleit solution (KHS). The solution consisted of (mM): NaCl 118.0, KCl 4.7, $NaHCO_3$ 25.0, $CaCl_2$ 1.8, NaH_2PO_4 1.2, $MgSO_4$ 1.2, dextrose 11.0. In some experiments, the endothelium of the aortic strips was removed by gentle rubbing of the endothelial surface with a disposable cotton applicator. The aorta was cleaned of loosely adhering fat and connective tissue and cut into helical strips 3 mm in width and 20 mm in length. The tissue was placed in a well-oxygenated (95% O_2, 5% CO_2) bath of 10 ml KHS at 37°C with one end connected to a tissue holder and other to a force-displacement transducer (Nihon Kohden TB-611T). The tissue was equilibrated for 60 min under a resting tension of 1.0 g. During this time the KHS in the tissue bath was replaced every 20 min.

After equilibration, each aortic strip was contracted by treatment with 10^{-7} M noradrenaline (NA). The presence of functional endothelial cells was confirmed by demonstrating relaxations to 10^{-5} M ACh, and aortic strips in which 85% relaxation occurred were regarded as tissues with endothelium. The removal of endothelial cells by rubbing was confirmed by loss of ACh-induced relaxation. The relaxation responses to ACh, atrial natriuretic peptide (ANP) and sodium nitroprusside (SNP) were expressed as a percentage of the decreased tension of contractile force induced by 10^{-7} M NA. Since the maximal contraction of aortic strips in response to NA was enhanced in diabetic rats (Kamata *et al.*, 1988a), for the relaxation studies, the aortic strips were precontracted with an equieffective concentration of NA (3×10^{-8}–10^{-7} M). This concentration produced 75–85% of the maximal response. Aortic strips, which were weighed at the end of each experiment, were precontracted with 3×10^{-8}–10^{-7} M NA so that the strips developed a tension of approximately 90 mg mg^{-1} tissue in both age-matched control and diabetic rats. When the NA-induced contraction reached a plateau, ACh (10^{-8}–10^{-5} M), ANP (10^{-9}–10^{-6} M) or SNP (10^{-9}–10^{-6} M) was added in a cumulative manner. Each aortic strip was exposed only to one relaxant agent.

Measurement of cyclic nucleotides

Basal concentrations of, or drug-induced changes in levels of cyclic nucleotide were measured in a separate series of experiments. Endothelium-intact aortic strips were allowed to equilibrate in tubes that contained KHS, gassed with 95% O_2, 5% CO_2, at 37°C for 60 min. After equilibration, the aortic strips were exposed to 10^{-7} M NA for 10 min. Then a single dose of ACh was added to the tubes. One minute after the addition of the drugs, tissues were frozen in liquid N_2, and then homogenized in 1 ml of 6% trichloroacetic acid, and centrifuged at 3000 *g* for 10 min. The supernatants were extracted three times in three volumes of water-saturated ether, and were then stored at −80°C until assayed for cyclic GMP. Following succinylation, levels of cyclic GMP were determined with radioimmunoassay kits as previously described by Kamata *et al.* (1988b). Protein was determined by the method of Lowry, with bovine serum albumin as standard (Lowry *et al.*, 1951).

Drugs

Streptozotocin, noradrenaline hydrochloride and sodium nitroprusside were purchased from Sigma Chemicals Co. (St. Louis, MO, U.S.A.). Acetylcholine chloride was purchased from Daiichi Pharmaceuticals (Tokyo, Japan). Atrial natriuretic peptide was purchased from Peptide Institute (Osaka, Japan). All drugs were dissolved in saline. All concentrations are expressed as final molar concentrations of the base in the organ bath. Radioimmnoassay kits for cyclic AMP and cyclic GMP were purchased from Yamasa Shoyu Co. (Choshi, Japan).

Statistical analysis

The contractile force of aortic strips from age-matched control and diabetic rats is expressed as mg tension mg^{-1} of tissue. Results are expressed as mean ± s.e. Significance was tested by the unpaired *t* test and considered significant if $P < 0.05$.

Results

Eight to ten weeks after treatment with Stz, the concentration of glucose in plasma was elevated significantly, from 113.6 ± 2.9 (mg dl^{-1}) in age-matched controls to 755.5 ± 44.0 (mg dl^{-1}) in diabetic rats, respectively ($n = 20$, $P < 0.01$).

When aortic strips were precontracted with NA, ACh relaxed the strips in a concentration-dependent manner. However, the concentration-response curve

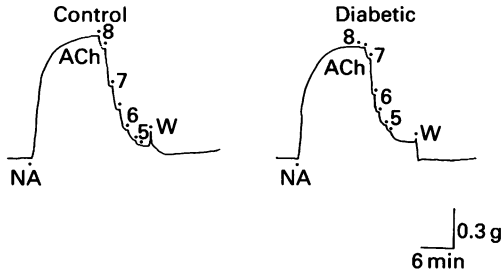


Figure 1 Relaxation responses to acetylcholine (ACh) of aortic strips with endothelium from age-matched control and diabetic rats. The aortic strips were precontracted with 3×10^{-8}–10^{-7} M noradrenaline (NA).

for ACh was shifted to the right in diabetic rats (Figure 1).

IC_{50} values for ACh were $4.57 \pm 0.67 \times 10^{-8}$ M and $1.00 \pm 0.87 \times 10^{-7}$ M in aortic strips from age-matched control and diabetic rats, respectively ($n = 6$, $P < 0.05$). In contrast to the effects of ACh, the concentration-dependent relaxation produced by ANP and SNP in diabetic rat aorta without endothelium was not different from the controls (Table 1).

Basal concentrations of cyclic GMP in aortic strips with endothelial cells were lower in strips from diabetic rats than in those from age-matched control rats (Table 2).

In both age-matched control and diabetic rats, ACh increased levels of cyclic GMP in isolated aortic strips with endothelium. The increase in levels of cyclic GMP (measured 1 min after addition of ACh) was greater in strips from age-matched control rats than in those from diabetic rats (Table 2). Basal levels of adenosine 3′:5′-cyclic monophosphate (cyclic AMP) in diabetic vessels were similar to those in age-matched control vessels (data not shown).

Discussion

It has been shown previously that the relaxation response of isolated aortic strips to ACh from spontaneously hypertensive rats and New Zealand hypertensive rats is reduced when compared to that in strips from age-matched, normotensive, Wistar Kyoto rats (Cheng & Shibata, 1981; Konishi & Su, 1983; Winquist *et al.* 1984a). In the present study, a similar result was observed in diabetic rats. However, the concentration-response curves for the relaxant effects of ANP and SNP, both endothelium-independent agents (Rapoport & Murad, 1983; Winquist *et al.*, 1984b), in aortic strips from diabetic rats, showed no shift and no depression of the maximal response. It is likely that the decrease in the relaxant response of the aortic strips with endothelium to ACh may be due to an impairment of endothelial cells but not smooth muscle guanylate cyclase activity in diabetic rats. Our observations are consistent with the findings of Oyama *et al.* (1986). The action of EDRF on blood vessels has been demonstrated to be mediated by increasing rates of formation of cyclic GMP in vascular smooth muscles (Rapoport & Murad, 1983).

Table 1 IC_{50} values for acetylcholine (ACh)-, atrial natriuretic peptide (ANP)- and sodium nitroprusside (SNP)-induced relaxation of aortic strips from age-matched control and diabetic rats

Drugs	*Control*	*Diabetic*
ACh	$4.57 \pm 0.67 \times 10^{-8}$ M	$1.00 \pm 0.87 \times 10^{-7}$ M*
ANP	$3.51 \pm 0.83 \times 10^{-9}$ M	$4.53 \pm 0.69 \times 10^{-9}$ M
SNP	$3.46 \pm 0.66 \times 10^{-9}$ M	$4.30 \pm 0.60 \times 10^{-9}$ M

Values are mean ± s.e.; $n = 6$ animals.
* Statistically different from age-matched control ($P < 0.05$).

Table 2 Basal and acetylcholine (ACh)-induced production of cyclic GMP in aortic strips from age-matched control and diabetic rats

Agents	*Control cyclic GMP* (pmol mg^{-1} protein)	*Diabetic cyclic GMP* (pmol mg^{-1} protein)
None	7.46 ± 2.02	1.28 ± 0.72*
NA (10^{-7} M)	7.30 ± 0.58	3.56 ± 1.13*
NA (10^{-7} M) + ACh (10^{-5} M)	43.88 ± 4.50	21.10 ± 1.32**

Values are mean ± s.e.; $n = 4$ animals. Significantly different from age-matched control, * $P < 0.05$, ** $P < 0.01$.
NA = noradrenaline.

In the present study, basal concentrations of cyclic GMP in aortic strips with endothelium were lower in strips from diabetic rats than in those from age-matched control rats. Furthermore, the production of cyclic GMP induced by ACh was also lower in strips from diabetic rats than in those from age-matched control rats. It is most likely, therefore, that decreases in basal and ACh-induced levels of cyclic GMP may be due to an impairment of the endothelium in diabetic rats. Changes in the responsiveness of vascular smooth muscle *in vitro* to ACh have been observed in pathological states, such as hypertension and atherosclerosis. In such cases, release of EDRF might be reduced (Konishi & Su, 1983; Winquist *et al.*, 1984a; Jayakody *et al.*, 1985).

In conclusion, ACh-induced endothelium-dependent relaxation of aortic strips precontracted with NA was significantly attenuated in strips from diabetic rats. Furthermore, basal levels of cyclic GMP and ACh-induced production of cyclic GMP were significantly decreased in strips of aorta from diabetic rats. However, relaxations produced by sodium nitroprusside in aortic strips from diabetic rats were similar to those in age-matched control vessels. This decreased production of cyclic GMP may be responsible for the decreased relaxation of the aorta induced by ACh.

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Depression of maximum rate of depolarization of guinea-pig ventricular action potentials by metabolites of encainide

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1 Standard microelectrode methods have been used to record action potentials from guinea-pig ventricular myocardium and dog Purkinje fibres, and to study the effects of the two major metabolites of encainide, O-desmethyl encainide (ODE) and 3-methoxy-O-desmethyl encainide (MODE).

2 In concentrations similar to those found in patients during chronic encainide therapy, neither ODE nor MODE produced significant depression of maximum rate of depolarization ($\dot{V}_{max}$) of action potentials in unstimulated tissue. Repetitive stimulation, however, was associated with depression of $\dot{V}_{max}$ which increased with increasing driving rates (rate-dependent block, RDB). At the fastest rate studied (interstimulus interval = 300 ms) ODE 1 μM depressed $\dot{V}_{max}$ by 47.5 $\pm$ 5.7% and MODE 1 μM, reduced $\dot{V}_{max}$ by 52.2 $\pm$ 12%.

3 The onset and offset kinetics of this rate-dependent block were very slow. Full development of RDB during a train required over 100 action potentials and the time constants of recovery of $\dot{V}_{max}$ from RDB were 86.4 $\pm$ 37 s for ODE and 100.4 $\pm$ 18 s for MODE. The amount of RDB and its rate of onset increased with drug concentration. The recovery time constants were independent of interstimulus interval or drug concentration. Both metabolites also produced rate-dependent depression of conduction velocity in canine Purkinje fibres, but no evidence of selective depression of conduction of interpolated premature potentials was seen.

4 Early afterdepolarizations occurred spontaneously in three preparations in the presence of MODE, 1 μM and one preparation in ODE, 1 μM.

5 It is concluded that these metabolites of encainide may play a role in producing both its antiarrhythmic and its proarrhythmic effects.

Introduction

Encainide is a new class 1 antiarrhythmic drug (Byrne *et al.*, 1977; Gibson *et al.*, 1978; Harrison *et al.*, 1980; Roden *et al.*, 1980). Recent trials have shown it to be at least as effective as disopyramide and quinidine in suppressing chronic ventricular arrhythmias (Caron *et al.*, 1985; Morganroth *et al.*, 1986). It is now known that some 90% of humans ('fast metabolizers'), rapidly transform encainide into two main metabolites, O-desmethyl encainide (ODE), and 3-methyoxy-O-desmethyl encainide (MODE). The other 10% produce very little or no ODE or MODE, but produce measurable amounts of third metabolite, N-desmethyl encainide (NDE). All of these metabolites possess potent class 1 antiarrhythmic actions of their own (Carey *et al.*, 1984; Davy *et al.*, 1986; Barbey *et al.*, 1988). Because they have longer elimination half-lives than encainide, MODE and ODE tend to accumulate in the plasma of patients on chronic oral therapy and reach concentrations well in excess of the parent compound

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(Kates *et al.*, 1982). Furthermore, it has been suggested that ODE in particular, may contribute significantly to the clinical effects of encainide (Carey *et al.*, 1984).

A number of recent studies have demonstrated that there are major differences between class 1 antiarrhythmic drugs in terms of their kinetics of interaction with the fast inward sodium channel, and these findings can explain a number of the clinically observed differences between the various subclasses, 1A, 1B and 1C, within this important group of compounds (Campbell, 1983a; Harrison, 1985). Encainide itself has been shown to have very slow kinetics (group 1C; Campbell, 1983b), so that at therapeutic concentrations, it tends to depress conduction of sinus beats and premature beats almost equally, thus prolonging intracardiac conduction times significantly (Pallandi & Campbell, 1988). MODE and ODE have been shown to produce rate-dependent depression of $\dot{V}_{max}$ (maximum rate of depolarization) of cardiac action potentials, as do all class 1 antiarrhythmic agents (Elharrar & Zipes, 1982), but these workers did not study the kinetics of onset and offset of these rate-dependent effects, nor their ability to depress $\dot{V}_{max}$ in the absence of prior stimulation ('resting block'). The present study was carried out to characterize the two major metabolites of encainide in terms of these parameters, at concentrations similar to those found in patients on chronic therapy with the parent drug.

Methods

Guinea-pig experiments

Guinea-pigs of either sex weighing 500–800 g were decapitated and their hearts quickly removed. Strips (approximately 5 mm × 3 mm × 1 mm) were cut from the right ventricular free wall and pinned to the base of a bath (volume 0.5 ml). The tissue was superfused at 3 ml min^{-1} with modified Locke solution gassed with 95% O_2 and 5% CO_2 and maintained at 37°C ± 0.2°C by a Peltier element. The composition of the Locke solution was as follows (mM): NaCl 125, KCl 5.6, $CaCl_2$ 2.16, $NaHCO_3$ 25, $MgCl_2$ 1.0, NaH_2PO_4 0.44, glucose 11 and the pH was 7.4. Preparations were allowed to equilibrate for 1 h before taking recordings. During this time and throughout the experiment, they were driven from bipolar platinum electrodes providing rectangular pulses of sufficient amplitude to limit variations in latency (between stimulus and action potential upstroke) to less than 1 ms (Walton & Fozzard, 1979). Action potentials were recorded from cells on the surface of the preparations by use of conventional glass microelectrodes filled with 3 M KCl, coupled to a high input impedance d.c. amplifier with negative capacitance compensation (Neurolog). To study rate-dependent effects the preparations were driven at interstimulus intervals (ISI) varying from 300–4800 ms. Trains of stimuli were of sufficient duration to achieve a stable level of effect and rest periods (unstimulated) of at least 5 min were interposed between trains to allow full recovery from rate-dependent effects. The time course of this recovery process was studied by adding a single extra-stimulus at varying intervals after a series of identical trains of stimuli at constant ISI (Campbell, 1982). The effective refractory period (ERP) was measured by interpolating single premature stimuli (S2), after every tenth basic drive stimulus (S1) at a constant interstimulus interval of 1000 ms. The S1-S2 interval was progressively decreased until no propagated action potential resulted from the S2.

Action potentials were monitored on an oscilloscope and sampled at 27 kHz using a DT 2801-A Analog-digital converter fitted to an IBM-PC. Analysis was performed by software developed within the Department (the absolute value of the maximum rate of depolarization being calculated by simple differencing of adjacent samples).

Dog experiments

The preparation has been described elsewhere (Pallandi & Campbell, 1988). Cardiac Purkinje fibres (8–15 mm long and 0.5–1.5 mm in diameter) were obtained from a total of 6 adult mongrel dogs anaesthetized with sodium pentobarbitone. The fibres were rapidly removed and mounted in the base of a tissue bath (volume 0.5 ml) and superfused at 3 ml min^{-1} with modified Locke solution gassed with 95% O_2 and 5% CO_2. They were driven from one end by stimuli from a Grass S11 Stimulator, delivered through a bipolar platinum electrode. The stimuli were rectangular pulses 4 ms long and of sufficient amplitude to maintain a constant latency between the stimulus and the upstroke of the action potential recorded at the proximal microelectrode. Two microelectrodes (5–12 mm apart) were used to record intracellular action potentials. These are referred to as proximal and distal, relative to the position of the extracellular stimulating electrode. They were coupled to a dual high input-impedance d.c. amplifier (WPI 750). Action potentials were monitored on an oscilloscope screen and analysed by digital techniques using a Rockwell AIM-65 8-bit microcomputer modified by the addition of a gain-matching amplifier, a sample-and-hold circuit and an

Analog Devices ADC 571 10-bit analog-to-digital converter. The analysis was as previously described (Campbell, 1987), with the addition of software capable of measuring the time between action potential upstrokes recorded in the proximal and then the distal microelectrode. This was converted into an estimate of conduction velocity by dividing the conduction time into the measured distance between the microelectrodes. The composition of the superfusate (kept at 37 ± 0.5°C by a Peltier element) was the same as for the guinea-pig experiments.

The samples of MODE and ODE were kindly provided by Bristol-Myers, Australia. All data are given as means ± s.d. and significance of differences between means estimated by Student's t test or the paired t test, as appropriate.

Results

Guinea-pig experiments

No obvious effects on action potential duration (APD) were noted for ODE or MODE but the experimental protocol involved frequent changes of stimulation rate and made accurate assessment of drug actions on this parameter impossible.

Class 1 effects, (reduction of maximum rate of depolarization), were evident within 5 min, and maximal within 15–20 min. They reversed completely with one hour of washing.

Resting block The ability of ODE and MODE 1 μM to depress $\dot{V}_{max}$ in the absence of prior stimulation, was studied by impaling single ventricular myocytes in control solution, and then superfusing with cumulative concentrations of drug. Single action potentials were stimulated after 60 min exposure time and their $\dot{V}_{max}$ measured and compared to the control value. Neither ODE ($n = 8$), nor MODE ($n = 6$) produced any statistically significant resting block.

Rate-dependent depression of $\dot{V}_{max}$ In contrast to the lack of resting block, repetitive stimulation produced large declines in $\dot{V}_{max}$ which slowly approached a new plateau value after 1–2 min of stimulation at a given interstimulus interval (ISI). The extent of this 'rate-dependent block' increased monotonically as the ISI was shortened from 4800 ms to 300 ms. It also increased with concentration (Figure 1). At the fastest stimulation rates (ISI = 300 ms), 1 μM ODE depressed $\dot{V}_{max}$ 47.5 ± 5.7% from control ($n = 10$ cells; $P < 0.0001$),

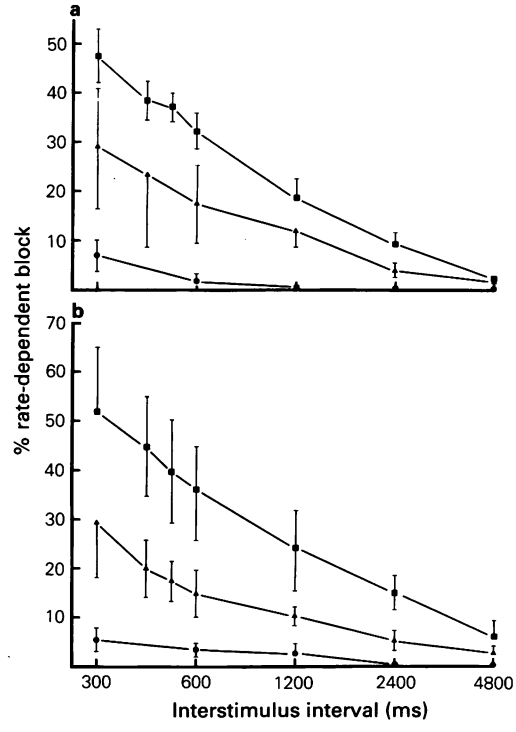


Figure 1 Relationships between stimulation rate (expressed as interstimulus interval) and the percentage rate-dependent reduction of $\dot{V}_{max}$ at two concentrations of O-desmethyl encainide (ODE, a) and 3-methyoxy-O-desmethyl encainide (MODE, b). (●) Control; effect of ODE (a) or MODE (b) (■) 1 μM and (▲) 0.25 μM. Each point represents the mean and vertical lines show s.e.mean.

and the corresponding value for 1 μM MODE was 52.2 ± 12.0% ($n = 6$; $P < 0.0001$). The actual baseline values (at ISI = 4800 ms) for $\dot{V}_{max}$ in control solution were 250 ± 25 V s^{-1} for the 10 cells later exposed to ODE and 286 ± 35 V s^{-1} for the six cells exposed to MODE.

Kinetics of onset of rate-dependent block The kinetics of onset of rate-dependent depression of $\dot{V}_{max}$ during a train of stimuli following a long rest period (5 min) were very slow, with equilibrium only occurring after 1–2 min of stimulation. The decline in $\dot{V}_{max}$ between the first and second beats of a train was always less than 5% of the final reduction achieved at equilibrium. Estimates were made of the rate at which $\dot{V}_{max}$ fell to its equilibrium value during stimulation at ISI = 300 ms. This process was fitted to a simple exponential and onset rate expressed in

Table 1 Conduction velocity ($m\,s^{-1}$) at stated interstimulus intervals

	Interstimulus interval (ms)					
	1000	*800*	*600*	*500*	*400*	*300*
Control ($n = 6$)	2.04 ± 0.36	1.94 ± 0.33	1.91 ± 0.35	1.91 ± 0.33	1.91 ± 0.33	1.91 ± 0.33
ODE 1 μM ($n = 6$)	1.74 ± 0.66	1.63 ± 0.61*	1.51 ± 0.58**	1.41 ± 0.51**	1.38 ± 0.58**	1.27 ± 0.58**
MODE 1 μM ($n = 6$)	1.42 ± 0.34**	1.40 ± 0.34**	1.29 ± 0.35**	1.19 ± 0.39†	1.12 ± 0.38†	1.03 ± 0.37†

Data shown are means ± s.e.mean. ODE = O-desmethyl encainide and MODE = 3-methoxyl-O-desmethyl encainide.
* $P < 0.05$; ** $P < 0.01$; † $P < 0.001$; significance of difference from controls.

terms of the slope of that exponential (Courtney, 1979). The value obtained for 1 μM MODE was $0.011 \pm 0.003\,AP^{-1}$ (AP = action potential), and for 1 μM ODE $0.012 \pm 0.004\,AP^{-1}$. The difference between the metabolites was not significant.

Kinetics of recovery from rate-dependent block Recovery of $\dot{V}_{max}$ towards its 'resting' level at the end of a train of stimuli followed an exponential time course that was independent of drug concentration or ISI. For ODE the time constant of recovery of $\dot{V}_{max}$ (τ_{re}) was 86.4 ± 37.4 s ($n = 15$), and for MODE, 100.4 ± 18.2 s ($n = 10$; NS).

Effects on refractory period Neither drug was able to prolong the effective refractory period (ERP), relative to the action potential duration (measured to 97% repolarization; APD_{97}). In fact, if anything there was a trend in the opposite direction which did not achieve statistical significance, possibly due to the large variation seen between preparations. The control value for ERP-APD_{97} for cells subsequently exposed to ODE was 13.4 ± 10.8 ms. ERP-APD_{97} in the presence of 1 μM ODE was 6.6 ± 12.8 ms ($n = 8$; NS). Corresponding figures for 1 μM MODE were 4 ± 12 ms (control) and −7 ± 11 ms (MODE; $n = 8$; NS).

Dog experiments

Purkinje fibres from six dogs were used to test the hypothesis that the kinetics of the actions of ODE and MODE on $\dot{V}_{max}$ in guinea-pig ventricle would correlate with the effects of these compounds on conduction velocity (at least in tissue which approximates reasonably well to a linear cable model).

Effects at steady-state In this series of experiments, the preparation was driven for at least 3 min at each of a series of increasing rates (ISI = 1000, 800, 600, 500, 400 and 300 ms). This was done first in control solution and then after 20 min exposure to ODE or MODE, 1 μM.

In the absence of drug, there was a small (non-significant) reduction in conduction velocity as stimulation rate increased (Table 1). Conduction velocity at ISI = 1000 ms was reduced in the presence of both drugs (only significant for MODE at this slowest rate) and these effects became greater as ISI was progressively shortened.

Rate of decline of conduction velocity after a reduction in interstimulus interval As was noted for $\dot{V}_{max}$, both drugs achieved a new steady-state value for conduction velocity very slowly after a step increase in stimulation rate, requiring > 100 beats to do so.

The (much smaller) falls in conduction speed seen during similar trains in control solution were complete within 2–3 beats.

Effects on conduction of single premature beats The second way in which the effects of sudden changes in rate were studied involved the interpolation of progressively more premature extrastimuli (S2) following every tenth basic drive stimulus (S1), at ISI = 1000 ms. Because of possible drug-induced variations in action potential duration, the diastolic interval was considered a more useful measure of degree of prematurity than the S1-S2 interval. The diastolic interval is the period between the end of the last action potential in the normal drive cycle (defined at 97% repolarization), and the premature extrastimulus (S2).

In control solution, the conduction velocity of the premature action potential remained exactly the same as that of the basic drive action potentials except where diastolic interval approached zero (i.e. premature action potential arising during repolariza-

tion of preceding potential; Pallandi & Campbell, 1988). Essentially the same behaviour was observed in the presence of 1 μM ODE and MODE. Values for conduction velocity were reduced in the presence of drug (as detailed in Table 1), but no significant reduction in the conduction velocity of the premature beat below that of the basic drive beat was seen at diastolic intervals above zero.

Early afterdepolarizations Interruption of repolarization, with transient repolarizations occurring during the plateau phase of action potentials, was observed as a chance finding at slow drive rates (ISI $\geq$ 1000 ms) on three occasions in the presence of 1 μM MODE (2 out of 8 guinea-pig ventricle and 1 out of 6 dog Purkinje fibre preparations), and on one occasion out of 8 during superfusion of guinea-pig ventricular cells with 1 μM ODE. In all cases full repolarization eventually occurred after 1–3 oscillatory cycles during the plateau, which led to extreme prolongation of the action potential. These early afterdepolarizations always disappeared quickly when the ISI was reduced to approximately 800–600 ms.

Discussion

The studies on guinea-pig ventricle confirm the findings of Elharrar & Zipes (1982), and provide in addition, much data on the kinetics of onset and offset of these actions. Elharrar & Zipes did not study the ability of ODE or MODE to reduce $\dot{V}_{max}$ in the absence of stimulation ('resting block' of the sodium channel). The ability to produce resting depression of $\dot{V}_{max}$ correlates well with lipophilicity for class 1 agents of relatively low molecular weight (Sada & Ban, 1981), but this relationship breaks down for drugs with molecular weights above about 350 (Campbell, 1983c; Courtney, 1987). Encainide which has a relatively high molecular weight (352) and is relatively lipid insoluble, shows a similar inability to produce resting block (Campbell, 1983b). It is not surprising therefore, that ODE and MODE which have similar molecular weights to encainide (337 and 396), and share its hydrophilicity, exhibit a similar lack of resting block.

The present study has confirmed the findings of Elharrar & Zipes (1982) that both ODE and MODE are significantly more potent than encainide at producing rate-dependent reduction of $\dot{V}_{max}$. In addition, the onset of this effect has been shown to be at least as slow as for encainide, requiring some 1–3 min stimulation or over one hundred beats to achieve equilibrium. Less than 5% of the final degree of rate-dependent block achieved during stimulation at a given ISI occurred between the first and second beats of a train. Corresponding figures for drugs such as lignocaine and mexiletine which exhibit fast onset kinetics (1B agents) are of the order of 90% and 60% (Campbell, 1983a). The offset kinetics of the two metabolites were significantly slower than for the parent compound (τ_{re} = 86.4 s for ODE and 100.4 s for MODE compared to 20.3 s for encainide in the same preparation; $P < 0.01$ in both cases; Campbell, 1983b).

It is not surprising therefore, that at concentrations comparable to those found in patients on chronic oral therapy with encainide (see below), neither ODE nor MODE had any significant effect on ERP relative to APD nor demonstrated any selective depression of conduction in premature extrasystoles. This last finding is consistent with the work of Dresel (1984), and confirms a correlation between kinetics of drug action and effects on conduction velocity as shown previously for other agents (Man & Dresel, 1979; Pallandi & Campbell, 1988).

Clinical relevance of concentrations studied

Kates *et al.* (1982) studied 13 patients on chronic encainide therapy and found mean plasma concentrations of encainide, ODE and MODE of respectively 56.3 ng ml^{-1} (159 nM), 214.6 ng ml^{-1} (500 nM) and 184.6 ng ml^{-1} (547 nM). Barbey *et al.* (1988) actually administered ODE and MODE to 9 patients with ventricular arrhythmias and found minimal effective concentrations of approximately 160 nM for ODE and 300 nM for MODE. Thus the concentrations used in the present study (250 nM and 1 μM) are clinically meaningful and the effects observed are likely to be comparable to those seen in patients on long-term treatment with encainide.

Significance of early afterdepolarizations

There is a rapidly increasing awareness of the potential ability of many antiarrhythmic agents, particularly class 1 drugs, to increase the frequency and/or severity of clinical arrhythmias in a minority of patients (Podrid, 1985; Zipes, 1988). Recent evidence has implicated early afterdepolarizations (such as those observed with ODE and MODE in the present study) in the pathogenesis of some of these 'proarrhythmic' effects, particularly in the genesis of a bizarre form of ventricular tachycardia characteristic of drug-induced arrhythmias, known as 'torsade de pointes' (Roden & Hoffman, 1985). It is interesting in this context to note that the incidence of

proarrhythmic effects in a number of studies of this phenomenon, has been higher for patients on encainide than for any other agent (Winkle *et al.*, 1981; DiBianco *et al.*, 1982; Podrid, 1985; Morganroth *et al.*, 1986; Zipes, 1988). Incidences of arrhythmia aggravation of 12–25% or more have been found for this agent.

It seems reasonable to speculate that one or both of these metabolites may play an important role not only in producing the antiarrhythmic effects of encainide, but in its proarrhythmic actions as well.

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Alterations of coronary vascular responses to noradrenaline, acetylcholine and isoprenaline during acute myocardinal ischaemia in dogs

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1 The reactivity of coronary arteries to noradrenaline (NA), acetylcholine (ACh) and isoprenaline (Iso) during acute myocardial ischaemia was studied in anaesthetized, open-chest dogs.

2 The left anterior descending coronary artery (LAD) was cannulated and perfused via an extracorporal circuit at constant flow. Ischaemia was produced by decreasing the perfusion blood flow of the LAD to 50% (moderate ischaemia) and 27% (severe ischemia) of normal. A dose of each agent which had no significant myocardial effects was chosen. Heart rate was held constant by ventricular pacing during ACh administration.

3 Under normal conditions, the infusion of NA, ACh and Iso into the LAD produced significant decreases in coronary vascular resistance ($P < 0.01$). However, under either moderate or severe ischaemic conditions intracoronary NA resulted in a marked increase in coronary vascular resistance ($P < 0.01$), which was completely blocked by phentolamine, an α-adrenoceptor blocker; intracoronary ACh and Iso did not have a significant effect on coronary vascular resistance ($P > 0.05$).

4 These results indicate that coronary arterial responsiveness to some intrinsic vasoactive substances, such as NA and ACh, is altered during acute myocardial ischaemia. This may be important in the pathophysiology of myocardial ischaemia.

Introduction

Through several decades of study, it has been well clarified that in the normal heart: (a) cardiac sympathetic nerve stimulation or the administration of noradrenaline (NA) produces coronary vasodilatation, which is mainly secondary to an increased metabolic activity of the myocardium (Berne *et al.*, 1965; Granata *et al.*, 1965; McRaven *et al.*, 1971). However, in the presence of a β-adrenoceptor blocker coronary vasoconstriction is observed (Feigl, 1967; Adam *et al.*, 1970). (b) Isoprenaline (Iso), a β-adrenoceptor agonist, causes coronary vasodilatation that is completely blocked by propranolol (Klocke *et al.*, 1965; Ross, 1974). (c) Stimulation of efferent vagal fibres to the heart or the administration of acetylcholine (ACh) in the dog results in coronary vasodilatation independent of the effects on heart rate and myocardial contractility (Berne *et al.*, 1965; Feigl, 1969; Levy & Zieske, 1969). Although the actions of sympathetic and parasympathetic nerves and their transmitters on the normal coronary vessels have been demonstrated, their actions on coronary vessels in myocardial ischaemia have not been clarified.

Recent studies have demonstrated that the vascular constrictor reaction in atherosclerotic arteries was augmented (Henry & Yokoyama, 1980; Yokoyama *et al.*, 1983; Heistard *et al.*, 1984). Other studies showed that after myocardial ischaemia or coronary ligation, coronary vascular responses to thrombin were changed (Ku, 1982; 1983) and the myocardial blood flow response to NA was profoundly modified (Moore & Parratt, 1973). All of these studies indicated that coronary artery reactivity is closely related to the state of the coronary arteries.

The purpose of the present investigation was to evaluate the effects of acute myocardial ischaemia on the responsiveness of canine coronary arteries to NA, ACh and Iso.

Methods

General preparation

Twelve dogs of either sex weighing 13–15 kg were anaesthetized with sodium pentobarbitone

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Table 1 Responses to noradrenaline (NA, 12 μg, i.c.)

	HR (beats min^{-1})	*P* (mmHg)	*Pp* (mmHg)	*CBF* (ml min^{-1})	*R* (mmHg ml^{-1} min)
Normal (n = 12)					
Control	184 ± 8	94 ± 4	118 ± 4	22 ± 1	5.53 ± 0.44
NA	182 ± 8	94 ± 3	107 ± 3*	22 ± 1	5.03 ± 0.36*
Moderate ischaemia (n = 10)					
Control	184 ± 8	86 ± 6	60 ± 3	11 ± 1	5.60 ± 0.65
NA	184 ± 8	85 ± 6	66 ± 3*	11 ± 1	6.24 ± 0.69*
Severe ischaemia (n = 7)					
Control	177 ± 10	73 ± 6	55 ± 6	6 ± 0.4	9.68 ± 1.39
NA	175 ± 9	73 ± 6	60 ± 6*	6 ± 0.4	10.65 ± 1.56*
Severe ischaemia after phentolamine (10 mg, i.c., n = 7)					
Control	178 ± 7	66 ± 4	56 ± 5	6 ± 0.2	9.42 ± 1.33
NA	179 ± 7	66 ± 4	56 ± 6	6 ± 0.2	9.37 ± 1.42

HR = heart rate, P = mean aortic pressure, Pp = mean perfusion pressure of the left anterior descending coronary artery (LAD), CBF = mean blood flow of the LAD, R = resistance of the LAD, i.c. = intracoronary infusion. Data are expressed as mean ± s.e.mean. *$P < 0.01$, control vs NA.

(30 mg kg^{-1} body weight, i.v.). Positive-pressure ventilation (model SC respirator, Shanghai) with an expiratory pressure of 0.5 kPa was adjusted to keep blood gases and acid/base balance within normal limits (Feigl & D'Alecy, 1972), as monitored by a blood gases and pH analyzer (BMS 3 Radiometer, Copenhagen). A left thoracotomy was performed through the 5th intercostal space and the heart suspended in a pericardial cradle. A short segment of the left anterior descending coronary artery (LAD) near its origin was carefully dissected free of periadventitial tissue. Ligatures were placed around the vessel. The vessel was incised and cannulated. Blood coagulation in the extracorporal circuit was prevented by infusion of heparin (750 u kg^{-1} plus 250 u kg^{-1} per hour, i.v.). Rectal temperature was held at 37°C with a heating pad.

Arterial pressure was measured with a polyethylene catheter advanced into the aorta through the right femoral artery. A standard lead II of the electrocardiogram was used to determine heart rate. All recordings were made on a Mingögraf-800 recorder.

Perfusion of the left anterior descending coronary artery

Blood was supplied to the LAD at constant flow through a cannula. The blood was obtained from the left femoral artery and passed through a constant flow roller pump (model HSP-A, Shazhou) and an extracorporal electromagnetic flow transducer (MF-27, Nihon Kohden). The electromagnetic flowmeter was fully calibrated at the end of each experiment with blood from the experimental animal. Occlusion zero flow determinations were repeated frequently throughout the experiment. Coronary perfusion pressure was measured through a polyethylene tube with an opening at the side of the distal end of the perfusion cannula. A pressure drop caused by the perfusion cannula was subtracted.

Initially, flow rate of the LAD was adjusted so that coronary perfusion pressure approximated systemic arterial pressure, i.e., about 100 mmHg (McRaven *et al.*, 1971); this flow rate was defined as normal in our experiments. Acute myocardial ischaemia (low-flow perfusion) was achieved by reducing the blood flow of the LAD to 50% (moderate ischaemia) and 27% (severe ischaemia) of normal for 60 min, respectively.

Protocol

After completing all of the surgical procedures, the dogs were allowed to stabilize for 30 min. The responses to a series of intracoronary NA, ACh and Iso infusions were recorded before and after acute myocardial ischaemia, i.e., first moderate ischaemia, and then severe ischaemia.

Intracoronary NA (12 μg ml^{-1}), ACh (20 μg ml^{-1}) and Iso (0.3 μg ml^{-1}) were administered in saline with a minute roller pump (model WSQ-B, Shazhou) at infusion rates of 12, 20 and 0.3 μg min^{-1}, respectively. Each agent was administered for 60 s after the effects of the previous administration had disappeared completely. A dose of each agent which had no significant myocardial effects was chosen. When ACh was infused, the ventricle was paced at a constant rate of 3.8 Hz. Phentolamine (10 mg) was infused slowly into the LAD before the last intracoronary NA infusion. Saline, at an infusion rate of

Table 2 Responses to acetylcholine (ACh 20 μg, i.c.) and isoprenaline (Iso 0.3 μg, i.c.)

	HR (beats min^{-1})	*P* (mmHg)	*Pp* (mmHg)	*CBF* (ml min^{-1})	*R* (mmHg ml^{-1} min)
Normal ($n = 11$)					
Control	227 ± 7	77 ± 5	104 ± 4	23 ± 2	4.89 ± 0.54
ACh	227 ± 7	74 ± 4	78 ± 4§	23 ± 2	3.62 ± 0.42§
Control	177 ± 6	91 ± 4	112 ± 7	23 ± 2	5.30 ± 0.62
Iso	176 ± 6	92 ± 4	95 ± 7§	23 ± 2	4.44 ± 0.49*
Moderate ischaemia ($n = 9$)					
Control	229 ± 9	75 ± 7	55 ± 3	11 ± 1	5.16 ± 0.61
ACh	229 ± 9	73 ± 6	57 ± 4	11 ± 1	5.36 ± 0.61
Control	184 ± 7	80 ± 6	57 ± 4	11 ± 1	5.38 ± 0.68
Iso	183 ± 7	80 ± 6	58 ± 3	11 ± 1	5.50 ± 0.64
Severe ischaemia ($n = 7$)					
Control	227 ± 9	63 ± 5	52 ± 5	6 ± 0.2	9.48 ± 1.18
ACh	227 ± 9	58 ± 6	53 ± 6	6 ± 0.2	9.60 ± 1.32
Control	176 ± 6	68 ± 6	54 ± 7	6 ± 0.2	9.85 ± 1.59
Iso	177 ± 6	67 ± 6	54 ± 8	6 ± 0.2	9.92 ± 1.73

Heart rate was held constant by pacing when ACh was infused.
* $P < 0.01$.
§ $P < 0.001$, control vs ACh or Iso. See Table 1 for explanation of abbreviations.

1 ml min^{-1} which was identical to the infusion rates of the drugs, was administered before and after myocardial ischaemia as a control.

At the end of the study, dogs were killed, and hearts removed for necropsy examination.

Drugs

The agents employed in this study were: noradrenaline (Tianjin Heping Pharmaceutical Manufactory), acetylcholine (S.A.F. Hoffman-LA Roche Co.), isoprenaline (Beijing Drugs Manufactory), phentolamine (Ciba-Geigy HG). All drug solutions used were prepared daily in saline.

Data analysis

Data were analysed from two time points associated with each agent infusion. Control values were read from the period immediately before infusion of each agent. Response values were read during the steady period near the end of each infusion. The calculation of coronary vascular resistance was as follows: R = P/Q, where, P is the mean perfusion pressure of the LAD (mmHg), Q is the mean blood flow of the LAD (ml min^{-1}), R is the resistance of the LAD (mmHg ml^{-1} min). Coronary venous pressure was assumed to be negligible.

All data are presented as means ±s.e.mean. The reactions to NA, ACh and Iso were analyzed by paired *t* test. *P* values < 0.05 were regarded as statistically significant.

Results

Intracoronary NA, ACh and Iso infusion did not alter systemic arterial pressure, and heart rate was unchanged by NA and Iso infusion and paced during ACh administration (Tables 1 and 2). With normal coronary perfusion blood flow, intracoronary NA, ACh and Iso infusion produced significant decreases in coronary vascular resistance (Tables 1 and 2). Under either moderate or severe myocardial ischaemic conditions, intracoronary NA infusion induced a marked increase in coronary vascular resistance (Table 1); neither intracoronary ACh nor intracoronary Iso had a significant effect on coronary vascular resistance (Table 2).

Tables 1 and 2 present data showing that reducing coronary flow from 11 to 6 ml min^{-1} does not lower significantly coronary perfusion pressure, suggesting that the coronary arteries have already been maximally dilated during moderate ischaemia. Also, under either moderate or severe myocardial ischaemia, NA infusions caused increases in coronary perfusion pressure to about the same extent, indicating that reducing the flow from 11 to 6 ml min^{-1} does not intensify the coronary constrictor effect produced by NA significantly. Iso infusions pro-

duced no decreases in coronary vascular resistance, demonstrating that activity on β-adrenoceptors was absent or near minimum during ischaemia.

In order to elucidate the mechanism involved in the altered response of ischaemic coronary arteries to NA, phentolamine was used. Intracoronary administration of phentolamine prevented the increase in coronary vascular resistance during NA infusion (Table 1). This indicated that constrictor response of ischaemic coronary arteries to NA was mediated by α-adrenoceptors.

Intracoronary infusion of saline at a rate of 1 ml min^{-1} had no obvious effect on coronary vascular resistance under either normal or ischaemic conditions.

Discussion

These experiments show that coronary arteries during acute myocardial ischaemia are significantly different from those under normal conditions in their responsiveness to NA, ACh and Iso. In ischaemic coronary arteries, the responsiveness to NA reverses to vasconstriction; and that to ACh and Iso appears to be reduced or absent. The paradoxical responses of ischaemic coronary arteries to NA, ACh and Iso could be due to the following mechanisms.

(a) The number and/or affinity of α-adrenoceptors in ischaemic coronary arteries may increase. It has been shown that α-adrenoceptors in both myocardium and vascular smooth muscle exist in two affinity states, high and low, and are subject to regulation by several factors, including guanine nucleotides, mono- and divalent cations, sex hormones and the NA content of myocardium (Colucci *et al.*, 1984). Thus, alterations of these factors due to myocardial ischaemia are probably associated with an increased affinity of the α-receptors. Other studies have demonstrated that during myocardial ischaemia α-adrenoceptor responsiveness is enhanced (Juhasz-Nagy & Aviads, 1976; Sheridan *et al.*, 1980; Corr & Witkowski, 1984) and that this change is associated with a two fold reversible increase in α-adrenoceptor number (Mukherjee *et al.*, 1980; Corr *et al.*, 1981). Accordingly, the number of α-adrenoceptors in ischaemic coronary arteries may possibly be increased.

(b) β-Adrenoceptor responses in coronary arteries were lost or near minimum during myocardial ischaemia, as evidenced by the lack of effect on coronary vascular resistance of Iso in these experiments. Consequently, α-adrenoceptor vasoconstriction is unopposed. With regard to this, Vlahakes *et al.* (1982) have suggested that tonic β-adrenoceptor activity might protect the coronary circulation from decreases in maximal flow produced by circulating catecholamines or increased sympathetic nerve activity.

(c) Coronary arterial endothelium may suffer injuries. It has been demonstrated that many vasodilators including ACh relax arteries indirectly by stimulating the release of endothelium-derived relaxant factor from the endothelium (Furchgott & Zawadzki, 1980; De Mey & Vanhoutte, 1981; Zawadzki *et al.*, 1981; Furchgott, 1983). Other experiments on isolated coronary arteries of the pig and the dog showed that NA and 5-hydroxytryptamine were significantly more powerful vasoconstrictors in the absence of endothelium (Cocks and Angus, 1983). A histological study of coronary arteries subjected to our experimental protocol showed that the coronary arterial endothelium was seriously damaged after acute low-flow perfusion (Sun *et al.*, 1986).

(d) With decreasing coronary blood flow, coronary reserve is so exhausted (Gould & Lipscomb, 1974; Gould *et al.*, 1975) that coronary vasodilator capacity is severely limited. In this situation, it is difficult to elicit further coronary vasodilatation and, therefore, vasoactive substances may elicit coronary vasoconstriction.

We can probably exclude mechanisms (c) and (d) as being major mechanisms for the constrictor responses of ischaemic coronary arteries to NA. Firstly, we found that phentolamine could abolish the coronary vasoconstriction produced by NA during myocardial ischaemia, this proved that NA constricted ischaemic coronary arteries via α-receptors rather than other mechanisms. Secondly, Vlahakes *et al.* (1982) have demonstrated that α-adrenoceptor stimulation with intracoronary phenylephrine produced little change in the pressure-flow relationship of the coronary arterial bed during maximal coronary vasodilatation produced by adenosine. We can not distinguish between mechanisms (a) and (b); each of these mechanisms may contribute, in part, to the constrictor response of ischaemic coronary arteries to NA.

As regards the reduced vasodilator responses of ischaemic coronary arteries to ACh and Iso, both (b) and (d) could contribute to the effect of Iso. Also (c) and (d) probably contribute to the effect of ACh.

In conclusion, during acute myocardial ischaemia in the dog, α-adrenoceptor mediated vasoconstrictor mechanisms become supersensitive, but β-adrenoceptor- and cholinoceptor-mediated vasodilator responses appear to be reduced or abolished. The sensitization of the ischaemic coronary vascular bed to vasoconstriction and impairment of its vasodilator capacity may be of great pathophysiological importance in myocardial ischaemia.

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Absence of P^2-purinoceptors in hippocampal pathways

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1 Many apparent actions of adenosine 5'-triphosphate (ATP) are mediated by adenosine produced by enzymatic hydrolysis of the nucleotide. Previously described actions of ATP in the CNS have been partly due to this phenomenon. In the present study analogues of ATP, which are not hydrolysed to adenosine, were used to seek responses to activating nucleotide (P_2) receptors in the hippocampus. The analogues used were L-adenosine-5'-(β,γ-methylene)-triphosphonate and 2-methylthioadenosine-5'-(β,γ-difluoromethylene)-triphosphonate.

2 Neither of the stable nucleotides had any effect on orthodromically evoked synaptic potentials in the CA1 region of rat hippocampal slices. Adenosine and ATP had inhibitory actions that could be prevented by the P_1-receptor blocker 8-phenyltheophylline.

3 The stable nucleotides had no consistent effects on the firing rate of single neurones in stratum pyramidale of the CA1 region, although adenosine and ATP produced a xanthine-sensitive inhibition.

4 Adenosine selectively reduced the sensitivity of CA1 neurones to microiontophoretically applied carbachol whereas stable nucleotides did not.

5 It is concluded that there are neither P_{2X}- nor P_{2Y}-receptors for adenine nucleotides on rat hippocampal CA1 pyramidal cells at the Schaffer collateral and commissural terminals in stratum radiatum.

Introduction

In the peripheral nervous system there is now substantial evidence to indicate that adenosine triphosphate (ATP) can be released as a neurotransmitter or cotransmitter together with acetylcholine or noradrenaline (Fedan *et al.*, 1981; Sneddon *et al.*, 1982; Westfall *et al.*, 1983; Sneddon & Burnstock, 1984a,b; Machaly *et al.*, 1988; Stjarne & Astrand, 1985). The nucleotide may then have direct actions on postjunctional sites (Burnstock, 1972; Stone, 1981a,b; Wiklund *et al.*, 1985; Burnstock & Kennedy, 1985; Gordon, 1986), but it is also rapidly metabolised to adenosine 5'-monophosphate (AMP) and adenosine (Cusack *et al.*, 1988), which can have a variety of actions both on the postjunctional surface (Stone, 1981a; Williams, 1987) and on presynaptic terminals (Paton *et al.*, 1978; Ribeiro, 1979; Fredholm & Hedqvist, 1980; Israel *et al.*, 1980; Su, 1983; Stone, 1981a; Schubert *et al.*, 1986). The latter responses have been attributed to activation of xanthine-sensitive nucleoside or P_1-receptors, while the effects of ATP have been ascribed to a distinct population of P_2-sites (Burnstock, 1978).

In the central nervous system most of the actions of nucleotides are sensitive to methylxanthine blockade, implying either the metabolism of the nucleotides to adenosine, or that the nucleotides are able to activate the P_1 population of receptors.

Thus the depressant effects of ATP and relatively stable analogues produced on spontaneous neuronal firing in the cerebral cortex can be prevented by locally applied theophylline (Stone & Perkins, 1981; Phillis & Wu, 1981; Stone, 1982).

However, responses to purine nucleotides which appear to reflect the involvement of P_2-receptors have been described, since they are not reproduced by adenosine and are not sensitive to blockade by methylxanthines. These effects include the excitatory action of ATP on some sympathetic and dorsal root ganglion cells and neurones in the brain stem (Jahr & Jessell, 1983; Salt & Hill, 1983; Salter & Henry 1985). The present experiments were therefore

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carried out to determine whether nucleotide receptors exist in the synaptic pathway from the stratum radiatum (Schaffer collateral and commissural fibres) to pyramidal neurones of the CA1 subfield of the rat hippocampus.

Advantage was taken of the development of two analogues of ATP, 2-methylthio-adenosine-5'-(β,γ-difluoromethylene)-triphosphonate (2-MeS-AMP-PCF_2P) and L-adenosine-5'-(β,γ-methylene)-triphosphonate (L-AMP-PCP), which are not hydrolysed to adenosine (Cusack & Hourani, 1984; Cusack *et al.*, 1987).

Methods

Four hundred μm thick transverse hippocampal slices were prepared from adult male rats which had been killed by stunning and cervical dislocation. Slices were cut using a McIlwain tissue chopper and stored in artificial CSF at room temperature under an atmosphere of water-saturated $95\%O_2/5\%CO_2$. After a minimum recovery period of one hour, individual slices were transferred to a perfusion chamber and superfused with warmed (30°C) artificial CSF containing (mM): NaCl 115, KCl 3.0, $MgSO_4$ 1.2, $CaCl_2$ 2.5, KH_2PO_4 1.2, $NaHCO_3$ 25 and D-glucose 10, gassed with 95% O_2/5% CO_2. The slices were completely submerged in the medium which flowed continuously from a gravity feed at a rate of approximately 3 ml min^{-1}.

The recorded signals were amplified and displayed on oscilloscopes and were processed on line via a Grafitek signal processing interface linked into a BBC microcomputer. The software package used (SAP, Harvard Instruments) allowed the positioning of cursors onto the graphical display of the recorded waveform in order to measure the amplitude and rate of rise of the population spike (Brooks & Stone, 1988). These amplitude values were printed on-line and subsequently plotted in histogram form. Potentials were evoked by a concentric bipolar electrode by use of stimuli of 0.1 ms duration and 5–200 μA amplitude delivered at 0.1 or 0.2 Hz.

Carbachol and NMDA were applied iontophoretically (5–100 nA, 5–25 s) at appropriate, constant time intervals to ensure recovery between applications. Purines and purine analogues were applied via the perfusate at known concentrations.

Responses of single cells were elicited by microiontophoretically administered compounds and recorded as described above using combined multibarrel pipettes and single recording electrodes. The multibarrel pipettes were constructed from 7-barrelled blanks which contained a glass fibre (Clarke Electromedical) and were filled immediately before use with a selection of the following solutions: carbachol, 10 mM, pH 6; kainic acid, 10 mM, pH 5; quisqualic acid 10 mM, pH 7; NMDA, 20 mM, pH 7.

Unit activity was recorded in the pyramidal cell layer of the CA1 region. Action potentials were amplified and monitored on oscilloscopes, gated and counted, and are presented in histogram form as records of cell firing rate in spikes per second. All calculations of response size are based upon a measure of total spike count during a drug response, expressed as a percentage of the total count obtained in the same period before drug application, determined using a computer programme written specifically for the purpose. Results are presented as means $\pm$ s.e. for n cells or slices. Statistical significance of differences was assessed by use of a two-tailed t test.

Drugs

Adenosine, ATP, adenosine 5'-(α,β-methylene)-triphosphonate (AMPCPP), adenosine 5'-(β,γ-methylene)-triphosphonate (AMP-PCP), 8-phenyltheophylline, carbamyl choline chloride (carbachol) and kainic acid were purchased from the Sigma Chemical Co, and quisqualic acid and N-methyl-D-aspartic acid (NMDA) obtained from Cambridge Research Biochemicals. L-Adenosine 5'-(β,γ-methylene)-triphosphonate (L-AMP-PCP) was synthesised as described by Cusack & Hourani (1984), 2-methylthioadenosine 5'-triphosphate (2-Mes-ATP) as described by Gough *et al.* (1973), and 2-methylthioadenosine 5'-(β,γ-difluoromethylene)-triphosphonate (2-MeS-AMP-PCF_2P) as described by Cusack *et al.* (1987).

Results

Adenosine itself inhibited orthodromic activation of CA1 pyramidal cells, as reflected by a depression of the evoked field postsynaptic potential (p.s.p.) and the population spike (Figure 1). A 50% reduction in the size of this potential was produced by



Figure 1 Records of the orthodromic population spike evoked in the CA1 region of a hippocampal slice. The records were taken before (a), during (b) and following (c) superfusion of the slice with adenosine (10 μM). Calibrations 2 mV and 5 ms.

22.4 ± 3.5 μM adenosine ($n = 12$). The effect of ATP was qualitatively similar though the concentration producing 50% inhibition was significantly greater (35.6 ± 3.2 μM, $n = 6$). Responses to both purines were totally abolished by superfusion with 8-phenyltheophylline (1 μM). Neither adenosine nor ATP had any effect on the antidromic population spike at concentrations up to 100 μM.

Superfusion with AMP-PCP produced a small depression of synaptic potentials, but even at 100 μM this was substantially less (21 ± 6%, $n = 3$) than that seen with ATP.

The other stable analogues of ATP tested, AMPCPP, 2-MeS-ATP and 2-MeS-AMP-PCF_2P, had no effect on the orthodromic or antidromic evoked potentials in the CA1 region when tested at concentrations up to 10 μM (Figure 2).

In separate experiments the various purines were tested on the single cell firing which occurred either spontaneously or was induced by NMDA or carbachol.

Spontaneous activity of CA1 pyramidal cells was inhibited by adenosine or ATP superfused at concentrations above 10 μM and 15 μM, respectively. These actions could be prevented by 8-phenyltheophylline at a concentration of 1 μM. In contrast AMPCPP (10 μM) had no effect on any of the 11 neurones tested (in different slices), although 2-MeS-ATP caused an increase of firing rate of 2 of 14 cells and 2-MeS-AMP-PCF_2P caused excitation of one and inhibition of one of the 12 cells (Figure 3). None of these effects could be repeated by subsequent applications of L-AMP-PCP or 2-MeS-ATP.

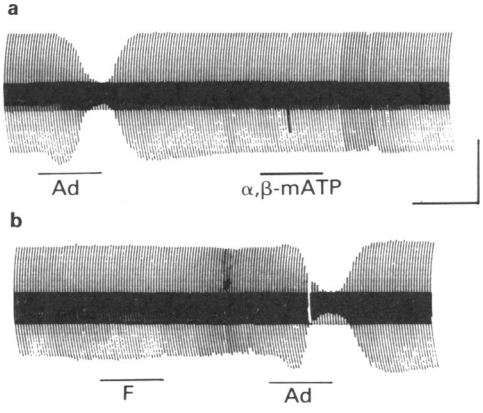


Figure 2 Chart records of the CA1 population spike potential plotted from a digital storage oscilloscope. The potential was almost abolished by adenosine (50 μM) (Ad), but unchanged by α,β-methylene ATP (10 μM) (α,β-mATP) or by 2-methylthioadenosine 5′-(β,γ-difluoromethylene)-triphosphonate (10 μM) (F). The two sections of recording are not consecutive but were made on the same slice. Calibrations 2 mV and 4 min.

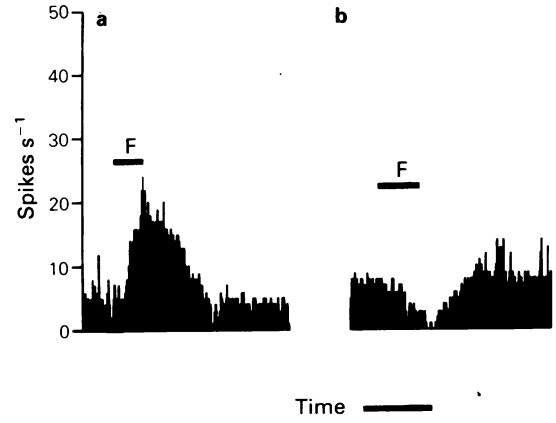


Figure 3 Records of the firing rate of 2 neurones in different slices of rat hippocampus. Neurone (a) is excited and cell (b) inhibited by superfusion with 2-methylthioadenosine 5′-(β,γ-difluoromethylene)-triphosphonate (10 μM) (F). Time bar 1 min.

Superfusion of slices with adenosine (20 μM) prevented excitation of neurones by carbachol applied by microiontophoresis, while sensitivity to the excitant amino acid NMDA was not significantly modified (5 cells) (Figure 4). None of the stable analogues, AMPCPP (4 cells), L-AMP-PCP (4 cells) and 2-MeS-AMP-PCF_2P (2 cells) changed neuronal responses to carbachol or NMDA when tested at a concentration of 10 μM.

Discussion

Of the nucleotide analogues tested here AMPCPP is a relatively stable compound in which the introduction of the α,β-methylene bridge into the phosphate side chain confers resistance to enzymic hydrolysis (Cusack *et al.*, 1988).

AMP-PCP is more susceptible to metabolism to adenosine and this probably explains the xanthine-sensitive effects of this compound on evoked potentials. Its enantiomer L-AMP-PCP which was synthesised and examined by Cusack & Hourani (1984), is also resistant to hydrolysis, but in any case would yield L-adenosine as a product. Unlike the naturally occurring (D) form, L-adenosine is unable to act on P_1-receptors (Cusack & Planker, 1979) and any effects of L-AMP-PCP can therefore be attributed to activation of P_2 sites. The failure of L-AMP-PCP, 2-MeS-ATP and 2-MeS-AMP-PCF_2P to affect evoked p.s.ps or population spikes, therefore, strongly implies the absence of P_2 nucleotide receptors capable of modulating either presynaptic transmitter release or pyramidal cell excitability.

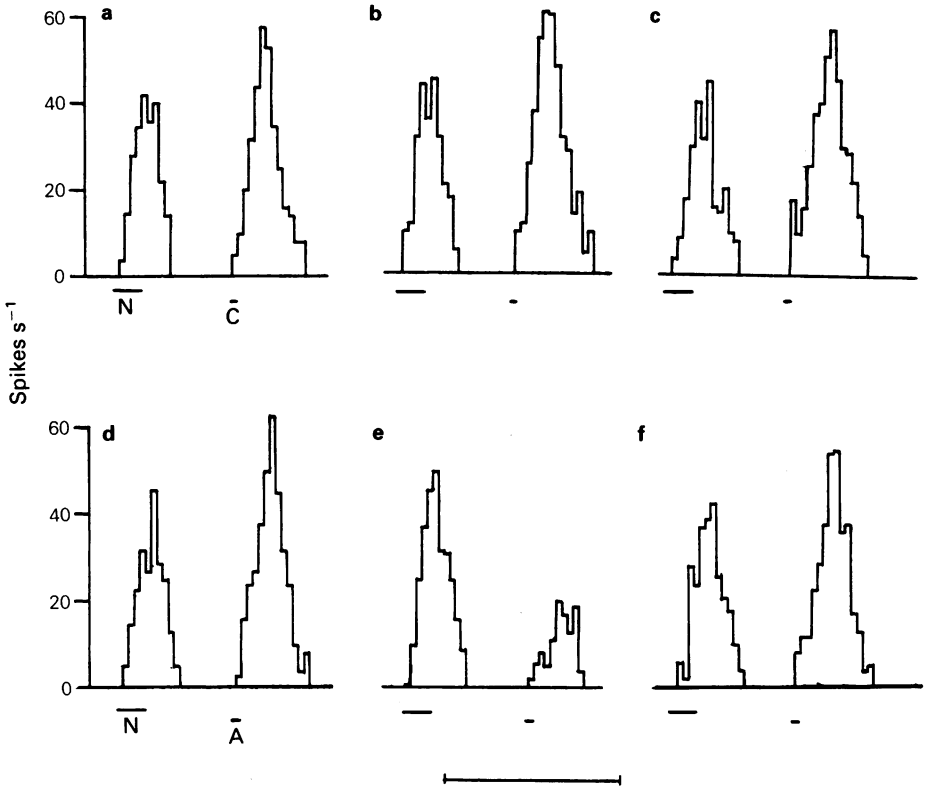


Figure 4 Records of the firing rate of a CA1 pyramidal neurone excited by the iontophoretic application of carbachol, 20 nA (C) and N-methyl-D-aspartate, 32 nA (N). (a) and (d) show control responses, and (c) and (f) recovery following superfusion with L-adenosine 5'-(β,γ-methylene)-triphosphonate, 10 μM (b) or adenosine, 20 μM (e). Adenosine selectively inhibits the excitation by carbachol whereas the nucleotide does not modify sensitivity to either agonist. Time bar: 1 min.

The ability of adenosine to suppress selectively pyramidal cell sensitivity to carbachol appears to be associated with an atypical adenosine receptor since, although the effect is xanthine-sensitive, it has characteristics which differentiate it from the usual A_1 or A_2 subtypes of the P_1 receptor (Brooks & Stone, 1988). Even here the nucleotide analogues had no apparent effect.

The changes of single cell firing rate are difficult to interpret. The restricted quantities of the analogues available precluded testing a larger population of neurones and the significance of unreproducible effects on 4 of 26 neurones must be questioned.

The P_2 population of nucleotide receptors has recently been reviewed by Burnstock & Kennedy (1985) and the proposal made that two subtypes may exist, the P_{2X} and P_{2Y} sites. The former may mediate contraction of a variety of smooth muscle tissues, including the vas deferens and urinary bladder of rats and guinea-pigs and the rat femoral artery. The P_{2Y} site on the other hand may mediate relaxation of the guinea-pig taenia coli and the rabbit portal vein. Of the compounds tested in the present study, L-AMP-PCP has been proposed as a specific agonist at the P_{2X}-receptor (Hourani *et al.*, 1985; Cusack *et al.*, 1987) and both 2-MeS-ATP and 2-MeS-AMP-PCF_2P are very potent at the P_{2Y} site. The present results therefore suggest the absence of either P_{2X}- or P_{2Y}-receptors in the CA1 subfield of rat hippocampus.

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